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Effective Treatment of Cephalosporin-Rifampin Combinations against Cryptic Methicillin-Resistant β-Lactamase-Producing Coagulase-Negative Staphylococcal Experimental Endocarditis

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The efficacy of cefazolin or cefpirome alone or combined with rifampin was compared with that of vancomycin alone or combined with rifampin in an experimental model of methicillin-resistant, β-lactamase-producing, coagulase-negative staphylococcal endocarditis. Phenotypically, the *mecA* gene-positive strain used in vivo did not exhibit methicillin resistance by the agar dilution or disk susceptibility method but was resistant in vitro (oxacillin MIC, 64 μg/ml) by the microtiter dilution method with 2% NaCl supplementation. Macrodilution broth susceptibilities at standard inocula failed to demonstrate cross-resistance of staphylococci to cefazolin (MIC, 8 μg/ml) or cefpirome (MIC, 4 μg/ml). In vivo, vancomycin and cefpirome had similar activities, and both regimens were more effective than was cefazolin alone. While the MIC of rifampin was low (0.031 μg/ml), monotherapy with rifampin resulted in a bimodal distribution of outcomes due to the expected emergence of resistant mutants. The results in vitro of time-kill synergy studies using rifampin in combination with cefazolin or cefpirome varied with the antimicrobial concentrations tested and did not reliably predict activities in vivo of rifampin–beta-lactam combination therapies. Cefpirome, but not cefazolin or vancomycin, in combination with rifampin was synergistic in vivo. Cefpirome in combination with rifampin was more effective than was cefazolin in combination with rifampin. Both cephalosporin-rifampin regimens were significantly more effective than was cephalosporin or vancomycin monotherapy and were as effective as vancomycin combined with rifampin. These data support further evaluation of rifampin–beta-lactam combinations as possible alternative therapies to vancomycin-containing regimens for selected methicillin-resistant coagulase-negative staphylococcal infections.

Previous studies have shown that rifampin–beta-lactam combinations may be antagonistic in methicillin-susceptible (MS) *Staphylococcus aureus* experimental endocarditis (9, 40). However, for the treatment of serious infections with coagulase-negative staphylococci (CoNS), especially bacteremia and infections of intravascular devices, prosthetic device implants, or foreign bodies, semisynthetic β-lactamase-resistant penicillins or narrow-spectrum cephalosporins combined with rifampin and/or gentamicin are recommended. Because resistance to methicillin is common among CoNS, beta-lactam-containing regimens should be used in the therapy of CoNS infections only when MS can be demonstrated conclusively (2, 8).

A unique penicillin-binding protein (PBP), PBP 2a, with a low affinity for beta-lactams is probably the critical target in staphylococci that mediates methicillin resistance (MR) (37). Production of PBP 2a is inducible by beta-lactams in all MR strains of staphylococci in which the β-lactamase plasmid is present, even though MR is independent from β-lactamase-mediated resistance (11, 21, 22). While the *mecA* gene, which encodes PBP 2a, is uniformly present in the cells of MR strains and all cells within an MR strain can produce PBP 2a, the phenotypic expression of MR is typically heterogeneic, expressed in as few as 1 in 10^5 to 10^8 organisms (11, 21, 22, 39). Thus, most cells in heterogenous MR strains remain susceptible to low concentrations of beta-lactams mediated by binding to PBPs 1 to 3. Within the MR subpopulation, however, PBP 2a can substitute for the functions of other PBPs, even at antimicrobial concentrations which inactivate PBPs 1 to 3.

While the genotypic detection of *mecA*-mediated resistance is currently the most accurate method of detecting MR (4), this technique is unavailable to most clinical laboratories. Routine methods such as disk diffusion or agar dilution susceptibility testing appear to be satisfactory for detecting most strains of CoNS which are MR despite the heterogeneity of such cultures. However, detection of cryptic MR strains with routine susceptibility tests remains controversial because some resistant strains may not be detected by this method, which could result in suboptimal therapy (18, 23–25, 29, 48).

