A mouse model of vancomycin-resistant *Enterococcus faecium* (VRE) intestinal colonization was used to study the effect of different subcutaneous antibiotics on persistence and density of VRE colonization. Gastric inoculation of a clinical VanB VRE isolate, in conjunction with oral vancomycin in drinking water (250 μg/mL), resulted in high-level VRE colonization (mean, 9.5 log_{10} cfu/g) in all 169 experimental mice. After discontinuation of oral vancomycin, the level of VRE in the stool specimens of mice receiving subcutaneous saline steadily decreased (mean, 3.59 log_{10} cfu/g at day 19). Subcutaneous vancomycin, clindamycin, piperacillin-tazobactam, ticarcillin-clavulanic acid, metronidazole, cefotetan, ampicillin, and ampicillin-sulbactam all promoted persistent high levels of stool VRE. Subcutaneous ceftriaxone, cefepime, ciprofloxacin, and aztreonam promoted increased VRE density to a lesser degree or not at all. Thus, in a mouse model, vancomycin and antibiotics with potent antianaerobic activity promoted persistent high-density intestinal VRE colonization, whereas antibiotics lacking potent antianaerobic activity did not.

Enterococci are part of the normal intestinal flora of humans and animals [1]. They have emerged as important nosocomial pathogens over the past 2 decades, at least in part because of their intrinsic and acquired resistance to many antimicrobial agents [2]. Enterococci are intrinsically resistant to aztreonam, cephalosporins, clindamycin, penicillinase-resistant penicillins, trimethoprim-sulfamethoxazole, and low levels of amino-glycosides. In addition, they have acquired resistance to penicillin (both by altered penicillin-binding proteins and by production of β-lactamase), erythromycin, gentamicin, streptomycin, tetracycline, and vancomycin. Studies in both humans and mice have demonstrated that selective pressure from antibiotic use can result in suppression of other flora and overgrowth of enterococci in the intestinal tract [3–6]. This overgrowth may predispose to the development of clinical infection [7–9]. Administration of cephalosporins, in particular, has been associated with enterococcal colonization and infection [6, 7, 10–12].

Vancomycin-resistant *Enterococcus faecium* (VRE) emerged as important nosocomial pathogens in the past decade. The risk of colonization or infection with VRE has been associated with use of various antibiotics, including oral and intravenous glycopeptides [13–16], third-generation cephalosporins [16–18], and antibiotics with potent antianaerobic activity [19, 20]. Because of these associations, alteration of hospital antimicrobial formularies has been suggested as a means of limiting the spread of VRE [21, 22]. Restriction of vancomycin use was not effective in reducing the incidence of VRE colonization in large polyclonal outbreaks [23]; however, Quale et al. [22] reported that VRE colonization was reduced from 47% to 15% over 9 months, when use of cefotaxime and clindamycin was significantly reduced and use of ampicillin-sulbactam and piperacillin-tazobactam were increased. They concluded that the change in antibiotics resulted in decreased VRE colonization, although concurrent control of a *Clostridium difficile* outbreak and increased infection control measures may also have played a role.

In Cleveland, we have noted an association between hospital purchases of certain classes of antibiotics and the prevalence of clinical VRE isolates [24]. Specifically, overall purchases of third-generation cephalosporins, ticarcillin-clavulanic acid, and clindamycin correlated positively with rates of VRE isolation, whereas purchases of ampicillin-sulbactam, piperacillin, and piperacillin-tazobactam correlated negatively. MICs for several clinical VRE isolates from Cleveland were very high for cephalosporins and ticarcillin (>10,000) compared with MICs for ampicillin and piperacillin (range, 312–1250). Therefore, we hypothesized that ampicillin-sulbactam and piperacillin-tazobactam result in lower rates of clinical VRE isolation because of partial inhibition of intestinal VRE. Here we report the results of experiments designed to compare the effects of different antibiotics on the persistence of VRE stool colonization in a mouse model.
Materials and Methods

Characterization of colonizing VRE strain. Enterococcus faecium C68 is a clinical wound isolate representing the most common pulsed-field gel electrophoresis (PFGE) genotype seen in a 1996 Cleveland area VRE outbreak [25]. It is phenotypically and genotypically VanB and expresses high levels of resistance to vancomycin. MICs for vancomycin and ampicillin were determined by standard agar dilution techniques using two-fold serial dilutions of antibiotic diluted in brain-heart infusion (BHI) agar. Single concentrations of antibiotics were used for agar susceptibility testing of chloramphenicol (10 µg/mL), erythromycin (10 µg/mL), tetracycline (10 µg/mL), gentamicin (500 µg/mL), and streptomycin (2000 µg/mL). MICs for high concentrations of ceftriaxone, cefepime, ticarcillin-clavulanic acid, pipercillin-tazobactam, and clindamycin were determined by a macrodilution technique in BHI broth [26].

