Inhibition of Vancomycin-Resistant Enterococci by an In Vitro Continuous-Flow Competitive Exclusion Culture Containing Human Stool Flora

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An in vitro anaerobic continuous-flow competitive exclusion (CFCE) culture model was used to study the ability of human stool flora to inhibit the growth of vancomycin-resistant (VR) enterococci (VRE). The CFCE culture was established from a stool sample obtained from a healthy adult. When 10³ or 10⁶ cfu/mL of VR Enterococcus faecium were added to the CFCE culture, the VRE were eliminated within 6 or 9 days, respectively. When 10⁷ cfu/mL of the CFCE culture was added to a continuous-flow culture that contained 10⁷ cfu/mL of VRE, the density of VRE was reduced but not eliminated. These data support the hypothesis that the indigenous intestinal flora inhibit growth of VRE and suggest that CFCE cultures may be a useful means to study interactions between the indigenous flora and VRE.

Materials and Methods

Parent CFCE culture. A parent CFCE culture was developed from a stool specimen collected from a healthy adult who had not received antibiotics for several years. The stool specimen was transferred immediately to an anaerobic chamber (Coy Laboratory Products) and was homogenized. A 4.86-g portion was incubated at 37°C for 6 h in 100 mL of Viande Levure (VL) broth [4], and then 100 mL was added to 650 mL of VL broth in a chemostat (New Brunswick Scientific), for a total culture volume of 750 mL. After incubation for 6 h, continuous flow of VL broth was begun at a rate of 0.52 mL/min (i.e., a vessel turnover time of 24 h). The culture vessel and the media reservoir were flushed with oxygen-free nitrogen to maintain anaerobic conditions. The culture was maintained at 37°C and was stirred at 200 rpm.

The culture pH, concentrations of lactic acid and volatile fatty acids (VFAs; acetic, propionic, butyric, and isobutyric acids), and bacterial densities were monitored [4, 5]. VFA and lactic acid concentrations were determined by gas chromatography, as described elsewhere [4]. The densities in cfu/mL of anaerobes were measured by transferring portions of the culture to the anaerobic chamber, serially diluting, plating onto Brucella agar (Anaerobe Systems), and incubation at 37°C for 48 h before counting colonies; to confirm that strict anaerobes were being measured, duplicate plates for selected samples were removed from the anaerobic chamber and were incubated simultaneously in room air. The samples were transferred from the anaerobic chamber and were plated onto Enterococcosel agar (Becton Dickinson) and MacConkey agar (Difco Laboratories), to measure densities of enterococci and aerobic and facultative gram-negative bacilli, respectively.
**Experimental design.** Two experiments for each inoculum (4 total experiments) were performed to determine the ability of a preestablished human CFCE culture to eliminate VRE. Effluent from the parent CFCE culture was collected anaerobically at 4°C and was used to start additional cultures. Bacterial densities and pH were monitored as noted above. VRE at 10³ or 10⁶ cfu/mL in 75 mL of PBS was added to each culture to give a final concentration of 10⁵ VRE cfu/mL. *E. faecium* strain C68 was used because it is a clinical isolate and because it was used in our mouse model of VRE colonization [2]. Samples were removed from each CFCE culture after 5 min and then at 24-h intervals for determination of pH, VFA and lactic acid concentrations, and density of VRE. VRE densities were measured by plating serially diluted samples onto Enterococcosel agar that contained vancomycin (6 µg/mL). Control cultures that contained VL broth were inoculated with 10², 10³, or 10⁴ cfu/mL of VRE.

Two experiments were performed subsequently to determine the ability of a human CFCE culture to eliminate a preestablished VRE continuous-flow culture. VRE at 10³ cfu/mL in 75 mL of PBS were added to the chemostat culture, to give a final concentration of 10⁶ VRE cfu/mL. This culture was allowed to grow for 6 days before the addition of 75 mL of CFCE culture at 10⁸ cfu/mL, to give a final concentration of 10⁹ CFCE cfu/mL. The cultures were monitored as described above.