Because all MR CoNS are considered resistant to beta-lactams, including newer aminothiazolyl cephalosporins with enhanced antistaphylococcal activity such as cefpirome, vancomycin alone or in combination with rifampin and/or gentamicin has become the regimen of choice for the treatment of serious MR CoNS infections (2, 8). The use of the latter antimicrobial agents is more expensive and requires more frequent monitoring for more severe adverse or toxic effects (43). Although most strains of CoNS have remained susceptible to vancomycin (6, 20), widespread use of vancomycin-containing regimens may lead to increased resistance to vancomycin (10), and in recent years, reports on glycopeptide-resistant strains of CoNS have become more frequent (5, 15, 41, 42, 47).

Even though rifampin has long been recognized as an active antistaphylococcal agent with preserved activities against MR strains (6), the usefulness of monotherapy with rifampin is

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limited because of the rapid emergence of resistance. However, in contrast to *S. aureus*, the few published data on the efficacy of rifampin in combination with cephalosporins against MR strains of CoNS have not demonstrated antagonism in vivo (3, 26, 27).

The purpose of this study was to determine the activities in vitro and in vivo of cephalosporin-rifampin combinations by using a narrow-spectrum cephalosporin (cefazolin) or an aminothiazolyl cephalosporin (cefpirome) in cryptic MR CoNS experimental endocarditis and to compare the activities of the combination regimens and monotherapy with cefazolin, cefpirome, rifampin, or vancomycin alone or vancomycin combined with rifampin.

MATERIALS AND METHODS

Antimicrobial agents. Cefpirome sulfate was provided by Roussel Uclaf, Romainville, France, for in vitro and in vivo studies. Oxacillin sodium salt, cefazolin sodium, and vancomycin hydrochloride (Sigma Chemical Co., St. Louis, Mo.) were used for in vitro testing; pharmaceutical preparations used in vivo were purchased from Geneva Pharmaceuticals, Broomfield, Colo. (cefazolin sodium), and Eli Lilly & Co., Indianapolis, Ind. (vancomycin hydrochloride [Vancocin Hydrochloride]). Rifampin (Rifadin 6000 iv) for in vitro and in vivo studies was supplied by Merrel Dow Pharmaceuticals, Inc., Cincinnati, Ohio.

Organism. A β-lactamase-producing clinical isolate of CoNS with cryptic MR was selected for in vivo studies (see methods below).

In vitro susceptibility testing. Oxacillin was used for all phenotypic susceptibility tests, since oxacillin reportedly detects more resistant strains than does methicillin (32, 48). Agar dilution susceptibility testing using a replicator system and disk susceptibility testing with 1 μg of oxacillin per disk were performed without added NaCl according to National Committee for Clinical Laboratory Standards standards (32, 33). Broth dilution procedures were performed as microtiter dilutions in cation-supplemented Mueller-Hinton broth with 2% NaCl, an inoculum of 10^5 CFU/ml, and an incubation of 24 h at 35°C as recommended by the National Committee for Clinical Laboratory Standards (33). To detect the *mecA* gene, a multiplex PCR was used (18). A macrodilution broth method was used for determining the effects of inoculum sizes of 5×10^5 and 5×10^7 CFU/ml on the MICs of the four antimicrobial agents used in vivo (33). In vitro testing for β-lactamase production was performed using the nitrocefin disk test (Cefinase; Becton Dickinson & Co., Cockeysville, Md.).

In vitro tests for synergy. Time-kill synergy studies were performed by diluting an overnight culture of the strain studied in vivo to 10^5 to 10^6 CFU/ml in a total volume of 25 ml of Mueller-Hinton broth for each culture. For antimicrobial combination studies, recommended subinhibitory concentrations at standard inocula (1/4× MIC and 1/2× MIC) (1, 13) and also inhibitory (MIC) and suprainhibitory (2× MIC and 4× MIC) concentrations at standard inocula of the antimicrobial agents were used. After 0, 6, and 24 h of incubation at 35°C in room air, samples were removed from each culture and serially diluted. Tests were performed in triplicate, and the results were expressed as mean $\log_{10}$ CFU per milliliter (13).