Quantification of stool organisms. Fresh stool specimens (1–2 pellets) were weighed, diluted in 800 µL of sterile normal saline, homogenized with a pestle, serially diluted in sterile saline, and plated on Enterococcosel agar (Becton Dickinson, Cockeysville, MD), supplemented with 6 µg/mL vancomycin for quantification of VRE. The number of VRE colonies was read at 72 h, and the number of colony-forming units/gram of stool was calculated. Selected stool samples were plated onto Enterococcosel agar without vancomycin for quantification of total enterococci and onto MacConkey agar (Difco Laboratories, Detroit) for quantification of total aerobic and facultative gram-negative rods.

Experimental model. A mouse model of VRE gastrointestinal colonization was developed based on the methods of Whitman et al. [14]. Female CF1 mice (Harlan Sprague-Dawley, Indianapolis), weighing 25–30 g, were used in all experiments. Mice were housed in individual cages and fed rodent chow and water ad libitum. Bedding was changed twice each week. VRE colonization was established in all animals by administering oral vancomycin (250 µg/mL) in drinking water for 5 days before and 7 days after gastric inoculation of E. faecium C68 (Figure 1). During oral vancomycin administration, water bottles were refilled with freshly prepared vancomycin solution every 2 days. On experimental day −7, gastric gavage of 0.5 mL of overnight culture, prepared by inoculating a single colony of E. faecium C68 into sterile BHI broth, was accomplished by a stainless steel feeding tube (Perfektum; Popper & Sons, New Hyde Park, NY). The bacterial inoculum was determined by serial dilution of the overnight culture. Stool colony counts of VRE and total enterococci were determined for a subset of mice on day −13 (prior to any intervention) and again on day −8 (after 4 days of oral vancomycin, prior to inoculation with oral VRE). On day −4, stool colony counts of VRE were determined for all animals, to confirm colonization with VRE.

On experimental day 0, oral vancomycin was discontinued in all but 1 experimental group (positive control), and subcutaneous injection of antibiotics was initiated at 12-h intervals. Each antibiotic dose was diluted in 0.2 mL of sterile normal saline. Total daily doses of antibiotics were equivalent to the total daily dose (per kilogram) recommended for healthy human adults (calculated for a mouse weight of 30 g). Antibiotic stock solutions were prepared every 24–48 h and stored at 4°C. Fresh stool (1–2 pellets) was collected from each mouse on days 4, 9, 14, and 19 of subcutaneous antibiotic administration for determination of VRE colony counts. To confirm that isolates recovered during the course of the experiment were identical to the initial C68 inoculum, agar dilution MICs were done on randomly selected stool isolates during the course of the experiment, and PFGE was performed on 5 stool isolates.

Initial antibiotic comparisons. In initial experiments, antimicrobial agents administered to individual groups of animals included sterile normal saline (negative control), cefepime (2.4 mg/day), ceftriaxone (2.4 mg/day), clindamycin (1.4 mg/day), pipercillin-tazobactam (8 mg/day), ticarcillin-clavulanic acid (12 mg/day), vancomycin (1.4 mg/day), and continued oral vancomycin (positive control). These antibiotics were chosen based on the basis of clinical studies, suggesting a positive or negative (for piperacillin-tazobactam) association with VRE. Cefepime has not specifically been identified as a risk factor for VRE; however, this cephalosporin is similar to cefazidine (which has been associated with VRE), in that it has limited activity against anaerobes and is primarily renally excreted. These experiments were performed twice. The first run of experiments consisted of 30 mice (4 in each treatment group, except for the oral vancomycin control group, which had 2 mice). The second run consisted of 50 mice (6 in each treatment group, except for the oral vancomycin control group, which had 8 mice).

