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# Synergistic Effect of Amoxicillin and Cefotaxime against *Enterococcus faecalis*

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**The antibacterial efficacy of the combination of amoxicillin and cefotaxime was assessed against 50 clinical strains of *Enterococcus faecalis*. For 48 of 50 strains, the MIC of amoxicillin that inhibited 50% of isolates tested decreased from 0.5 μg/ml (range, 0.25 to 1 μg/ml) to 0.06 μg/ml (range, 0.01 to 0.25 μg/ml) in the presence of only 4 μg of cefotaxime per ml. Alternatively, the MIC of cefotaxime that inhibited 50% of isolates tested decreased from 256 μg/ml (range, 8 to 512 μg/ml) to 1 μg/ml (range, 0.5 to 16 μg/ml) in the presence of only 0.06 μg of amoxicillin per ml. For JH2-2, a reference strain of *E. faecalis*, the MICs of amoxicillin, cefotaxime, and amoxicillin in the presence of cefotaxime (4 μg/ml) were 0.5, 512, and 0.06 μg/ml, respectively. By using a penicillin-binding protein (PBP) competition assay, it was shown that with cefotaxime, 50% saturation of PBPs 2 and 3 was obtained at very low concentrations (<1 μg/ml), while 50% saturation of PBPs 1, 4, and 5 was obtained with ≥128 μg/ml. With amoxicillin, 50% saturation of PBPs 4 and 5 was obtained at 0.12 and 0.5 μg/ml, respectively. Therefore, the partial saturation of PBPs 4 and 5 by amoxicillin combined with the total saturation of PBPs 2 and 3 by cefotaxime could be responsible for the observed synergy between these two compounds.**

Enterococci are isolated with increasing frequency from a wide variety of nosocomial infections (7, 15). Epidemics with multiply resistant strains of *Enterococcus faecalis* and *Enterococcus faecium* have recently been reported in the United States (10, 11, 19). Few antibiotics remain effective against *E. faecalis*, such as amoxicillin, piperacillin, and imipenem, to which *E. faecalis* is only moderately susceptible compared with the susceptibilities of streptococci. Glycopeptides are recommended as the drugs of choice when a significant penicillin allergy or when infections caused by high-level ampicillin- and penicillin-resistant strains are to be treated. However, an increasing rate of glycopeptide resistance in U.S. hospitals has been reported (4, 9, 14, 16).

To extend the spectrum of activity, amoxicillin plus cefotaxime might be an alternative to other antibiotic combinations for the treatment of mixed infections containing enterococci and gram-negative bacteria. Thus, it was of interest to study the in vitro effect of the combination of cefotaxime with amoxicillin against *E. faecalis*.

## MATERIALS AND METHODS

**Bacterial strains.** Fifty recent clinical isolates of *E. faecalis*, EF 6370, a tolerant strain, and JH2-2, a reference strain (13), were studied. The bacteria were identified by usual procedures (6). Strains were cultivated at 37°C in Mueller-Hinton broth or in brain heart infusion broth (Difco Laboratories, Detroit, Mich.).

**Antibiotics.** Cefotaxime was provided by Roussel Uclaf (Romainville, France), amoxicillin was provided by SmithKline Beecham (Paris, France), gentamicin was provided by Schering-Plough (Levallois-Perret, France), and benzylpenicillin was provided by Laboratoire Roussel-Diamant (Romainville, France). [$^3$H]benzylpenicillin (0.66 TBq/mmol) was synthesized at the Service des Molecules Marquées, Commissariat à l'Energie Atomique (Gif-sur-Yvette, France) and was generously provided by Rhône-Poulenc Recherche.

**Antibiotic synergy testing.** Potentiation between amoxicillin and cefotaxime was investigated qualitatively by the disk diffusion method on brain heart infusion agar with disks containing 25 μg of amoxicillin and 30 μg of cefotaxime.

**MIC and FIC determinations.** For the clinical strains, MICs were determined on Mueller-Hinton agar containing antibiotics serially diluted twofold. Plates were inoculated with a Steers-type device ($10^4$ CFU per spot) and were incubated at 37°C for 18 h. The MICs of amoxicillin were determined alone and in association with a fixed concentration of 4 μg of cefotaxime per ml. The MICs of cefotaxime were determined alone and in association with a fixed concentration of 0.06 μg of amoxicillin per ml. For strains JH2-2 and EF 6370, the MICs of amoxicillin, cefotaxime, and the combination were similarly determined in brain heart infusion broth. The fractional inhibitory concentration (FIC) index was determined for strain JH2-2 by a checkerboard method.