In vivo studies. Catheter-associated aortic valve experimental infective endocarditis was established in New Zealand White rabbits (weight > 2.5 kg) by a modification of the method described by Garrison and Freedman (17) as described previously (44). Catheters remained in place for the duration of the experiment. Forty-eight hours after catheter placement, rabbits were infected through the arterial catheter with 5×10^8 CFU of staphylococci per ml. Antimicrobial therapy was initiated 48 h after infection and continued for 3 days. Rabbits that died before the first dose of treatment were excluded from the experiment. The antimicrobial dosages (in milligrams per kilogram of body weight) used were chosen to result in peak concentrations in serum in rabbits similar to those reported in humans receiving recommended therapeutic doses (35). For each antimicrobial agent, concentrations in the serum were assayed 30 min after the first dose on the second day of treatment in the respective monotherapy treatment groups. A bioassay technique was used for all antimicrobial assays (12).

Rabbits were randomly assigned to one of the following eight treatment groups: (i) as controls, 24 rabbits received no treatment; (ii) in the rifampin group, 13 rabbits received rifampin (5 mg/kg) subcutaneously twice daily; (iii) in the cefpirome group, 13 rabbits received cefpirome (40 mg/kg) subcutaneously three times daily; (iv) in the cefpirome-plus-rifampin group, 12 rabbits received cefpirome as described above and rifampin as described above; (v) in the cefazolin group, 13 rabbits received cefazolin (50 mg/kg) intramuscularly three times daily; (vi) in the cefazolin-plus-rifampin group, 12 rabbits received cefazolin as described above and rifampin as described above; (vii) in the vancomycin group, 13 rabbits received vancomycin (15 mg/kg) intravenously twice daily; and (viii) in the vancomycin-plus-rifampin group, 13 rabbits received vancomycin as described above and rifampin as described above.

Surviving animals were sacrificed at least 12 h after administration of the last dose of antimicrobial agents. Aortic valve vegetations were removed aseptically, and vegetations were weighed, homogenized, and cultured quantitatively by a pour plate method. Results were expressed as $\log_{10}$ CFU of staphylococci per gram of valve vegetation. Portions of homogenized valve vegetations were resuspended in Mueller-Hinton broth and by the agar dilution replicator system (33) screened for subpopulations of MR CoNS which had developed in vitro resistance to ≥2 μg of rifampin per ml during treatment with rifampin alone.

Statistical analysis of results. The overall null hypothesis that no differences existed among any of the treatment groups was analyzed with the Kruskal-Wallis test to estimate the per-experiment type I error rate. Individual pairwise comparisons were performed only if the preliminary Kruskal-Wallis test indicated significant differences among treatment groups at the $\alpha = 0.05$ level (36). The reported *P* values for individual treatment group comparisons therefore reflect the comparisonwise type 1 statistical error rate conditional on an experimentwise error rate of $P \leq 0.05$ (36). Differences between pairwise comparisons were considered significant at the $\alpha = 0.05$ level. Twenty-eight different pairwise comparisons between treatment groups were performed with the Wilcoxon rank sum test. If Bonferroni correction for multiple comparisons is applied, the corrected significance level is $\alpha = 0.001786$. Applying the Bonferroni correction reduces type I error but increases type II error. A minimum of 12 animals per treatment arm provided an a priori power of ≥80% to detect differences in means among treatment groups of ≥1.2 standard deviations.

