Optimal Combination and Concentration of Antibiotics in Media for Isolation of Pathogenic Fungi and Nocardia asteroides

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Strains of Blastomyces dermatitidis, Sporothrix schenckii, Histoplasma capsulatum, Cryptococcus neoformans, Nocardia asteroides, and Coccidioides immitis were tested for in vitro susceptibility to polymyxin, gentamicin, kanamycin, chloramphenicol, and neomycin at concentrations of 1, 2, 4, 8, and 16 μg/ml. Polymyxin was the most inhibitory and gentamicin was the least inhibitory of the five antibiotics. Two Histoplasma mycelial strains were partially inhibited by 2 and 8 μg of gentamicin per ml and showed at least a 2+ growth at the higher antibiotic concentration. Kanamycin and neomycin produced significant inhibition of N. asteroides but otherwise were noninhibitory. A combination of chloramphenicol and kanamycin, each at 16 μg/ml, and gentamicin, at 4 μg/ml, was noninhibitory to the strains tested except for N. asteroides. Chloramphenicol at 16 μg/ml was not inhibitory for N. asteroides. The results suggest that the optimal antibiotic combination to use in the isolation of fungi and higher bacteria is chloramphenicol, 16 μg/ml, and gentamicin, 4 μg/ml. Addition of sheep blood (5%) had no effect on antibiotic susceptibility of the organisms studied.

Since 1945 at least six articles have been published on the evaluation of antibiotics in media used for the isolation of pathogenic fungi. In 1945, Thompson (7) reported that penicillin and streptomycin in a selective medium did not affect the growth of the pathogenic fungi but would inhibit a wide spectrum of bacteria commonly encountered at that time. Our experience has substantiated his report, but, in more recent years, gram-negative bacilli have become more numerous in clinical specimens and are resistant to penicillin and streptomycin.

In 1954, Georg and associates (3) showed that cycloheximide at a concentration of 0.5 mg/ml did not inhibit the fungi which produce subcutaneous or systemic disease in man except for Cryptococcus neoformans, Aspergillus fumigatus, and Allescheria boydii. Further studies from this group (4, 5) showed that the yeast phase of dimorphic fungi infecting man were sensitive to cycloheximide at 0.5 mg/ml and chloramphenicol at 0.05 mg/ml when incubated at 37 C but were not affected when incubated at 25 C, allowing conversion to the mycelial phase.

Taplin's study (6) in 1965 was primarily concerned with the comparison of peptone-glucose-agar containing chloramphenicol and cycloheximide with and without gentamicin for the isolation of dermatophytes and Candida albicans. The combination of all three antibiotics was more efficacious than the combination of chloramphenicol and cycloheximide for the isolation of dermatophytes, but this study did not show a significant difference in the isolation of C. albicans. Also, he did not study the effect of the antibiotics on the other fungi. In Cohen's work (2) reported in 1969, only C. albicans, C. parapsilosis (para-krusel), and bacteria were studied, and the effect of neomycin and polymyxin on the isolation of the majority of pathogenic fungi was not evaluated.

MATERIALS AND METHODS

Test organisms. All of the organisms used in this study were isolated from clinical specimens. These included five strains of mycelial and yeast phases of Blastomyces dermatitidis and Sporothrix schenckii, five strains of the mycelial phase and three strains of the yeast phase of Histoplasma capsulatum, five strains each of C. neoformans and Nocardia asteroides, and five strains of the mycelial phase of Coccidioides immitis. These were identified by conventional morphological and biochemical techniques (1).
Antibiotics. Three of the antibiotics were obtained from the manufacturer for use as standards for antibiotic susceptibility testing. These were: gentamicin (Schering Corp., Bloomfield, N.J.), kanamycin (Bristol Laboratories, Syracuses, N.Y.), and chloramphenicol (Parke, Davis & Company, Detroit, Mich.). Neomycin was obtained commercially as Myecifradin sulfate, sterile powder (The Upjohn Company, Kalamazoo, Mich.), and polymyxin sulfate was obtained as Aerosporin, sterile powder (Burroughs Wellcome & Co., Tuchahoe, N.Y.).

Stock solutions (1,000 µg/ml) were prepared and frozen until used. The final concentrations used in the testing of growth were 1, 2, 4, 8, and 16 µg/ml. These were chosen because they represented the range at which the commonly isolated bacteria are inhibited.

