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# High-Content Aminoglycoside Disks for Determining Aminoglycoside-Penicillin Synergy against *Enterococcus faecalis*

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**We investigated the use of high-content aminoglycoside disks for determining *Enterococcus faecalis* susceptibility to aminoglycoside-penicillin synergy. The susceptibility of the organisms to synergy was established by 24-h time-kill studies performed with streptomycin, kanamycin, amikacin, gentamicin, and tobramycin, alone and in combination with penicillin. A total of 20 isolates that were susceptible to all drug combinations and 20 strains that were resistant to each aminoglycoside-penicillin combination were selected for testing against high-content disks. Disk-agar diffusion was performed on Mueller-Hinton agar, with and without 5% sheep blood, by using disks that contained either 300 or 2,000 μg of streptomycin and either 120 or 2,000 μg of kanamycin, amikacin, tobramycin, or gentamicin. Zone size results obtained for each aminoglycoside, except amikacin, could be used to differentiate between synergy-susceptible and -resistant isolates. No overlap occurred between the zone sizes of susceptible and resistant strains. Susceptibility to amikacin-penicillin synergy could reliably be tested with kanamycin, but not amikacin, disks. When the disks containing 120 μg were tested, a narrow zone size range of 6 to 7 mm could be used to identify all resistant strains. In contrast, when the disks containing 2,000 μg were used, the zone size ranges for resistant isolates varied widely with the aminoglycoside being tested. The presence of blood in the medium did not appreciably affect the disk test results. To detect resistance to every aminoglycoside-penicillin combination that may be considered for therapy, *E. faecalis* isolates need to be tested against a maximum of three different high-content disks (i.e., streptomycin, gentamicin, kanamycin). The disk-agar diffusion test performed with high-content aminoglycoside disks can provide laboratories with a convenient and reliable method for detecting *E. faecalis* isolates that are resistant to aminoglycoside-penicillin synergy.**

*Enterococcus faecalis* strains may acquire transferable plasmids that encode for the production of aminoglycoside-modifying enzymes. Strains that produce these enzymes exhibit high-level resistance (i.e., MIC, ≥2,000 μg/ml) to various aminoglycosides and, as a result, are resistant to the synergistic activity that would occur between penicillin, or other cell wall-active agents, and an aminoglycoside (3, 5, 10). An accurate disk diffusion test that could be used to detect this synergy resistance would provide laboratories with a convenient and reliable method for testing clinically significant *E. faecalis* isolates.

The aminoglycoside disks recommended for routine disk-agar diffusion tests (11) are not reliable for detecting high-level resistance among *E. faecalis* isolates (12, 14). However, Rosenthal and Freundlich (14) showed that if disks containing high drug concentrations are used, isolates that exhibit high-level aminoglycoside resistance could be detected by disk-agar diffusion. Although their results indicated that the disk test may be an accurate method for detecting resistance to synergy, their study included only three enterococcal strains that were highly resistant to gentamicin and four that were resistant to tobramycin. In addition, none of the disk test results were compared with results of time-kill synergy studies. To further investigate the accuracy of the disk method, we selected several *E. faecalis* strains that, based on results of time-kill synergy studies, were already characterized as being either resistant or susceptible to the synergy of various aminoglycoside-penicillin combinations. These strains were tested against high-content aminoglycoside disks, and the resulting inhibition zone sizes were correlated with the results obtained by the time-kill studies. In addition, because it has been shown in other studies (4, 13) that the presence of blood can affect the in vitro susceptibility of enterococci to aminoglycosides, disk tests were performed on Mueller-Hinton agar with and without 5% sheep blood.