TABLE 1. In vitro susceptibilities of the CoNS strain studied in vivo

Antimicrobial agent	MIC (μg/ml) at:	
	Standard inoculum (10^5 CFU/ml)	High inoculum (10^7 CFU/ml)
Cefazolin	8	64
Cefpirome	4	16
Vancomycin	2	8
Rifampin	0.031	0.062

RESULTS

In vitro studies. Neither the agar dilution nor the disk susceptibility test detected MR (MIC, ≤2 μg of oxacillin per ml). By the microdilution method with 2% NaCl, the MIC of oxacillin for the strain studied in vivo was 64 μg/ml. MICs of the agents used in vivo are shown in Table 1. Comparing standard and high inoculum sizes, a greater inoculum effect was noted for cefazolin (eightfold) than for cefpirome (fourfold). The nitrocefin disk test revealed β-lactamase production in the strain studied in vivo. Results of time-kill studies of the strain used in experimental endocarditis for cefpirome-rifampin combinations are shown in Fig. 1. Similar results were obtained for cefazolin combined with rifampin. Occasional regrowth of cultures containing only rifampin at concentrations at or above the MIC was attributed to rifampin-resistant mutants (31). These overgrown cultures were excluded from the evaluation of in vitro susceptibility.

In vivo studies. The concentrations in serum of antimicrobial agents measured 30 min after administration of the first dose on the second day of treatment were as follows: 156 mg of cefazolin per liter, 61 mg of cefpirome per liter, 3.3 mg of rifampin per liter, and 48 mg of vancomycin per liter. Table 2 shows the results of treatment of experimental endocarditis in rabbits. Preliminary overall analysis revealed a significant difference among treatment groups. *P* values for the individual pairwise comparisons among treatment groups are given in Table 3. All treatment regimens were significantly more effective than no treatment. Cefpirome ($P < 0.000030$) or vancomycin ($P < 0.001719$) was more effective than was cefazolin, and both cephalosporin-rifampin combinations were significantly more effective (cefazolin-rifampin, $P = 0.000999$; cefpirome-rifampin, $P = 0.000064$) than was vancomycin alone. The combination of cefpirome and rifampin was synergistic in vivo