Comparison of antibiotics with various levels of anti-anaerobic activity. A second set of experiments was performed to further examine the role of anti-anaerobic activity in the promotion of persistent VRE colonization in this model. The experimental model followed was identical to the initial experiments, except that stool samples were collected on experimental days 5, 10, 15, and 20. Antimicrobial agents administered to individual groups of animals included sterile normal saline (negative control), cefepime (2.4 mg/day), cefotetan (3.0 mg/day), aztreonam (3.0 mg/day), metronidazole (8.0 mg/day), ciprofloxacin (0.4 mg/day), ampicillin (4 mg/day), and ampicillin-sulbactam (6 mg/day). These experiments were also done twice. The first run of experiments consisted of 47 mice (5 in each treatment group, except for the oral vancomycin group, which had 7 mice, and the saline group, which had 4 mice). The
second run of experiments consisted of 42 mice (5 in each treatment group, except for the metronidazole group, which had 8 mice, and the saline group, which had 4 mice; no oral vancomycin control group was included).

Metronidazole was given in a higher dose than the other antibiotics, because we observed during the first run of experiments that the human equivalent dose (0.9 mg/day) did not promote high density of VRE colonization. However, when metronidazole was given at 10× the initial dose after experimental day 20, the density of VRE markedly increased (data not shown). The higher dose of metronidazole (4 mg/day) selectively eliminates strictly anaerobic cecal bacteria in mice, with subsequent increased growth of aerobic and facultative species, including enterococci [4]. Promotion of VRE after day 20 did not occur when ciprofloxacin, aztreonam, or cefepime were given in similar high doses (data not shown).

Statistical analysis. Statistical analysis for the presence of significant differences in VRE counts among groups at each experimental time point was done by analysis of variance. Linear regression was performed to determine which experimental groups were significantly different from negative controls. For the first set of experiments, linear regression was used to compare clindamycin, piperacillin-tazobactam, ticarcillin-clavulanic acid, and vancomycin groups with cefepime and ceftriaxone groups. For the second set of experiments, paired t tests (P < .05) were used to compare antibiotic groups with greater anti-anaerobic activity (cefotetan, metronidazole, and ampicillin-sulbactam) with antibiotic groups with less anti-anaerobic activity (cefepime, ciprofloxacin, aztreonam, and ampicillin). Paired t tests (P < .05) were done to compare levels of stool gram-negative organisms from experimental groups with those of the saline control group at experimental days 19 or 20 and to compare the level of total enterococci (VRE plus vancomycin-susceptible enterococci) with the level of VRE for each group at day 19 or 20.

Results

MICs for E. faecium C68 were 512 µg/mL for vancomycin, 1 µg/mL for teicoplanin, <10 µg/mL for chloramphenicol, >10 µg/mL for erythromycin, >10 µg/mL for tetracycline, >2000 for streptomycin, >500 for gentamicin, 256 for ampicillin, 1250 for piperacillin-tazobactam, >10,000 for ceftriaxone, >10,000 for cefepime, >10,000 for ticarcillin-clavulanic acid, and >10,000 for clindamycin. Antibiotic susceptibilities of 14 selected isolates, recovered from the stool of mice during antimicrobial administration, were identical to those determined for the original strain. The 5 stool isolates subjected to PFGE analysis had restriction patterns identical to E. faecium C68.

Initial antibiotic comparisons. At day –13, prior to any intervention, 17/17 mice tested were colonized with vancomycin-susceptible enterococci, with counts of 4.16–8.95 log10 cfu/g of stool (mean, 5.48). None of these 17 animals were colonized with detectable levels of VRE. On experimental day –8 (after 4 days of oral vancomycin, prior to inoculation of VRE), 11/12 mice had no detectable enterococci (lower limit of detection <3.02 to <3.43 log10 cfu/g) and 1/12 had 3.11 log10 cfu/g. None of these 12 mice had detectable VRE.

The VRE inoculum administered by gavage was 1 × 10⁹ cfu for the first group of mice and 4 × 10⁸ cfu for the second group. Three days after VRE inoculation (experimental day –4), all 80 mice were colonized with high levels of VRE (8.84–11.15 log10 cfu/g). The effect of subcutaneous antibiotics on persistence of VRE colonization is shown in figure 2. For mice receiving saline injections (negative controls), the level of VRE stool colonization steadily decreased during the 19 days of the experiment. For mice receiving continued oral vancomycin (positive controls), persistent high levels of VRE colonization were seen throughout the 19 days of the experiment. The level of VRE colonization in negative and positive controls over time was similar to the results reported by Whitman et al. [14].