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## MATERIALS AND METHODS

The *E. faecalis* strains selected for disk-agar diffusion studies were previously subjected to time-kill synergy tests that were performed as described elsewhere (7, 15). The drugs used for synergy tests included streptomycin (25 μg/ml; Sigma Chemical Co., St. Louis Mo.), gentamicin (5 μg/ml; Schering Corp., Bloomfield, N.J.), tobramycin (5 μg/ml; Eli Lilly & Co., Indianapolis, Ind.), kanamycin (20 μg/ml; Bristol Laboratories, Syracuse, N.Y.), or amikacin (20 μg/ml; Bristol Laboratories). These aminoglycosides were tested alone or in combination with 10 U of penicillin per ml (Sigma). By this method, synergy susceptibility was defined as a ≥100-fold increase in killing by the aminoglycoside-penicillin combination over the killing accomplished by the most active of the two drugs when tested separately. Synergy resistance was defined as a <100-fold increase in killing. All *E. faecalis* strains studied were clinical isolates obtained from patient specimens submitted to the University of Chicago Clinical Microbiology Laboratories.

The high-content aminoglycoside disks for disk-agar dif-

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fusion testing were prepared by applying 25 μl of aminoglycoside stock solution containing 40 times the final desired drug potency to sterile blank disks (diameter, 6 mm; Difco Laboratories, Detroit, Mich.) and allowing them to air dry. For each aminoglycoside, high-content disks of two different concentrations were prepared. The drug concentrations were selected based on the concentrations used by Rosenthal and Freundlich (14) and on the aminoglycoside concentrations used in agar dilution screens (8). Streptomycin disks contained either 300 or 2,000 μg of drug, whereas tobramycin, gentamicin, kanamycin, and amikacin disks each contained either 120 or 2,000 μg of drug. Disk-agar diffusion tests were performed by recommended procedures (11) by using Mueller-Hinton agar supplemented (SMH) and not supplemented (MH) with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.).

For each *E. faecalis* isolate, the disk-agar diffusion tests were performed in triplicate, and the inhibition zone diameters were averaged. To determine whether discrete zone size ranges for separating synergy-susceptible and -resistant isolates could be established, zone sizes were correlated with results that were obtained with the time-kill synergy studies.

## RESULTS

In all, 20 *E. faecalis* isolates that were susceptible to every aminoglycoside-penicillin combination and 20 strains that were resistant to each of the drug combinations were selected for the disk-agar diffusion studies. The results obtained by testing synergy-susceptible and -resistant *E. faecalis* isolates against the disks containing 120 μg (300 μg of streptomycin) and 2,000 μg of aminoglycoside are given in Table 1. For every aminoglycoside disk tested, except amikacin, a substantial difference was observed between the zone size ranges obtained with susceptible strains and those obtained with resistant strains. The ranges of synergy-susceptible isolates did not overlap with those of the synergy-resistant isolates. For amikacin disk test results, considerable overlap was noted between the zone size ranges of susceptible and resistant isolates. When kanamycin disks were used to predict amikacin-penicillin synergy, however, there was a substantial difference between zone size ranges obtained with strains that were susceptible to amikacin-penicillin synergy and those that were resistant to this drug combination. In addition, no overlap in zone size ranges occurred between resistant and susceptible isolates.

TABLE 1. Ranges of inhibition zone diameters obtained with aminoglycoside disks tested against synergy-susceptible and -resistant *E. faecalis* isolates[a]

| Aminoglycoside disk and content (μg) | Inhibition zone diam (mm) with growth on[b]: | | | |
|---|---|---|---|---|
| | SMH | | MH | |
| | SYN-S | SYN-R | SYN-S | SYN-R |
| Streptomycin | | | | |
| 300 | 14–18 | 6 | 12–22 | 6 |
| 2,000 | 20–25 | 6–14 | 18–26 | 6–10 |
| Tobramycin | | | | |
| 120 | 22–26 | 6–10 | 14–20 | 6 |
| 2,000 | 30–34 | 6–22 | 22–30 | 6–14 |
| Gentamicin | | | | |
| 120 | 20–26 | 6 | 15–22 | 6–7 |
| 2,000 | 30–34 | 6–20 | 20–29 | 6–15 |
| Kanamycin[c] | | | | |
| 120 | 20–24 | 6 | 10–20 | 6 |
| 2,000 | 28–32 | 6–10 | 21–28 | 6–17 |
| Amikacin | | | | |
| 120 | 12–20 | 6–18 | 6–14 | 6 |
| 2,000 | 20–28 | 10–30 | 6–24 | 6–14 |

[a] For each aminoglycoside, 20 synergy-susceptible and 20 synergy-resistant strains were tested.