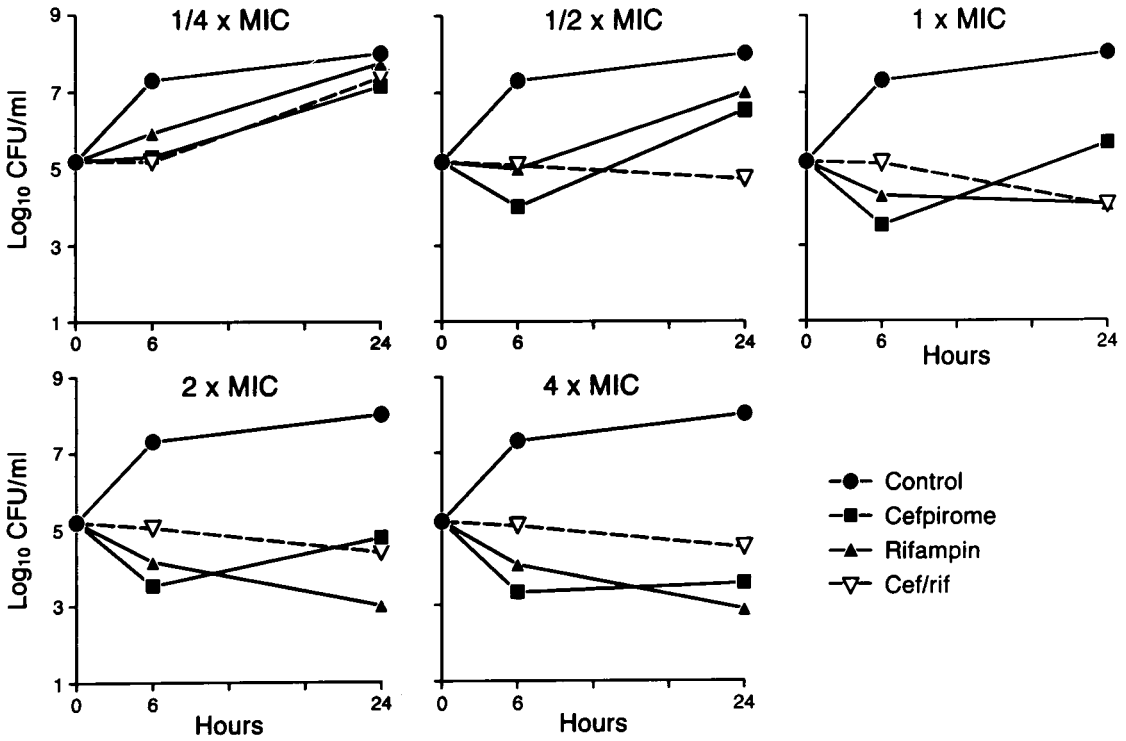

FIG. 1. Results of time-kill synergy studies of cefpirome with rifampin at concentrations lower than, equal to, or greater than the MICs as assessed at an inoculum of 10^5 CFU/ml. The inoculum density used in the time-kill studies was 10^5 to 10^6 CFU of the CoNS strain studied in vivo per ml. Cef/rif, cefpirome plus rifampin.

(cefpirome, $P = 0.000025$; rifampin, $P = 0.01657$) and more effective ($P = 0.04638$) than cefazolin plus rifampin.

In the 12 surviving animals treated with rifampin alone, rifampin-resistant (MIC, ≥4 μg/ml) strains were recovered from 7 animals and an intermediately rifampin-resistant strain (MIC, 2 μg/ml) was recovered from 1 animal. Detection of rifampin-resistant subpopulations of CoNS corresponded with high colony counts in cardiac vegetations among the bimodal distribution of all animals treated with rifampin alone.

DISCUSSION

In CoNS, factors which enhance phenotypic expression of MR, such as NaCl, prolonged incubation, or high inocula, seem to affect primarily the MICs for MR but not MS strains (22, 25, 45), although these factors may enhance β-lactamase production and may cause susceptible strains of *S. aureus* to appear borderline or falsely resistant (22). Results in vitro with our strain studied in vivo are consistent with previous findings (25) that for the detection of MR subpopulations in CoNS (which are even fewer in MR CoNS than in MR *S. aureus* [39]), the microdilution method with 2% NaCl supplementation as recommended by the National Committee for Clinical Laboratory Standards (33) is more reliable than either the disk diffusion or agar dilution method.

TABLE 2. Results of treatment of experimental endocarditis in rabbits

Treatment	No. of rabbits that died/no. treated	Vegetation (log_{10} CFU/g)	
		Median	Range, 25th–75th percentile
None	3/24	10.57	10.03–10.73
Cefazolin	1/13	9.95	9.95–10.05
Vancomycin	0/13	8.82	7.70–8.87
Cefpirome	0/13	8.57	8.30–8.94
Rifampin	1/13	7.03	3.40–8.41
Cefazolin + rifampin	0/12	3.77	2.76–6.88
Vancomycin + rifampin	2/13	3.58	3.05–6.04
Cefpirome + rifampin	0/12	2.95	2.57–3.31

Although the susceptibility tests with standard inocula suggested that the MR strain used in vivo was susceptible to cefazolin or cefpirome, at higher inocula resistance to cefazolin and cefpirome corresponded with the rather minimal effect of cephalosporin monotherapy in our study compared with the reported effectiveness of beta-lactam monotherapy in susceptible strains of CoNS (7). Previous studies (3, 30, 46) have demonstrated cross-resistance in vivo of many beta-lactams with methicillin for MR CoNS, although a previous retrospective clinical study (26) reported a higher cure rate among patients receiving beta-lactam monotherapy for MR CoNS endocarditis than expected. The superior efficacy of cefpirome compared with that of cefazolin in this study may be due to differences in the affinity for cephalosporinase and the rate of hydrolysis (19) or to differences in the affinity or induction of PBP 2a in the MR subpopulation (34) or in the affinity for PBPs 1 to 3 in the MS subpopulation (11).