**Bactericidal effects of antibiotics.** For strains JH2-2 and EF 6370, the bactericidal effect of amoxicillin, either alone or combined with a fixed concentration of cefotaxime (4 μg/ml), was determined by the tube macrodilution method in brain heart infusion broth at $10^8$ and $10^7$ CFU/ml, respectively; each tube was incubated for 24 h at 37°C, and then 0.1 ml from each tube was diluted and plated onto brain heart infusion agar and the numbers of CFU were counted after 24 h of incubation at 37°C.

**Analysis of PBPs of JH2-2.** Bacteria were grown without shaking in brain heart infusion broth at 37°C. One milliliter of an exponential-phase culture (optical density at 650 nm, 0.55) was rapidly chilled on ice, centrifuged (4,000 × *g* for 10 min) at 4°C, and resuspended in 30 μl of ice-cold 50 mM sodium phosphate buffer. Competition assays were carried out as follows. The samples (30 μl) were first incubated for 20 min at 37°C with 10 μl of various concentrations of nonradioactive amoxicillin or cefotaxime to obtain concentrations of 0.03 to 2 μg/ml and 1 to 1,024 μg/ml, respectively. When the drugs were combined, a fixed final concentration of 4 μg of cefotaxime per ml was used with concentrations of amoxicillin of 0.06 or 0.12 μg/ml. Finally, 10 μl of [$^3$H]benzylpenicillin at a final concentration of 20 μg/ml was added. Samples were incubated for an additional 20 min, and then unlabelled benzylpenicillin (2,000 μg/ml) was added. Lysis was obtained after resuspension in 20 μl of phosphate buffer containing 10 μg of lysozyme, 10 μg of M1-muramidase, and 0.1% (wt/vol) Triton X-100. Analysis of penicillin-binding proteins (PBPs) was performed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (18). The exposure times of the fluorograms were 4 to 10 days. The intensities of the bands were determined by scanning densitometry (Helen Laboratories, Beaumont, Tex.).

## RESULTS

**Antibiotic synergy testing.** As shown as an example in Fig. 1, the disk diffusion assay revealed a bacteriostatic synergistic effect between amoxicillin and cefotaxime. This was confirmed by MIC testing. For 48 of the 50 strains, the MIC of amoxicillin decreased from 0.25 to 1 μg/ml to 0.01 to 0.25 μg/ml in the

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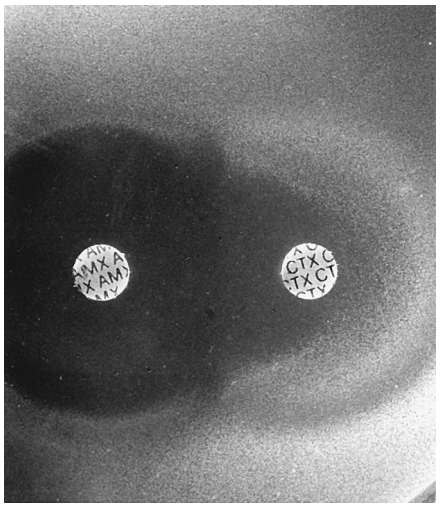

FIG. 1. Synergistic effect between amoxicillin and cefotaxime against JH2-2 on brain heart infusion agar. AMX, amoxicillin; CTX, cefotaxime.

presence of only 4 μg of cefotaxime per ml (Fig. 2A). Alternatively, the MIC of cefotaxime decreased from 8 to 512 μg/ml to 0.5 to 16 μg/ml in the presence of only 0.06 μg of amoxicillin per ml (Fig. 2B). For the last two strains, for which amoxicillin and cefotaxime MICs were 2 and 512 μg/ml, respectively, only