**Statistical analysis.** Three samples were collected for each data measurement, and means were calculated. Data from replicate experiments were pooled for analysis. A Duncan’s multiple range comparison was performed by use of PC-SAS commercial statistical software (version 6.02; SAS Institute), to compare the density of VRE both before and after the addition of CFCE culture to a pre-established VRE culture.

**Results**

**Characteristics of the parent CFCE culture.** A steady state was reached for bacterial densities and pH within 5–7 days. The densities of total anaerobes (~9 log₁₀ cfu/mL) and aerobic and facultative gram-negative bacilli (~7 log₁₀ cfu/mL) remained stable over time. Incubation of samples on Brucella agar under anaerobic conditions consistently resulted in ~1–2 log₁₀ cfu/mL higher bacterial concentrations than did incubation under aerobic conditions. The density of enterococci increased by 5 log₁₀ cfu/mL over the initial 7 days of culture, before stabilizing at ~7 log₁₀ cfu/mL. Bacterial densities and pH of the parent chemostat have remained stable for 6 months (data not shown).

The concentrations of VFAs in the parent chemostat reached a steady state within 1 day. The total VFA concentration for 72 samples obtained between 1 and 108 days was 731.8 ± 79.6 µmol/mL (mean ± SD). Propionic acid (96.8 ± 15.9 µmol/mL), acetic acid (313.3 ± 59.3 µmol/mL), and butyric acid (228.8 ± 35.1 µmol/mL) were the predominant VFAs present, whereas isobutyric acid (28.6 ± 12.5 µmol/mL), isovaleric acid (13.7 ± 13.5 µmol/mL), and valeric acid (13.9 ± 15.7 µmol/mL) were present in lower concentrations. Lactic acid was present in concentrations of 36.8 ± 19.6 µmol/mL.

**Ability of the CFCE culture to eliminate VRE inocula.** The ability of the CFCE culture to eliminate VRE inocula of 10³ or 10⁶ cfu/mL is shown in figure 1. For both inocula, the density of VRE decreased to an undetectable level within 6–9 days. Culture pH, VFA concentrations, and vancomycin-susceptible

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**Figure 1.** Vancomycin-resistant enterococcus (VRE) densities after inoculation of VRE into a human continuous-flow competitive exclusion (CFCE) culture or a continuous-flow culture that contained sterile media (controls). ◊, Control 10⁵ cfu/mL VRE inoculum; ○, control 10⁶ cfu/mL inoculum; □, control 10⁶ cfu/mL VRE inoculum into CFCE culture; ■, 10⁶ cfu/mL VRE inoculum into CFCE culture.
enterococcal concentrations were not affected by inoculation of VRE (data not shown). Inoculation of $10^2$, $10^3$, or $10^4$ cfu/mL of VRE into control continuous-flow cultures resulted in growth to $7 \log_{10}$ cfu/mL of VRE (figure 1). The control VRE cultures maintained a pH $<5$, and $253.9 \pm 63.3$ $\mu$mol/mL of lactic acid was produced.

Displacement of preestablished VRE. An anaerobic continuous flow culture that contained $10^7$ VRE/mL was maintained for 6 days. After the addition of $10^7$ cfu/mL of the CFCE culture at day 0 (figure 2), the density of VRE decreased to $<4.0 \log_{10}$ cfu/mL over 14 days. In comparison with days $-6$ to 0, the density of VRE was significantly reduced from days 3–14 ($P < .05$, for all time points). Total anaerobes and total facultative and aerobic gram-negative bacilli were established at concentrations comparable to those of the previous experiments (data not shown). After the addition of CFCE culture, the concentrations of VFAs rose to concentrations similar to those of the parent chemostat, but the pH remained $<5$.

Discussion

We used an in vitro anaerobic continuous-flow culture model to study the ability of human stool flora to inhibit growth of VRE. A culture derived from a stool specimen obtained from a healthy adult eliminated VRE inocula of $10^3$ or $10^6$ cfu/mL over several days and significantly reduced the density of a preestablished culture of VRE. These data support the hypothesis that the indigenous intestinal flora inhibits growth of VRE and demonstrate that CFCE cultures may be a useful means to study interactions between the indigenous flora and VRE.