Media. The Brain Heart Infusion (BHI)-agar was prepared by dissolving 37 g of the prepared medium (Bioquest, Cocksley, Md.) and 17 g of agar in 1 liter of distilled water with moderate heating; it was autoclaved at 120 C for 15 min. An appropriate quantity of each antibiotic was added to a measured quantity of medium cooled to 55 C, and the mixture was placed in sterile plastic dishes (100 by 15 mm). Media with and without 5% sterile defibrinated sheep blood (Climost Laboratories, Shaker Heights, Ohio) were used. The organisms were maintained on BHI-5% sheep blood-agar slants.

Procedure. Inocula from all of the organisms, except for the yeasts, were made by carefully scraping the growth from the surface of an agar slant and grinding it into a sterile conical glass grinder. This homogenate was made into a suspension with sterile saline and adjusted to an absorbance of 0.20 in a spectrophotometer at 600 nm wavelength. Inocula from the yeasts were not ground but were directly made into a similar suspension. A 0.1-ml portion of this suspension was placed at one edge of an agar plate. A sterile cooled loop was used to streak the inoculum in divided thirds of the plate, by using a conventional streaking technique. This produced areas of heavy, moderate, and light inoculation. The plates were incubated at 30 C for 7 days. One plate of BHI with 5% sheep blood without antibiotics was inoculated similarly with each strain and used as the control plate. Duplicate BHI plates containing the same concentration of antibiotics but with and without 5% sheep blood were inoculated and studied in parallel. The amount of growth was graded 1+ through 4+ based on comparison with the control plate without antibiotics.

RESULTS

Table 1 shows the antibiotic concentrations at which there was a 3+ or greater growth of the various strains tested. The growths on BHI with and without 5% sheep blood at the various antibiotic concentrations were essentially similar; therefore, only the results of growth on BHI with 5% sheep blood are recorded in this table.

It is evident that polymyxin was the most inhibitory and gentamicin was the least inhibitory of the four antibiotics recorded in Table 1. Two

| Organism          | Straina | Antibiotic conc (µg/ml) | Polymyxin | Gentamicin | Kanamycin | Neomycin |
|-------------------|---------|-------------------------|-----------|------------|-----------|----------|
| Histoplasma capsulatum | 1-M+    | 2 16 16 16 16 | 2 16 16 16 16 | 2 16 16 16 16 | 2 16 16 16 16 | 2 16 16 16 16 |
| Blastomyces dermatitidis | 24-M    | 1 2 16 16 16 | 1 2 16 16 16 | 1 2 16 16 16 | 1 2 16 16 16 | 1 2 16 16 16 |
| Cryptococcus neoformans | 35-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |
| Nocardia asteroides | 36-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |
| Coccidioides immitis | 37-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |
| Sporothrix schenckii | 38-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |

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|-------------------------|-----------|------------|-----------|----------|
| Histoplasma capsulatum | 1-M+      | 2 16 16 16 16 | 2 16 16 16 16 | 2 16 16 16 16 | 2 16 16 16 16 |
| Blastomyces dermatitidis | 24-M    | 1 2 16 16 16 | 1 2 16 16 16 | 1 2 16 16 16 | 1 2 16 16 16 | 1 2 16 16 16 |
| Cryptococcus neoformans | 35-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |
| Nocardia asteroides | 36-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |
| Coccidioides immitis | 37-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |
| Sporothrix schenckii | 38-M    | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 | 4 16 16 16 16 |

* M = mycelial phase; Y = yeast phase.
* 2+ Growth.

Histoplasma mycelial strains partially inhibited by gentamicin showed at least 2+ growth at the higher concentrations of antibiotic. Kanamycin and neomycin both produced significant inhibition of Nocardia but otherwise were uninhibitory. S. schenckii appeared to be the organism least affected by the antibiotics recorded in Table 1. B. dermatitidis, C. neoformans, and C. immitis were unaffected by the antibiotics studied except for polymyxin.

Table 2 records the results of a comparison of chloramphenicol alone and in two other antibiotic combinations against the five strains of N. asteroides. All of the organisms included in this
Table 2. Growth of Nocardia asteroides on media containing chloramphenicol alone and in two antibiotic combinations

| Strain | Control plate | C | C + K | C + K + G |
|--------|---------------|---|-------|----------|
| 15     | 4+            | 4+ | 1+    | 1+       |
| 31     | 4+            | 4+ | 3+    |          |
| 61     | 4+            | 3+ | 3+    |          |
| 63     | 4+            | 4+ | 1+    |          |
| 78     | 4+            | 4+ | 1+    |          |

* C = chloramphenicol, 16 μg/ml; K = kanamycin, 16 μg/ml; and G = gentamicin, 4 μg/ml.