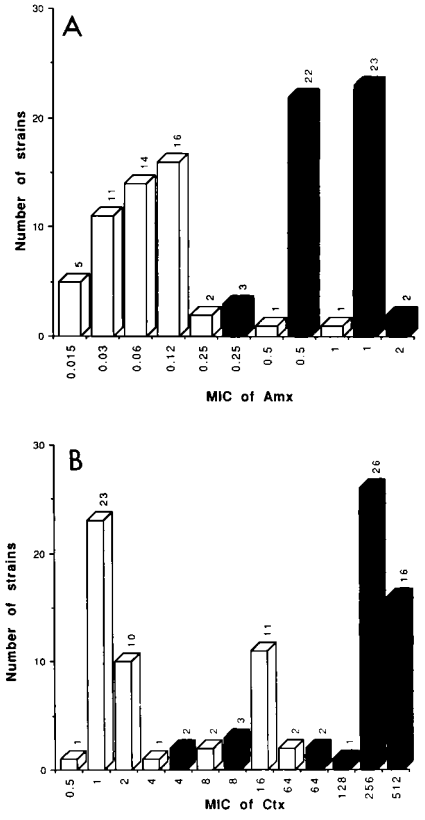


FIG. 2. (A) MICs of amoxicillin (Amx) alone (■) or in combination with a fixed concentration (4 μg/ml) of cefotaxime (□). (B) MICs of cefotaxime (Ctx) alone (■) or in combination with a fixed concentration (0.06 μg/ml) of amoxicillin (□).

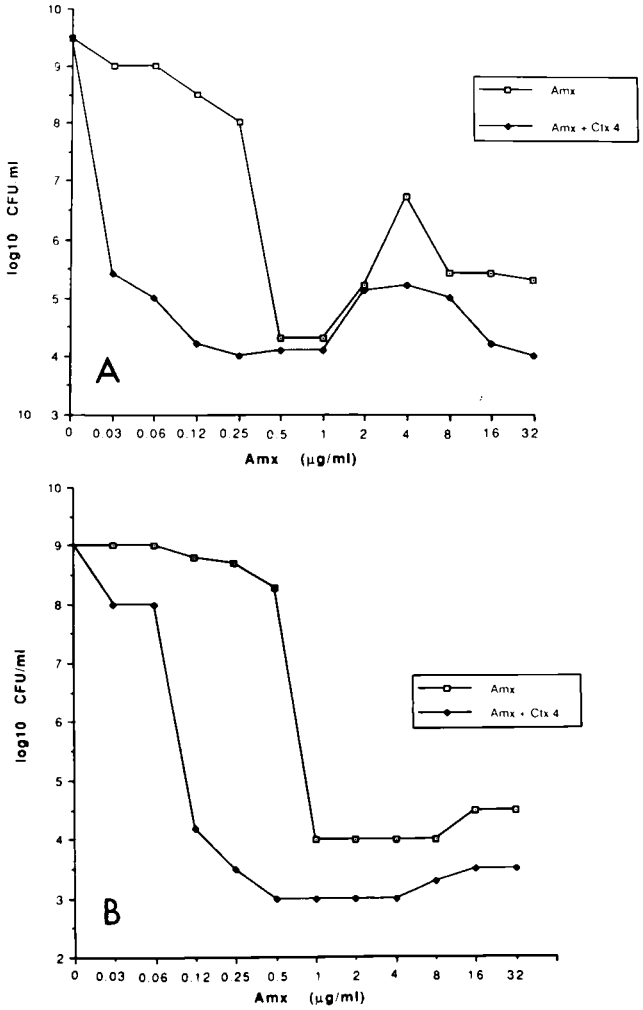


FIG. 3. (A) Bactericidal effect at 24 h of amoxicillin alone (Amx) or combined with a fixed concentration (4 μg/ml) of cefotaxime (Amx + Ctx 4) against JH2-2. (B) Bactericidal effect at 24 h of amoxicillin alone (Amx) or combined with a fixed concentration (4 μg/ml) of cefotaxime (Amx + Ctx 4) against EF 6370.

a one- to twofold dilution reduction of the MICs of amoxicillin and cefotaxime was observed.