[b] Abbreviations: SMH, Mueller-Hinton agar plus 5% sheep blood; MH, Mueller-Hinton agar without blood; SYN-S, synergy susceptible; SYN-R, synergy resistant.

[c] Kanamycin disk results were used to predict both kanamycin-penicillin synergy and amikacin-penicillin synergy.

For every 120-μg aminoglycoside disk tested, other than amikacin, synergy-resistant strains showed a very narrow zone size range, with most zones seldom exceeding 6 mm (Table 1). In contrast, the zone sizes obtained for synergy-susceptible strains tested against 120-μg disks were never less than 10 mm and frequently were much greater. The zone size ranges given by synergy-resistant strains tested against the different 2,000-μg aminoglycoside disks varied considerably and were not as narrow as when 120-μg disks were used. Many zones were >10 mm and some were ≥20 mm in diameter.

Although the type of medium did not appreciably affect the zone sizes observed with synergy-resistant strains tested against the 120-μg disks, zone size ranges obtained with the synergy-susceptible organisms tended to be slightly greater on SMH than on MH (Table 1). The zone size range for synergy-resistant strains tested against disks containing 120 μg of tobramycin on SMH (diameter, 6 to 10 mm) overlapped with the range given by synergy-susceptible strains tested against disks containing 120 μg of kanamycin on MH (diameter, 10 to 20 mm). Regardless of the 2,000-μg aminoglycoside disk being tested, the type of medium used (SMH or MH) did not notably affect the zone size ranges. However, overlap did occur between zone size ranges obtained with synergy-resistant strains tested against tobramycin and gentamicin on SMH (diameters, 6 to 22 and 6 to 20 mm, respectively) and the ranges obtained with synergy-susceptible strains tested against these two drugs on MH (diameters, 22 to 30 and 20 to 29 mm, respectively).

## DISCUSSION

By direct correlation of disk-agar diffusion results with data that were obtained by time-kill synergy studies, we have shown that high-content aminoglycoside disks can be used to accurately differentiate between *E. faecalis* isolates that are resistant or susceptible to the synergy of various aminoglycoside-penicillin combinations. Our findings are consistent with those of an earlier study by Rosenthal and Freundlich (14), who tested a few resistant enterococcal isolates against high-content aminoglycoside disks and were able to correlate a lack of zone of inhibition (i.e., zone diameter, 6 mm) with high-level (i.e., MIC, ≥2,000 μg/ml) aminoglycoside resistance.

For each aminoglycoside tested, except amikacin, both the 120-μg (300-μg for streptomycin) and the 2,000-μg disks differentiated between synergy-susceptible and -resistant strains. However, 120-μg disks provided more clear-cut results than 2,000-μg disks. Zone size diameters obtained with 120-μg disks were almost always limited to 6 mm when synergy-resistant strains were tested and reached 10 mm only when tobramycin and amikacin disks were tested on

SMH. The zone size diameters given by synergy-susceptible isolates were always ≥10 mm. Therefore, with the 120-μg gentamicin and kanamycin disks and the 300-μg streptomycin disk, a narrow zone diameter of 6 to 7 mm could be used as a reliable indicator of synergy resistance. Zone diameters of ≥10 mm indicated synergy susceptibility. In contrast, the zone sizes obtained for synergy-resistant strains tested against 2,000-μg disks varied considerably with each aminoglycoside. Thus, if 2,000-μg disks were used, a different maximum zone size range for defining synergy resistance would need to be established for each drug. The single range breakpoint (zone diameter, 6 to 7 mm) for all aminoglycosides, which was afforded by the use of 120-μg disks, is preferable to the multiple breakpoints that would be required if 2,000-μg disks were used. In addition, with the 2,000-μg gentamicin and tobramycin disks, the zone size ranges obtained with synergy-resistant strains on SMH overlapped with zone sizes of synergy-susceptible strains tested on MH (Table 1). These zone size overlaps did not occur with 120-μg disks; therefore, if the lower-content disks are used, the breakpoints for synergy resistance need not be changed according to the blood content of MH.

