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Synergy Characterization for *Enterococcus faecalis* Strains Displaying Moderately High-Level Gentamicin and Streptomycin Resistance

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Synergy of 14 *Enterococcus faecalis* strains displaying moderately high-level aminoglycoside resistance (MICs, 500 and 256 to 1,000 μg/ml for gentamicin and streptomycin, respectively) was characterized by time-kill studies. All strains proved resistant to penicillin plus the respective aminoglycoside. Strains with moderately high-level aminoglycoside resistance should be considered to exhibit high-level resistance in severe infections.

Aminoglycoside and penicillin combinations exhibit bactericidal synergy against enterococci (4), and they are recommended as the therapy of choice in severe enterococcal infections (3). However, this synergistic antienterococcal activity is abolished in the presence of high-level aminoglycoside resistance (i.e., resistant to ≥2,000 μg/ml) (10).

Several methods to determine penicillin-aminoglycoside synergy resistance in *Enterococcus faecalis*, by high-level aminoglycoside resistance detection, have been proposed as routine screening tests, including agar dilution (9), broth dilution (11), and agar disk diffusion (2). Although it is generally assumed that a 2,000-μg/ml and a 500- or 2,000-μg/ml single concentration of streptomycin (STR) and gentamicin (GEN), respectively, are reliable to detect high-level resistance (10, 11), rare strains for which aminoglycoside MICs are moderately high (512 and 2,56 to 1,024 μg/ml for GEN and STR, respectively) have been described (2).

The present study was undertaken to detect *E. faecalis* strains displaying moderately high-level resistance (MHLR) to STR and GEN and to characterize their synergy by time-kill studies. In addition, a single concentration (64 and 128 μg/ml for GEN and STR, respectively) agar screening test to determine presumptive penicillin-aminoglycoside synergy was evaluated.

Two hundred and nineteen unique consecutive clinical isolates of *E. faecalis* were identified according to the conventional test scheme recommended by Facklam et al. (1) and Ruoff et al. (8).

MIC determinations of penicillin G (PEN), vancomycin, STR (Lepetit S.A., Buenos Aires, Argentina), and GEN (Schering-Plough S.A., Buenos Aires, Argentina) were performed (twice) for all 219 isolates by a dilution method with Mueller-Hinton agar (Laboratorios Britania S.A., Buenos Aires, Argentina), following National Committee for Clinical Laboratory Standards recommendations (6).

To perform the single-concentration agar screening test, STR and GEN were incorporated into Mueller-Hinton agar to final concentrations of 128 and 64 μg/ml, respectively. Plates were inoculated with each isolate in the log phase, in order to reach a final plate inoculum of roughly 10^6 CFU. Strains showing any evidence of growth at such concentrations were assumed to be resistant.

Media lacking antibiotic supplement were used as positive growth controls. All plates were incubated for 18 to 24 h in an ambient atmosphere at 35°C. *E. faecalis* ATCC 29212 was used as the control in all susceptibility tests performed.

Time-kill studies were carried out with Mueller-Hinton broth (Laboratorios Britania S.A., Buenos Aires, Argentina) by a method based on Sahm and Torres' procedure (9). Briefly, drug concentrations were as follows: STR, 25 μg/ml; GEN, 5 μg/ml; and PEN, 10 U/ml. Each tube was inoculated with the organism in the log phase to reach a final viable bacterial concentration of roughly 5×10^6 CFU/ml. Inoculated broths were incubated in an ambient atmosphere at 35°C. At zero hour and at 4- and 24-h intervals after inoculation, a 0.1-ml portion was removed from each tube and diluted, and 0.1 ml of diluent was plated on Trypticase soy agar supplemented with 5% sheep blood. By using the viable counts determined at each interval, a 24-h time-kill curve was established for each strain. Susceptibility to penicillin-aminoglycoside synergy (positive synergy) was defined as a ≥100-fold increase in killing by the drug combination over that accomplished by the more active of the two antibiotics when tested separately. Resistance to synergy (negative synergy) was taken as a <100-fold increase in killing (4). All time-kill curves were performed twice.

In all, 14 strains displaying MHLR to aminoglycosides (11 for STR and 3 for GEN) were found. STR MICs for strains showing MHLR to STR were 256 μg/ml (three strains), 500 μg/ml (four strains), and 1,000 μg/ml (four strains). The GEN MIC for all three strains with MHLR to GEN was 500 μg/ml.