The report were evaluated with chloramphenicol and kanamycin alone and combined with gentamicin at the same concentrations, but none was inhibited except for four strains of N. asteroides. However, chloramphenicol at 16 μg/ml alone does not appreciably inhibit any of the five N. asteroides strains.

DISCUSSION

Since the frequency of isolation of gram-negative bacilli is increasing in the diagnostic microbiological laboratory and also since these organisms usually grow in 24 hr, it has become increasingly apparent that antibiotics more effective against these organisms must be used in media for the isolation of the slow-growing fungi and N. asteroides. The five antibiotics used in this study have bacterial susceptibility patterns which suggested that they may be useful for this purpose.

The present results suggest that gentamicin might be a useful agent to inhibit bacteria but not fungi or N. asteroides. In vitro studies in our laboratory have shown the following percentages of commonly isolated strains susceptible to gentamicin at 5 μg/ml: Staphylococcus aureus, 100%; S. epidermidis, 100%; Escherichia coli, 100%; Klebsiella sp., 100%; Enterobacter sp., 100%; Serratia sp., 100%; Salmonella sp., 100%; Citrobacter sp., 100%; Proteus sp., 100%; Providence sp., 100%; Pseudomonas sp., 96%; Herellea sp., 80%; Mima sp., 81%; and Alcaligenes sp., 50%. These organisms include the majority of those found to be overgrowing fungi cultures.

It was decided to test chloramphenicol alone only against N. asteroides for the following reasons. First, McDonough et al. (4) had shown that the mycelial phases of B. dermatitidis, H. capsulatum, and S. schenckii are not inhibited by this antibiotic at concentrations up to 200 μg/ml. Second, other studies (Dolan and Woodward, Amer. J. Clin. Pathol., in press) have shown that 50 strains of C. neoformans and 15 strains of other Cryptococcus were not inhibited by chloramphenicol at concentrations up to 125 μg/ml. Third, I have found (unpublished data) that the mycelial phase of C. immitis is not inhibited by chloramphenicol at these concentrations. Studies of chloramphenicol alone (Table 2) showed that it does not appreciably inhibit the five strains of N. asteroides. Thus, the results in Tables 1 and 2 show that gentamicin and chloramphenicol do not inhibit the five strains of N. asteroides tested, but kanamycin does.

In vitro susceptibility testing in our laboratory has shown that only about 10% of the group D streptococci isolates would be inhibited by gentamicin at 4 μg/ml. However, about 90% or more of these isolates would be inhibited by chloramphenicol at 16 μg/ml. Thus, the addition of this antibiotic would enhance the spectrum of bacterial inhibition. Also, since strains of fungi and N. asteroides susceptible to the concentrations of chloramphenicol and gentamicin recommended here could conceivably occur in clinical specimens, it is imperative to inoculate a medium not containing antibiotics along with a medium containing these antibiotics.

In this study, no attempt was made to compare the yeast conversion of the dimorphic fungi on antibiotic-containing medium. The addition of antibiotics to media used for yeast conversion usually is not necessary unless an attempt is made to isolate the yeast phase directly from the clinical specimen. The majority of laboratories prefer to isolate the fungus in the mycelial phase and then convert isolated colonies to the yeast phase. A comparable study of Candida sp., Torulopsis sp., Aspergillus sp., and other fungi is in progress.

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LITERATURE CITED

1. Ajello, L., L. K. Georg, W. Kaplan, and L. Kaufman. 1963. Laboratory manual for medical mycology. Public Health Service (Publication 994), National Communicable Disease Center, Atlanta, Georgia.
2. Cohen, S. N. 1969. Modified Sabouraud medium containing neomycin and polymyxin. Appl. Microbiol. 17:486-487.
3. Georg, L. K., L. Ajello, and C. Papageorge. 1954. Use of cycloheximide in the selective isolation of fungal pathogenic to man. J. Lab. Clin. Med. 44:422-428.
4. McDonough, E. S., L. Ajello, L. K. Georg, and S. Brinkman. 1960. In vitro effects of antibiotics on yeast phase of Blastomyces dermatitidis and other fungi. J. Lab. Clin. Med. 55:116-119.
5. McDonough, E. S., L. K. Georg, L. Ajello, and S. Brinkman. 1960. Growth of dimorphic human pathogenic fungi on media containing cycloheximide and chloramphenicol. Mycopathol. Mycol. Appl. 13:113-120.
6. Taplin, D. 1965. The use of gentamicin in mycology media. J. Invest. Dermatol. 45:549-550.
7. Thompson, L. 1945. Note on a selective medium for fungi. Proc. Staff Meet. Mayo Clin. 20:248-249.