**MIC, FIC index, and bactericidal effects of antibiotics for *E. faecalis* JH2-2 and EF 6370.** The MICs of amoxicillin alone, cefotaxime alone, and amoxicillin in the presence of 4 μg of cefotaxime per ml for strain JH2-2 were 0.5, 512, and 0.06 μg/ml, respectively, and those for strain EF 6370 were 1, 512, and 0.12 μg/ml, respectively. For JH2-2, the FIC index was 0.13. For amoxicillin alone at concentrations of 0.5 and 1 μg/ml, a marked bactericidal effect (>3 logs) was noted for JH2-2 after 24 h (Fig. 3A). Therefore, this strain was considered not tolerant to β-lactams. However, at concentrations of amoxicillin of ≥2 μg/ml, an Eagle effect was observed, as has been described previously for many *E. faecalis* isolates (8). A synergistic bactericidal activity was observed between cefotaxime, which was used at 4 μg/ml, and amoxicillin at concentrations ranging from 0.03 to 0.25 μg/ml. Neither cefotaxime nor amoxicillin alone, in this range of MICs, had an effect on the growth curve at 4 or 24 h relative to growth in medium alone (data not shown). For EF 6370, a reduction of ≤3 logs was observed with amoxicillin alone (Fig. 3B). This strain was

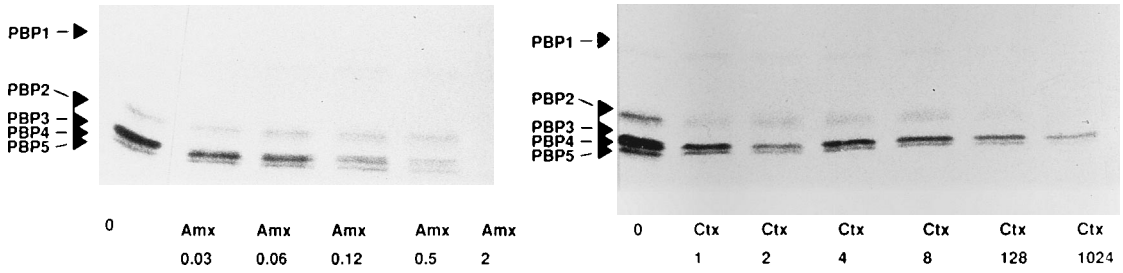


FIG. 4. Saturation of PBPs of JH2-2 by amoxicillin or cefotaxime. Competition experiments with radioactive benzylpenicillin were carried out with increasing concentrations (in micrograms per milliliter) of amoxicillin or cefotaxime. Amx, amoxicillin; Ctx, cefotaxime.

therefore considered relatively tolerant to β-lactams. Nevertheless, similar to what was observed with JH2-2, a synergistic bactericidal effect was observed when cefotaxime (4 μg/ml) was combined with amoxicillin over a range of concentrations (0.12 to 0.5 μg/ml).

**Analysis of PBPs of strain JH2-2.** Saturation (50%) of PBPs 2 and 3 with cefotaxime was obtained at concentrations of less than 1 μg/ml, while 50% saturation of PBPs 1, 4, and 5 was obtained at concentrations of 128 μg/ml or greater (Table 1 and Fig. 4). With amoxicillin, 50% saturation of PBPs 4 and 5 was obtained at 0.12 and 0.5 μg/ml, respectively (Table 1 and Fig. 4). When the combination was tested, as expected from the competition experiments, a partial saturation of PBPs 4 and 5 (25%) with 0.06 μg of amoxicillin per ml and the total saturation of PBPs 2 and 3 with 4 μg of cefotaxime per ml were observed (Fig. 5).

## DISCUSSION

This is the first report of a study demonstrating a synergistic effect of amoxicillin and cefotaxime against clinical strains of *E. faecalis*. The synergy was found for low concentrations of each antibiotic, particularly of cefotaxime, regardless of the susceptibility of the *E. faecalis* isolate to aminoglycosides (2 of 50 strains were gentamicin resistant).

The PBP patterns of strain JH2-2 were identical to those found in previous studies (17, 18). Considering the MIC of cefotaxime (512 μg/ml), only PBPs 1, 4, and 5 had high 50% saturation values (≥128 μg/ml), while PBPs 2 and 3 were saturated at very low concentrations. The MICs of amoxicillin correlated better with the saturation of PBPs 4 and 5. Therefore, we infer that PBPs 4 and 5 are the most likely candidates for essential PBPs, confirming that the low-molecular-weight PBPs in *E. faecalis*, as in other *Enterococcus* spp., play this role (1, 12, 17).

