

Importance of Medium in Demonstrating Penicillin Tolerance by Group B Streptococci

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A total of 30 clinical isolates of group B streptococci were studied for penicillin tolerance *in vitro*. Minimal inhibitory and bactericidal concentrations of penicillin were determined simultaneously in three test media which have been used for group B streptococci, tryptose phosphate, Mueller-Hinton, and Todd-Hewitt broths, using a logarithmic-phase inoculum of 10^5 colony-forming units per ml. Minimal inhibitory concentrations in the three media did not differ significantly. However, minimal bactericidal concentrations were significantly higher in tryptose phosphate broth (mean, 1.04 $\mu\text{g/ml}$) than in Mueller-Hinton broth (0.22 $\mu\text{g/ml}$) or Todd-Hewitt broth (0.15 $\mu\text{g/ml}$). Similarly, ratios of minimal bactericidal to minimal inhibitory concentrations were significantly greater in tryptose phosphate broth than in Mueller-Hinton or Todd-Hewitt broth. After incubation in tryptose phosphate broth for an additional 24 h, the minimal bactericidal concentration consistently fell to levels which were only twice or equal to the minimal inhibitory concentration. This study illustrates the importance of the medium in the demonstration of penicillin tolerance and of controlling laboratory variables in the susceptibility testing of group B streptococci with penicillin.

The group B streptococci are recognized as a major cause of serious neonatal infections. Poor results with penicillin therapy, despite consistent susceptibility to this group of antimicrobial agents, have prompted studies of the *in vitro* susceptibility of these streptococci to antibiotics. There are several reports of penicillin tolerance (minimal bactericidal concentration [MBC] significantly higher than minimal inhibitory concentration [MIC]), but the methodology has varied from study to study. In this report, we describe the importance of the test medium in the demonstration of this phenomenon.

MATERIALS AND METHODS

A total of 30 strains of group B streptococci were obtained from the clinical microbiology laboratory at Harbor/UCLA Medical Center; 12 were fresh isolates, and 18 were previous isolates which had been stored at -70°C in 10% sterile skimmed milk (Difco Laboratories, Detroit, Mich.) for up to 12 months. Each strain was from a different patient. They were identified as group B streptococci by the method of Lancefield (8) with sera provided by the Center for Disease Control, Atlanta, Ga. Twelve isolates were from throat cultures, five were from cervical swabs, two were from cerebrospinal fluid, two were from umbilical cultures, and one each was from blood, gastric aspirate, urine, stool, conjunctiva, nose, external ear, endometrium, and scalp lesion.

Five colonies were subcultured from sheep blood agar to 10 ml of tryptose phosphate (TP) broth (Difco), which was incubated at 37°C for 6 h. This was diluted in fresh TP broth to a concentration of approximately 10^5 colony-forming units per ml, as determined by measuring the light scattering of the suspension with a Coleman model 9 nephelometer (Coleman Instruments, Oak Brook, Ill.). Colony counts performed with each experiment showed that this represented a bacterial concentration of 0.9×10^5 to 3.3×10^5 colony-forming units per ml. Identical dilutions of the logarithmic-phase culture were made in fresh Todd-Hewitt (TH) and Mueller-Hinton (MH) broths (Difco).

The penicillin susceptibility of each strain was measured simultaneously in the three test broths (TP, TH, and MH). Potassium penicillin G (Bristol Laboratories, Syracuse, N.Y.) at a potency of 1,595 U/mg was reconstituted to 1 mg/ml in sterile distilled water, dispensed in aliquots, stored at -70°C , and used within 2 weeks of freezing. The penicillin was thawed immediately before use, and twofold dilutions were made in each test medium. Control tubes without penicillin were included in each series of dilutions. Equal volumes (0.5 ml) of penicillin solution and of the culture dilution were mixed and incubated for approximately 18 h at 37°C . The MIC was defined as the lowest concentration of penicillin at which there was no turbidity on visual inspection. From each tube 0.1 ml was pipetted to an antibiotic-free blood agar plate and incubated at 37°C overnight to determine the MBC, which was defined as the lowest concentration of

penicillin resulting in ≤ 10 colony-forming units per ml (≤ 1 colony per plate). For each strain of *Streptococcus*, the relationship between the MBC and MIC was expressed as the MBC/MIC ratio.