Although rifampin therapy was more effective than was either cefazolin or cefpirome in our study, rifampin alone was no more effective than was vancomycin. Emergence of rifampin resistance was common (7 of 12 animals), even though the treatment period of 3 days was relatively short. Our results are consistent with those of previous studies reporting the emergence of rifampin-resistant strains of CoNS during 3 or 7 days of therapy with rifampin alone in experimental endocarditis (3, 38, 46). One study (46), however, did not find the emergence of rifampin-resistant strains after only 2 days of treatment and

TABLE 3. Results of pairwise comparisons among treatment groups

Treatment	*P* value[a] of:						
	Cefpirome + rifampin	Vancomycin + rifampin	Cefazolin + rifampin	Rifampin	Cefpirome	Vancomycin	Cefazolin
None	0.000003*	0.000005*	0.000003*	0.000092*	0.000003*	0.000008*	0.008136
Cefazolin	0.000035*	0.000053*	0.000035*	0.000573*	0.000030*	0.001719*	
Vancomycin	0.000064*	0.000262*	0.000999*	0.078	1		
Cefpirome	0.000025*	0.000039*	0.000195*	0.008338			
Rifampin	0.01657	0.131588	0.340779				
Cefazolin + rifampin	0.04638	0.6891					
Vancomycin + rifampin	0.06943						

[a] Asterisks indicate P values that remained significant after the Bonferroni correction for 28 different comparisons.

reported a superior efficacy of monotherapy with rifampin compared with that of monotherapy with vancomycin or gentamicin.

Monotherapy with vancomycin was no more effective than was cefpirome in our study. The increased activity in vivo of the combination of vancomycin and rifampin compared with that of vancomycin monotherapy is in agreement with results of previous studies, which demonstrated that the combination of vancomycin with rifampin and/or gentamicin in experimental MR CoNS endocarditis was more effective than monotherapy with vancomycin (16, 28). Other studies have reported that the triple combination of vancomycin, rifampin, and gentamicin was not significantly more effective than was therapy with vancomycin plus rifampin alone for MR CoNS experimental endocarditis (16, 30, 38).

Results of in vitro synergy studies for rifampin-cephalosporin combinations against the study strain varied with the concentrations of both agents, similar to results previously observed with *S. aureus* (9, 49). Although for *S. aureus*, rifampin–beta-lactam combinations with suprainhibitory concentrations were reported to be a better indicator of treatment outcome than were those with subinhibitory concentrations (9), for CoNS in the present study, in vitro results with suprainhibitory concentrations did not correspond with in vivo outcome and caution should be exercised when interpreting the interaction in vitro of rifampin-cephalosporin regimens against CoNS.

In contrast to results of previous studies (3), in our study, both cephalosporin-rifampin combination regimens were significantly more effective than was the respective cephalosporin monotherapy. Although rifampin alone is not recommended for the treatment of staphylococcal infections because of the rapid emergence of resistant strains, the addition of rifampin to a beta-lactam may render MR strains more susceptible to beta-lactams by decreasing the production of PBP 2a. Thus, the observed increased synergistic activity of cefpirome and rifampin compared with that of cefazolin and rifampin may be due to the better antistaphylococcal activity of cefpirome rather than to the different ability to prevent emergence of rifampin resistance as previously described in vitro for different cell wall-active antimicrobial agents for *S. aureus* (14). Previous studies in our laboratory, however, demonstrated antagonism with the same rifampin-cephalosporin combinations in vivo in *S. aureus* experimental endocarditis (9). Therefore, findings from studies against *S. aureus* with rifampin-cephalosporin combinations cannot be easily generalized to CoNS infections, and further studies are necessary to evaluate strain-specific effects on rifampin–beta-lactam therapy against staphylococci.

Because rifampin–beta-lactam combinations were more effective than was vancomycin monotherapy and as effective as combination therapy of vancomycin and rifampin against MR CoNS in this study, our data contrast with those from retrospective clinical studies (26, 27) which observed that cure rates only of those patients with MR-CoNS infections treated with vancomycin, but not with beta-lactams, were enhanced by the addition of rifampin to therapy. Moreover, since cryptic MR of CoNS may be difficult to detect with routine susceptibility tests, rifampin–beta-lactam combinations deserve further evaluation as a possible treatment regimen for CoNS infections when in vitro MS of the organism cannot be ensured.

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