To characterize penicillin-aminoglycoside synergy by time-kill studies in the above-described 14 strains with MHLR, another 32 were selected as controls for low-level resistance (LLR) (MICs, ≤64 and ≤128 μg/ml for GEN and STR, respectively) or high-level resistance (HLR) (MICs, >500 and >2,000 μg/ml for GEN and STR, respectively). The 46 strains were grouped as follows: group I, 7 strains with LLR to both STR and GEN; group II, 11 strains with HLR to both STR and GEN; group IIIa, 6 strains with MHLR to STR (MIC, 256 to 1,000 μg/ml) and LLR to GEN;

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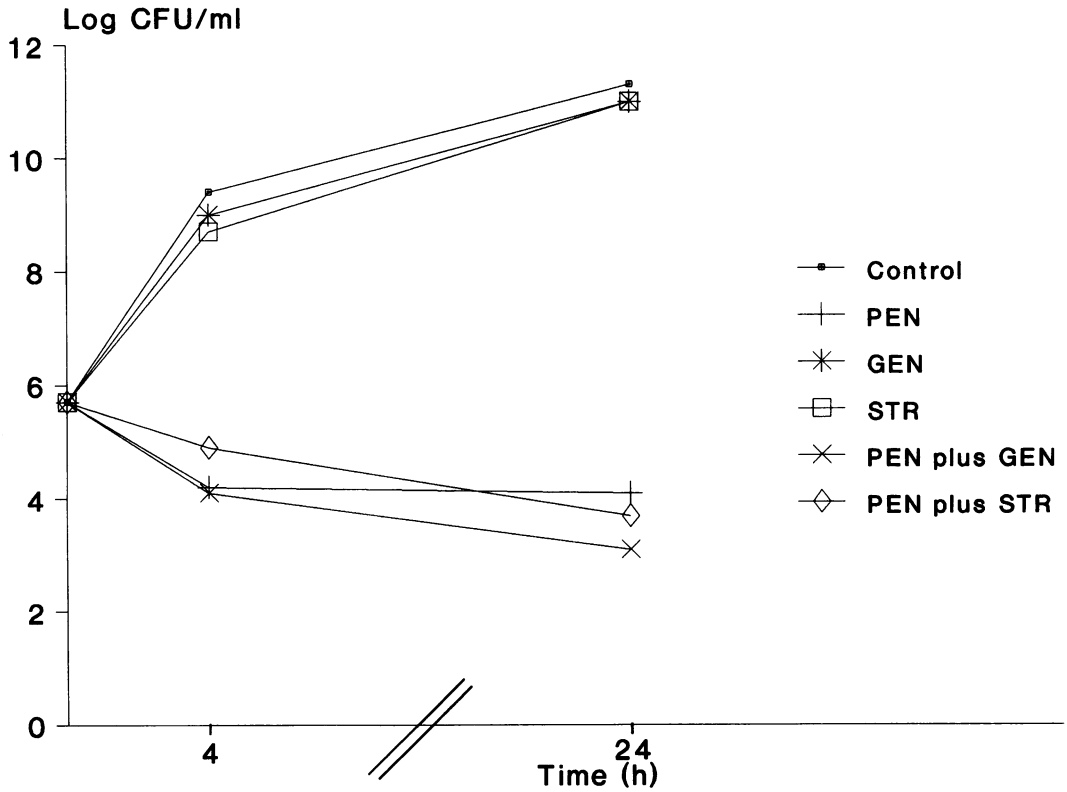

FIG. 1. Time-kill curve of an *E. faecalis* strain displaying MHLR to GEN (MIC, 500 μg/ml) and HLR to STR (MIC, 10,000 μg/ml).

group IIIb, 5 strains with MHLR to STR and HLR to GEN; group IV, 3 strains with LLR to STR and HLR to GEN; group V, 3 strains with MHLR to GEN (MIC, 500 μg/ml) and HLR to STR; and group VI, 11 strains with LLR to GEN and HLR to STR.

Both STR and GEN exhibited positive synergy and negative synergy against all strains showing LLR and HLR, respectively. All strains displaying MHLR to either STR or GEN were resistant to bactericidal synergic action by PEN plus the respective aminoglycoside, with agreement between duplicate determinations. No cross-resistance to synergy was observed in group IIIa, IV, or VI with GEN and STR. Illustrative time-kill curves for two strains displaying MHLR to GEN and STR are given in Fig. 1 and 2, respectively.

PEN MICs for 90% of the 46 selected strains were 2 μg/ml (range, 0.5 to 4 μg/ml). Vancomycin MICs were ≤2 μg/ml for all isolates.

No discrepancies between GEN or STR MICs and the single-concentration agar screening test were observed in any of the 219 strains.

E. faecalis is the enterococcal species most frequently associated to human infections (1, 8). The emergence of strains exhibiting HLR to aminoglycosides can significantly limit the therapeutic choices for severe infections caused by this organism (3, 5).