On experimental day 4, all experimental groups and saline controls had high levels of stool VRE (all means, >8.0 log10 cfu/g). On days 9, 14, and 19, all antibiotics, except cefepime, promoted higher density of VRE colonization than did saline controls (P < .005). On days 9, 14, and 19, subcutaneous clindamycin, piperacillin-tazobactam, ticarcillin-clavulanic acid, and vancomycin promoted more high-density VRE colonization than did subcutaneous cefepime or ceftriaxone (P < .005). The level of VRE colonization maintained by treatment with subcutaneous clindamycin, piperacillin-tazobactam, ticarcillin-clavulanic acid, and vancomycin was similar to that observed with continued administration of oral vancomycin. Variation in VRE counts within treatment groups was low at all time points (SD, <2.0 log10 cfu/g for all groups except the negative control group on day 9; SD, <1.0 log10 cfu/g for 66% [21/32] of all groups). No significant or persistent diarrhea was observed in any treatment group.

For the 50 mice in the second run of experiments, assessment of total enterococci on day 19 revealed significant recolonization with vancomycin-susceptible enterococci in the saline (P < .001), cefepime (P = .029), and clindamycin (P = .004) treatment groups. Assessment of total lactose-fermenting aerobic or facultative gram-negative organisms in these same animals revealed significant suppression of gram-negative organisms in the cefepime (P = .003) and piperacillin-tazobactam (P = .004) groups, compared with saline controls, and increased growth of gram-negative organisms in the clindamycin (P = .001) and oral vancomycin (P = .005) groups.

Comparison of antibiotics with varying antianaerobic activity. Prior to any intervention, 24/24 mice tested were colonized with vancomycin-susceptible enterococci (3.5–7.5 log10 cfu/g of stool), and none were colonized with VRE. On day –8 (after 4 days of oral vancomycin, prior to inoculation of VRE), 24/24 mice tested had no detectable enterococci. The inoculum of VRE C68 administered by gavage was 10⁷ cfu for both runs of these experiments. Three days after VRE inoculation (day –4), all 89 mice were colonized with VRE (4.83–10.16 log10 cfu/g). The effect of these subcutaneous antibiotics on VRE C68 colonization is shown in figure 3. The density of VRE colonization in saline controls and in oral van-
Vancomycin-Resistant *E. faecium* in Mice

Figure 2. Persistence and density of vancomycin-resistant *Enterococcus faecium* (VRE) intestinal colonization in mice. Mice were given different antibiotics subcutaneously (sc), as shown in legend (10 mice/group), every 12 h. All 80 mice were colonized with VRE on day 0 (mean, 9.5 log₁₀ cfu/g stool). Negative control mice received sc saline; positive controls received oral vancomycin (250 μg/mL) in drinking water. Stool VRE levels were quantified on days 4, 9, 14, and 19. For comparisons between groups, analysis of variance and linear regression were done. pip, piperacillin; tic, ticarcillin.

Assessment of total lactose-fermenting aerobic or facultative gram-negative organisms on day 20 revealed significant suppression of gram-negative organisms in the cefotetan, ciprofloxacin, and aztreonam groups (*P* < .05). A trend toward decreased gram-negative organisms was observed in the cefepime, ampicillin, and ampicillin-sulbactam groups, and a trend toward increased gram-negative organisms was observed in the metronidazole group, but these changes were not statistically significant. Assessment of total enterococci on day 20 revealed significant recolonization with vancomycin-susceptible enterococci in the saline (*P* = .03), cefepime (*P* = .01), aztreonam (*P* = .03), metronidazole (*P* < .005), and ciprofloxacin (*P* = .049) treatment groups, but not in the oral vancomycin, ampicillin, or ampicillin-sulbactam groups.

