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Screening for Penicillin Tolerance in Viridans Streptococci by a Simple Disk Method

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A disk method was developed for demonstrating penicillin tolerance in viridans streptococci. This was achieved by the substitution of the penicillin disk used for susceptibility testing by a disk containing penicillinase. After reincubation, penicillin-tolerant strains exhibited new growth in the area adjacent to this disk, providing a rapid screening test.

Tolerance to the lethal action of penicillin has been demonstrated in the streptococcus viridans group (2, 4) and recently in Lancefield group B (5), group C (9), and group G (7) haemolytic streptococci. The phenomenon of tolerance is characterized by a discrepancy between the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC) when this ratio is greater than 32 (9, 13). Based upon this definition, the method used to detect tolerance is the broth dilution susceptibility test with subsequent subculturing onto blood agar plates. Performing time-killing kinetics in the presence of penicillin is another method of demonstrating tolerance (4, 5, 9). In this method, a reduction in viable count of less than 10^3 colony-forming units per ml after 24 h of incubation is taken to denote tolerance (1). These two methods for detection of tolerance are both time-consuming and not easily applicable in the routine diagnostic bacteriological laboratory. Since the disk method is commonly used for susceptibility testing in such laboratories, we have attempted to use disks to demonstrate tolerance to penicillin.

Viridans streptococci were isolated from the sulcus gingivalis flora of children with cardiac disease who risked developing endocarditis. The strains were identified as previously reported (3) and were preserved at −20°C in skim milk before being tested for their susceptibility to penicillin by the serial broth dilution method. Five penicillin-susceptible strains (MIC, <0.8 U of penicillin per ml; MBC/MIC ratio <32) and five penicillin-tolerant strains (MBC/MIC ratio, ≥32) were chosen, grown in Todd-Hewitt broth (Oxoid Ltd., London, England), at 37°C for 18 h and subcultured onto sheep blood (5% vol/vol) agar plates (Oxoid Ltd.). The plates were incubated at 37°C for 48 h in 10% CO_2, since some strains were found to be CO_2 dependent. Colonies of each strain were suspended in physiological saline to a density of McFarland no. 0.5 turbidity standard, and 0.1 ml of the solution was spread onto blood agar plates. Disks containing 2 U of penicillin (Oxoid Ltd.) were placed in the center of these plates. After incubation at 37°C for 24 h in 10% CO_2, the inhibition zones were measured, the penicillin disks were carefully removed with sterile forceps, and disks (Bacto disk, Difco Laboratories, Detroit, Mich.) containing sufficient penicillinase to counteract 50 U of penicillin were substituted. These disks were prepared as follows: 0.05 ml of a 1,000-U/ml solution of penicillinase (Gist Brocades, Delft, The Netherlands) was pipetted onto the blank disks, providing a concentration of 50 U of penicillinase per disk. These disks were used immediately. After incubation at 37°C for 24 to 48 h in 10% CO_2, the initial zones of inhibition had not changed, but plates inoculated with penicillin-tolerant strains exhibited new growth in the area adjacent to the penicillinase disk (Fig. 1). This new growth never occurred in the case of penicillin-susceptible strains (Fig. 2), whereas both susceptible and tolerant strains displayed large inhibition zones with the penicillin disk. To study the effect of the moment of substitution of the penicillin disk on the occurrence of bacterial regrowth within the center of the initial zone of inhibition, disks were removed after 4, 6, 12, 18, 24, 32, and 48 h of incubation, and a penicillinase-containing disk was substituted. After reincubation at 37°C for 24 to 48 h in 10% CO_2, growth reoccurred within the initial zones of inhibition for all penicillin-tolerant strains irrespective of the time of substitution of the penicillin disks (Table 1). For the susceptible strains, the number of colonies present within the inhibition zones decreased as the time of incubation

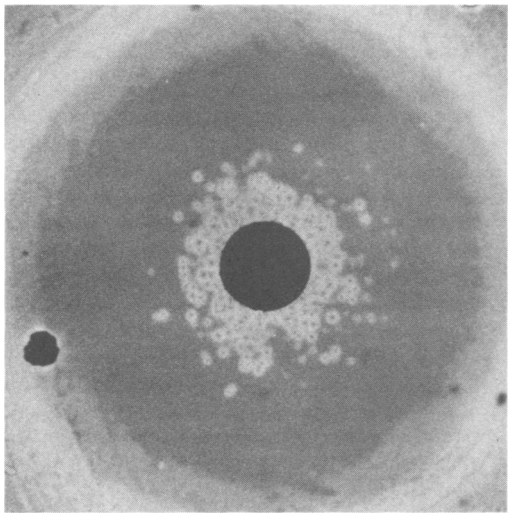

FIG. 1. Regrowth of colonies of a penicillin-tolerant *Streptococcus sanguis* II strain adjacent to a penicillinase (50 U)-containing disk which had been placed in the center of a zone of inhibition due to penicillin. The original penicillin disk was replaced by the penicillinase-containing disk after 24 h of incubation at 37°C in 10% CO_2. The blood agar plate was reincubated for 24 h at 37°C in 10% CO_2.