Several screening tests to determine penicillin-aminoglycoside synergy have been proposed, all based on the detection of high-level aminoglycoside resistance, and a 2,000-μg/ml MIC has so far been adopted as a reliable breakpoint (5, 10, 11). However, rare strains displaying moderately high aminoglycoside MICs (512 and 256 to 1,024 μg/ml for GEN and STR, respectively) have recently been described by Leclercq et al., who emphasized the need to rule out synergy resistance for such strains and proposed their inclusion in an indeterminate category but failed to perform time-kill studies (2).

Strains displaying MHLR to STR and GEN have been isolated at our institution with 5 and 1.4% frequencies, respectively (data not shown). In agreement with other authors (2), aminoglycoside MICs remained unchanged after a 48-h incubation (data not shown). Influence of a higher inoculum density was not evaluated in our study, but Leclercq et al. found no variations due to inoculum size in their strains with MHLR (2).

Ounissi et al. demonstrated that strains displaying MHLR could produce aminoglycoside-inactivating enzyme, since their DNA hybridizes with the specific probes (7). Time-kill results suggest that our strains are resistant to penicillin-aminoglycoside synergy, so they should be considered to exhibit HLR in severe infections when time-kill studies cannot be performed. Further studies with specific DNA probes for genes that mediate aminoglycoside resistance will be of interest to characterize MHLR more thoroughly.

Aminoglycoside concentrations currently employed in conventional screening tests to determine presumptively penicillin-gentamicin and penicillin-streptomycin synergy should be regarded with caution. Although the concentrations assayed in our single-concentration agar screening test were able to detect strains with MHLR, their routine use requires further evaluation on the basis of data from a large number of strains displaying MHLR.

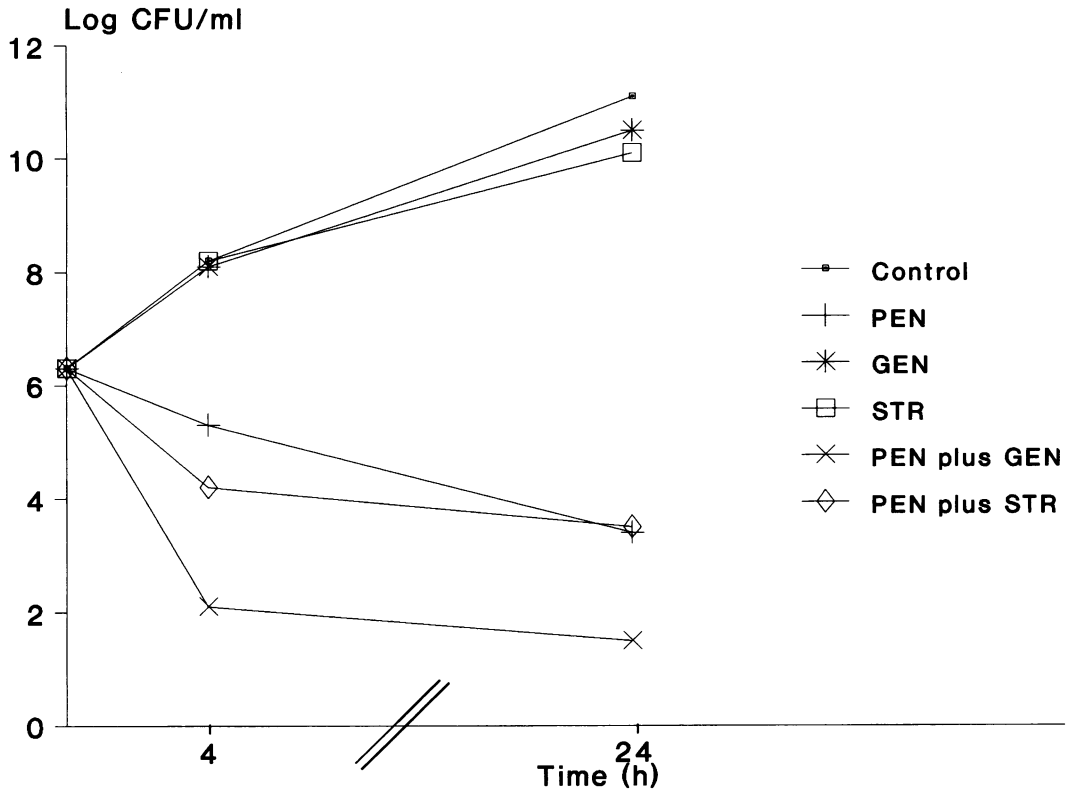

FIG. 2. Time-kill curve of an *E. faecalis* strain displaying MHLR to STR (MIC, 256 μg/ml) and LLR to GEN (MIC, ≤32 μg/ml).

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