Discussion

Case-control studies have shown an association between VRE colonization or infection and certain classes of antibiotics,
including vancomycin [13–16], third-generation cephalosporins [16–18, 22], and antibiotics with antianaerobic activity [19, 20]. To our knowledge, this is the first published study to directly compare the effects of different antibiotics on persistence and density of VRE intestinal colonization. A higher density of VRE fecal colonization may significantly increase the risk of transmission of VRE strains to the environment and to other patients [27]. In this mouse model of VRE intestinal colonization, subcutaneous vancomycin, clindamycin, piperacillin-tazobactam, ticarcillin-clavulanic acid, metronidazole, cefotetan, ampicillin, and ampicillin-sulbactam promoted persistent high-density VRE colonization. Cefepime, ceftiraxone, aztreonam, and ciprofloxacin promoted VRE to a lesser degree or not at all. Our results support the clinical studies that have shown an association between VRE colonization or infection and antibiotics with potent antianaerobic activity.

Antibiotics may influence VRE intestinal colonization directly by inhibiting VRE or indirectly by inhibiting other intestinal flora that compete with VRE, thereby promoting VRE overgrowth. It is not known whether inhibition of certain components of the competing bowel flora, such as anaerobes or gram-negative aerobes, results in greater promotion of VRE overgrowth than suppression of other components. Previous studies in mice and humans have suggested that the intestinal anaerobic flora is most important in providing colonization resistance against colonization of the bowel by potentially pathogenic microorganisms [5]. In addition to the intrinsic antimicrobial activity of antibiotics, factors such as the amount of biliary excretion and intestinal degradation of an antibiotic are important in determining the effect of an antibiotic on intestinal flora.

The results of this study suggest that antimicrobial activity against anaerobes is the most important factor for promoting persistent high-density VRE stool colonization in mice. Piperacillin-tazobactam, ticarcillin-clavulanic acid, clindamycin, metronidazole, ampicillin-sulbactam, and cefotetan all have potent

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Figure 3. Persistence and density of vancomycin-resistant Enterococcus faecium (VRE) intestinal colonization in mice treated with subcutaneous (sc) antibiotics with variable activity against anaerobes. All 74 mice were colonized with VRE on day 0 (mean, 9.5 log₁₀ cfu/g stool). Mice received sc injections of antibiotics, as shown in legend, every 12 h (no. of mice/group in parentheses). Negative control mice received sc saline; positive control mice received oral vancomycin. Stool VRE levels were quantified on days 5, 10, 15, and 20. For comparisons between groups, analysis of variance and linear regression were done. amp, ampicillin.
activity against anaerobes and resulted in sustained high levels of stool VRE. Ampicillin has activity against intestinal anaerobic species that do not produce β-lactamases and promoted high-density VRE colonization. Ceftriaxone has less-potent anti-aerobic activity than cefotetan and promoted increased VRE density to a lesser degree. Cefepime, aztreonam, and ciprofloxacin have minimal activity against anaerobes and affected VRE density minimally or not at all. Inhibition of aerobic gram-negative bacilli was neither necessary (clindamycin-treated mice had increased levels of aerobic gram-negative rods) nor sufficient (cefepime-, ciprofloxacin-, and aztreonam-treated mice had decreased levels of aerobic gram-negative rods) for the promotion of increased VRE density. Antimicrobial activity against vancomycin-susceptible enterococci was not necessary for the promotion of increased VRE density (clindamycin- and metronidazole-treated mice had significant regrowth of vancomycin-susceptible enterococci).

Unlike the other antibiotics that promoted stool VRE, vancomycin does not have in vitro activity against Bacteroides fragilis or other gram-negative anaerobes. Although Edlund et al. [28] reported that oral vancomycin treatment markedly decreased the level of Bacteroides species in human volunteers through unclear mechanisms, our study of oral vancomycin treatment of mice did not suppress Bacteroides organisms in stool (data not shown). Vancomycin has activity against gram-positive anaerobic organisms (e.g., clostridia) and aerobic gram-positive organisms. Antimicrobial activity against gram-positive anaerobic organisms is a common feature of all antibiotics in this study, which promoted high-density VRE colonization. Also, it should be noted that subcutaneous administration of vancomycin promoted intestinal VRE as much as oral vancomycin. In humans, intestinal levels of vancomycin are relatively low after intravenous administration (6–10 µg/mL), but elimination of Clostridium species and elimination or reduction of E. faecalis in feces have been demonstrated [29]. The level of vancomycin present in the intestinal tract of mice after parenteral administration is not known. The absence of regrowth of vancomycin-susceptible enterococci in mice treated with subcutaneous vancomycin suggests that the active drug was present in the intestinal tract.