RESULTS

The means and ranges of the MICs and MBCs in the three media are summarized in Table 1. The MICs varied over a narrow range (two or less) of twofold dilutions and did not differ significantly among the three broths. However, the MBCs were noticeably higher and varied over a wider range (five to six dilutions in each medium). Moreover, the MBCs were significantly higher in TP broth than in MH or TH broth ($P < 0.001$).

Figure 1 shows the distribution of MBC/MIC ratios for 30 strains tested simultaneously in the three media. The distribution of ratios in TP broth was shifted to the right compared with the ratios in MH and TH broths, reflecting higher MBCs and higher MBC/MIC ratios in TP broth. In this medium, 26 strains (86%) showed an MBC/MIC ratio of ≥ 16 . In contrast, only two strains (6%) in MH broth and one strain (3%) in TH broth showed a ratio of 16.

Ten strains were reexamined in TP broth by the method described above but with an additional determination of MBCs after another 24 h of incubation. All MIC and MBC results

agreed within one dilution with earlier determinations. The MBC/MIC ratio varied from 2 to 64, with a mean of 24.6. However, the MBC fell sharply during the additional incubation period, and the MBC/MIC ratio decreased to 1 for nine strains and to 2 for one.

DISCUSSION

Although group B streptococci appear to be consistently susceptible to penicillin (2, 3), unsatisfactory results have often followed penicillin treatment of septic infants, including reoccurrence of disease after apparently successful therapy (4, 13, 14), persistence of viable streptococci in the cerebrospinal fluid after several days of treatment (6, 7), and frank bacteriological and clinical relapse of meningitis during therapy (5). The reasons for these poor responses are undoubtedly complex, but one proposed explanation is penicillin tolerance by *Streptococcus*, defined as delayed or diminished killing by growth-inhibiting concentrations of the antibiotic. In vitro penicillin tolerance, usually demonstrated by significantly higher MBCs than MICs, has been studied extensively in other streptococcal species by Tomasz et al. (12) and in staphylococci by Sabath et al. (10).

Differences between penicillin MICs and MBCs of eightfold or greater have been described in several studies of group B streptococci (1, 9, 11, 14), but the media, growth phase of the bacterial inoculum, and quantitative definition of MBC usually varied. In the present reexamination of tolerance, penicillin susceptibility was examined by using three different media (TP, MH, and TH broths), an exponential-phase inoculum, and a definition of MBC as the lowest penicillin concentration which reduced the viable count from 10^5 to 10^1 colony-forming units per ml during overnight incubation. The most striking finding was the effect of the medium. Although the MICs did not differ significantly in the three media, the MBCs and consequently the MBC/MIC ratios were substantially higher in TP broth than in the other two broths. Thus, penicillin tolerance of group B streptococci was not demonstrated in TH or MH broth, but, when the arbitrary definition of an MBC/MIC ratio of ≥ 16 was used, 26 strains (87%) were tolerant when studied in TP broth. Preliminary observations of reincubation of strains with antibiotic in TP broth for an additional 24 h suggest that a killing effect of penicillin was delayed but eventually did occur.

TABLE 1. Comparison of MICs and MBCs in three media

Medium	MIC ($\mu\text{g/ml}$)		MBC ($\mu\text{g/ml}$)	
	Mean	Range	Mean	Range
TP	0.04	0.02-0.08	1.04 ^a	0.08-2.5
TH	0.05	0.04-0.08	0.15	0.04-1.25
MH	0.04	0.02-0.08	0.22	0.02-1.25

^a $P < 0.001$ (by paired *t* test) when the MBCs in TP broth were compared with those in TH and MH broths. Otherwise, there were no significant differences among MICs and MBCs.

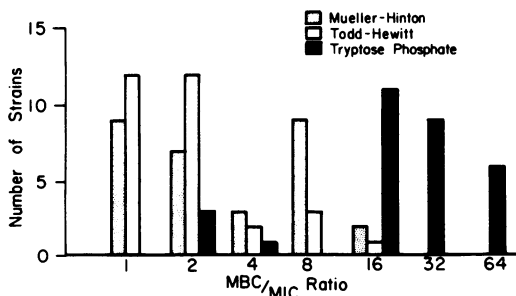


FIG. 1. Distribution of the MBC/MIC ratios for 30 strains of group B streptococci in three test media. The various ratios are plotted along the x-axis and the number of strains is plotted along the y-axis.

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