The synergy between amoxicillin and cefotaxime could be explained, at least for strain JH2-2, by the partial saturation of PBPs 4 and 5 by amoxicillin at 0.06 μg/ml combined with the total saturation of PBPs 2 and 3 by cefotaxime at 4 μg/ml. This would suggest that if PBPs 2 and 3 are not essential targets, they might participate in building the cell wall, particularly when the low-molecular-weight PBPs begin to be inactivated, which could be the case when the latter are partially saturated with amoxicillin.

Little is known about the detailed functions of the PBPs of *E. faecalis*. It has been shown that resistant mutants selected in vitro expressed increased quantities of PBPs 1, 4, and 5 (1, 12). For *Enterococcus hirae*, it was demonstrated that a balance exists between PBPs 1, 2, 3, and 5 as far as the physiology of cell growth is concerned (3). By analogy, one can hypothesize that a similar balance exists in *E. faecalis* between high-molecular-weight PBPs 2 and 3 and low-molecular-weight PBPs 4 and 5, as reflected by the synergy that was observed. In contrast to what was observed for *E. faecium*, for which complete saturation of the low-molecular-weight PBP 5 was necessary to obtain lysis (2), it would appear that in *E. faecalis*, partial saturation of PBP 4 or 5 in association with total saturation of PBPs 2 and 3 would be sufficient to cause lysis.

To test if such a synergistic effect could exist with *E. faecium*, preliminary experiments were done with three strains of *E. faecium* for which the cefotaxime MIC was 512 μg/ml and the amoxicillin MICs were 4, 16, and 64 μg/ml, respectively. No bacteriostatic synergistic effect of the combination even at one-quarter of the MICs of the respective compounds was observed. Thus, the synergistic effect observed with *E. faecalis* might not hold true for *E. faecium*. The reason for such a difference is not known at present, but it could relate to the relative difference between the PBPs of *E. faecalis* and *E. faecium* and, possibly, to the different interactions of these

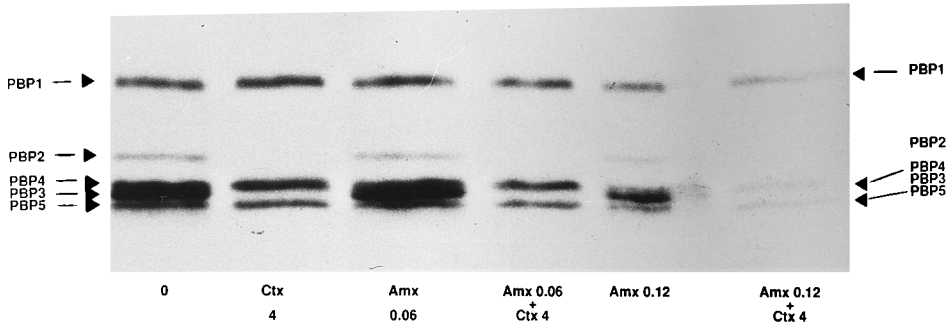


FIG. 5. Saturation of PBPs of JH2-2 with amoxicillin (0.06 and 0.12 μg/ml) and cefotaxime (4 μg/ml) alone or in combination. Radioactive benzylpenicillin was used. On this gel, which was run at 4°C, PBP 4 has migrated above PBP 3. Amx, amoxicillin; Ctx, cefotaxime.

TABLE 1. Fifty percent saturation of PBPs of strain JH2-2 with cefotaxime and amoxicillin

| PBP | Concn μg/ml | |
|---|---|---|
| | Cefotaxime | Amoxicillin |
| 1 | >128 | 1 |
| 2 | <1 | 1 |
| 3 | <1 | 1 |
| 4 | >128 | 0.12 |
| 5 | 128 | 0.5 |
| MIC | 512 | 0.5 |

PBPs with the various compounds as well as their natural substrate (peptidoglycan precursor).

In conclusion, as far as *E. faecalis* is concerned, the enhanced activity of combining amoxicillin with cefotaxime, thereby decreasing the MIC of each antibiotic, might be of interest in the clinical situation in which borderline concentrations of either compound are usually achieved at the site of infection. This is the case for amoxicillin concentrations in the prostate or heart valve vegetations. By decreasing the MICs, a much longer time of coverage above the MIC will be obtained, and this is expected to be linked to a better clinical outcome (5). A similar argument could apply for highly aminoglycoside-resistant strains. These results suggest that further animal and clinical studies are warranted.

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