Our hypothesis that piperacillin-tazobactam is associated with less clinical VRE isolation in the Cleveland area, because of its intrinsic activity against intestinal VRE, was not supported by this study. This may be because the model we used examined the effect of antibiotics on established VRE colonization rather than on initial development of colonization. After high levels of intestinal colonization have been established, promotion of VRE by inhibition of anaerobic flora may outweigh any direct suppressive effect of piperacillin-tazobactam on VRE itself. In humans, piperacillin achieves levels in bile (>1000 µg/mL after a 4-g intravenous dose [30]) that are above the MICs of many VRE isolates. In clinical settings, intestinal colonization may result from ingestion of small inocula of VRE. We hypothesize that these small inocula may be inhibited by the level of piperacillin achievable in the human small intestine, resulting in less VRE colonization in patients treated with this antibiotic. Further studies are planned, to determine whether administration of piperacillin will prevent establishment of VRE colonization in mice when small inocula are ingested.

In contrast to our results, several case-control studies have demonstrated an association between VRE colonization or infection and third-generation cephalosporins that lack potent in vitro activity against anaerobes [16–18]. This discrepancy may be due to differences between the model we used and the clinical setting or to differences between mouse and humans. Cephalosporins lacking potent activity against anaerobes may promote establishment of VRE colonization in clinical settings without causing high-density colonization. Fecal overgrowth with E. faecium in oncology patients, however, has been associated with preceding treatment with third-generation cephalosporins, including ceftriaxone, cefotaxime, cefazidime, and cefotaxime [6]. Some cephalosporins that lack potent anti-aerobic activity in vitro can inhibit intestinal anaerobes in humans, with significant variation between individual antibiotics [31]. Although many case-control studies have grouped third-generation cephalosporins together as a risk factor for VRE, these antibiotics may promote VRE to different degrees in humans, on the basis of their widely varying levels of biliary excretion and activity levels against anaerobes.

The applicability of this mouse model to human VRE colonization is potentially limited by several factors. Like human intestinal flora, rodent flora is predominantly anaerobic, but individual species differ significantly [32]. It is unclear whether these differences in flora affect the relationship between antibiotic therapy and VRE colonization. Also, although the strain of VRE used in these experiments is representative of the majority of VRE isolates in the Cleveland area, many outbreaks in the United States have been polyclonal, with a wide range of levels of resistance to various antibiotics. The effect of antibiotics on VRE colonization may vary for strains with differing levels of antibiotic resistance. In addition, we assessed the effect of antibiotics after establishing colonization with high levels of VRE. As noted previously, some antibiotics may influence the establishment of VRE colonization.

The pharmacokinetics of many antibiotics differ significantly in mice and humans. In mouse treatment models, more frequent and higher relative doses of antibiotics are required to achieve tissue levels similar to those in humans. In this model, we gave mice antibiotic doses that were equivalent to human doses on the basis of weight. Our goal was to approximate (on a milligram per kilogram basis) the daily quantity of antibiotic seen in the gastrointestinal tracts of humans. Percentage of biliary excretion of ticarcillin-clavulanic acid (SmithKline Beecham Pharmaceuticals, Van Nuys, CA) is similar for mice and humans; however, no data were available on biliary excretion of the remaining antibiotics in mice. After parenteral administra-
tion, the fraction of ceftiraxone that is present in the intestinal tract of mice and humans is similar [33]. The fraction of cefepime that is excreted into the intestinal tract of mice and humans also appears to be similar, although precise information about the concentration of cefepime in human feces is not known [33]. The demonstrated effect of several antibiotics on the mouse colonic flora suggests that significant levels of antibiotics were achieved in this study.

In conclusion, our data demonstrate that antibiotics with potent activity against anaerobes promote sustained high-density VRE stool colonization in mice. These findings suggest that once persons are colonized with VRE, administration of antibiotics with potent antianaerobic activity may promote sustained high-density colonization. Further research is necessary to identify the mechanisms by which antibiotics influence VRE intestinal colonization. If some classes of antibiotics promote VRE intestinal colonization more than other equally effective alternative antibiotics, then alteration of antimicrobial formulations may provide a means of limiting the spread of these organisms.

References