Using a continuous-flow culture system maintained inside an anaerobic chamber, Freter et al. [3] demonstrated that the culture reproduced the numeric balance among 37 strict anaerobes present in the original mouse cecal inoculum. Inoculation of germ-free mice with the culture contents resulted in normalization of cecal size and reduction of an overgrown Escherichia coli population to the same density as that in normal mice [3]. The benchtop chemostat CFCE model of chicken cecal contents contains a mixture of 14 strict anaerobes and 15 facultative anaerobes [4]. Previous studies of continuous-flow cultures of human stool have demonstrated similar mixtures of facultative bacteria and strict anaerobes [6, 7]. Although detailed microbiologic studies were not performed on our culture, the available data suggest that strict anaerobes were present in higher concentrations than facultative gram-negative bacilli or enterococcal species. Bacteroides species and Veillonella species have been shown to be present in concentrations of $8–9 \log_{10}$ cfu/mL (data not shown). The production of acetic acid, propionic acid, and butyric acid by the CFCE culture is consistent with previous data for human stool [8] and for a human continuous-flow culture [7].

The elimination of VRE by the indigenous flora present in the CFCE culture is consistent with other studies of mice [9] and humans [1, 10]. Laboratory mice that have not received antibiotic treatment develop only transient low-density intestinal colonization after oral gavage of VRE, whereas mice pretreated with antianaerobic antibiotics with minimal activity against enterococci develop high-density colonization [9]. Similarly,
healthy human volunteers who do not receive antibiotic therapy develop only transient stool colonization after ingestion of 10^5 cfu of VRE [10]. A CFCE culture that contained porcine intestinal microflora also has been shown to eliminate VRE in vitro at a rate similar to that observed in this study [11].

The in vitro anaerobic continuous-flow culture model has been used successfully in other studies to examine microbial interactions in the intestinal tract [3, 4, 7, 12, 13]. For example, competition for nutrients has been shown to be an important mechanism by which the indigenous flora control the growth of several organisms, including *E. coli*, *Eubacterium* species, *Fusobacterium* species, *Salmonella* species, and *Clostridium difficile* [3, 12, 13]. In addition, an in vitro anaerobic culture model potentially could be used as a means to examine how antibiotics disrupt the mechanisms that control the growth of pathogens in the intestinal tract.

The chicken CFCE culture developed by researchers with the US Department of Agriculture has been shown to prevent colonization with *Salmonella* species in broiler chickens [4]. A porcine CFCE culture developed by the same group has been shown to inhibit *Salmonella typhimurium* colonization [5]. Theoretically, this technique also could be used to develop biotherapeutic agents that could be used to inhibit intestinal colonization with VRE or other potentially pathogenic organisms. For example, a CFCE culture could be administered after a patient has completed antibiotic therapy, to restore protective flora that would inhibit the establishment of colonization with VRE. For patients who already are colonized with VRE, administration of a CFCE culture might reduce the density of colonization, thus possibly decreasing the number of organisms shed into the environment [1].

Our study has several limitations. We studied only 1 strain of VR *E. faecium*. In other studies of mice [14] and humans [1], however, different strains of VR *E. faecium* were eliminated at similar rates after the discontinuation of antibiotics. It is not known whether colonization with vancomycin-resistant *E. faecalis* strains differs from *E. faecium* strains; however, most VRE strains are *E. faecium*. In addition, elimination of *E. faecalis* JH2-SS, a laboratory strain, from a human CFCE culture has been demonstrated elsewhere [15]. The factors that facilitate persistence of some enterococcal strains in the intestinal tract or in CFCE cultures are unknown and deserve further study. The CFCE culture model we used may not reproduce all the relative bacterial densities present in the original stool inoculum, as exemplified by the fact that the density of vancomycin-susceptible enterococci was higher in the culture than in the stool inoculum. As was noted above, detailed microbiologic studies were not performed; therefore, it is not known whether the predominant strict anaerobes in the CFCE culture are those present in the original stool inoculum.

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**References**