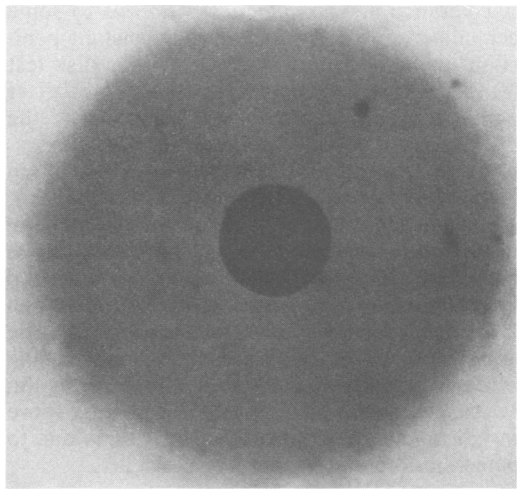

FIG. 2. Inhibition zone of a penicillin-susceptible *Streptococcus sanguis* II strain. The original penicillin disk was removed after 24 h of incubation and substituted by a penicillinase (50 U)-containing disk. The blood agar plate was reincubated for 24 h at 37°C in 10% CO_2.

with the penicillin disk in place was prolonged.

The regrowth of colonies of penicillin-tolerant strains within the inhibition zones after the penicillin disk has been replaced by a penicillinase-containing disk is due to the fact that penicillin acts as a bacteriostatic agent on these strains and that inactivation of the antibiotic by penicillinase which diffused into the medium results in growth of inhibited bacterial cells. Using the agar dilution method to determine MICs and MBCs of β-lactams for various microorganisms, Masuda et al. (6) found that when a potent β-lactamase solution was sprayed on the agar surface, growth was absent in the area where microorganisms had been killed and reoccurred where microorganisms had been inhibited, but not killed.

In the broth dilution test, we have found that the addition of penicillinase to the subculture plates is imperative for preventing the effect of growth inhibition of surviving streptococcal cells due to the carry-over of penicillin from the tubes, thus providing a false low MBC. The disk screening test for penicillin tolerance among streptococci can be easily performed.

In an additional 45 viridans streptococcal strains tested, we have found no false negatives compared with their MBC/MIC ratio. However, false positives can occur when the medium is inoculated with 10^7 microorganisms. Of the strains tested so far, 20% showed the phenomenon of penicillin tolerance in both the screening test and broth dilution test and in killing curves.

TABLE 1. Effect of moment of substitution of the penicillin disk by a penicillinase disk on regrowth within the center of the initial inhibition zone

Viridans streptococci	Time of substitution (h of incubation) of penicillin disk[a]						
	4	6	12	18	24	32	48
Penicillin susceptible (n = 5)	++++	++	+	±	−	−	−
Penicillin tolerant (n = 5)	++++	++++	+++	+++	+++	+++	++

[a] Symbols: ++++, colonies grew in almost the entire zone of inhibition; +++, many colonies were present, but not in the entire zone of inhibition; ++, about 100 colonies were present around the penicillinase disk; +, 10 to 100 colonies were present around the penicillinase disk; ±, 2 to <10 colonies were present around the penicillinase disk; and −, 0 to 2 colonies were present around the penicillinase disk.

Tolerance in *Staphylococcus aureus* to some cephalosporins and β-lactamase resistant penicillins has also been determined with a disk test (8). The results were found to correlate better with clinical outcome than the results of the broth dilution susceptibility testing (8). The clinical significance of tolerance has been discussed (12), but no conclusions have been reached, although cases of meningitis and bacteremia due to penicillin-tolerant group B streptococci (5) and endocarditis due to penicillin-tolerant group C streptococci (10) and viridans streptococci (11; unpublished data) have been noted. The application of a rapid, reliable, and simple screening test for the detection of penicillin tolerance in streptococci can probably give more insight into the occurrence of infections due to penicillin-tolerant streptococcal strains.

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