In general, our results indicate that MH with or without blood may be chosen for disk tests and that the adverse effects of blood or blood products on susceptibility test results obtained with standard-concentration aminoglycoside disks (4, 13) are not evident when high-content disks are used. However, when 120-μg tobramycin disks were tested on MH against synergy-resistant isolates, all zone sizes were 6 mm in diameter, but on SMH zones ranged from 6 to 10 mm in diameter, which overlapped with the 10-mm zones on kanamycin disks obtained with synergy-susceptible isolates on MH (Table 1). As will be discussed below, problems that this zone range overlap might cause for establishing a single breakpoint for all aminoglycosides tested may be avoided by using gentamicin disks, which consistently gave zone size diameters of 6 mm, to predict tobramycin-penicillin synergy.

Because the only enzyme that is known to mediate *E. faecalis* resistance to gentamicin-penicillin synergy (2″-phosphotransferase–6′-acetyltransferase) also mediates resistance to penicillin synergy with kanamycin, amikacin, tobramycin, and netilmicin (3), 120-μg gentamicin disks can be used to detect resistance to synergy between penicillin and any of these aminoglycosides. Regardless of the medium used or the potency of the disks, our results showed that amikacin cannot be used to determine *E. faecalis* susceptibility to amikacin-penicillin synergy. In contrast, the kanamycin disks proved to be an accurate and reliable substitute. These findings are consistent with those of others (1, 2, 15) who have shown by agar dilution techniques that kanamycin more accurately predicts amikacin-penicillin synergy than does amikacin. Based on the results of this study and the aminoglycoside-modifying enzymes that are currently known to mediate synergy resistance among *E. faecalis* isolates (3, 15), a maximum of three different high-content disks (streptomycin, gentamicin, and, occasionally, kanamycin) are needed to determine *E. faecalis* resistance or susceptibility to every aminoglycoside-penicillin combination that may be considered for therapy. Kanamycin would need to be tested only in the unlikely situation that either kanamycin or amikacin are being considered for therapy against gentamicin-susceptible isolates.

In summary, in this investigation we have shown that high-content aminoglycoside disks (120 μg for gentamicin and kanamycin; 300 μg for streptomycin) can be used with the disk-agar diffusion method to accurately determine the susceptibility of *E. faecalis* isolates to aminoglycoside-penicillin synergy. The high-content disks can be produced in large numbers, stored at either 4 or −20°C for extended periods of time without significant loss of activity (data not shown), and retrieved for use as needed. This method, therefore, offers a convenient technique by which many laboratories would be able to screen clinically significant *E. faecalis* isolates for synergy resistance. In our laboratory we have used high-content disks for a year and have found that disks stored at 4°C for as long as 4 months have not shown any appreciable loss of activity. If this procedure is adopted in clinical laboratories, however, we recommend that the quality of the disks be examined periodically by testing known synergy-susceptible and -resistant *E. faecalis* isolates. Finally, because of the greater resistance of *Enterococcus faecium* to both beta-lactam and aminoglycoside antibiotics (6, 9), the disk-agar diffusion results presented here should be applied only to *E. faecalis* isolates and not to *E. faecium* strains. Further studies are required to determine the reliability of the disk test for detecting synergy resistance among important clinical isolates of *E. faecium* and other *Enterococcus* spp. Until such studies are completed, laboratories should follow appropriate identification procedures to identify an enterococcal isolate as *E. faecalis* before disk tests are performed.

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