Clinical Evaluation of the Addition of Gentamicin to Commercially Prepared Mycological Media

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A simple procedure is presented whereby an antibiotic solution can be added to prepared agar media for conversion to a selective medium to isolate fungi. Gentamicin solution was deposited onto slants of a variety of previously prepared agar media, allowed to diffuse overnight, and then the slants were inoculated with clinical specimens. Control media without gentamicin included a cycloheximide-chloramphenicol medium (CC), Sabouraud dextrose agar (SDA), and brain heart infusion agar (BHI). Of 75 specimens originating from the respiratory tract, the fungi isolated were predominantly yeast; 35, 39, and 43 were positive on CC, SDA, and SDA with gentamicin, respectively, incubated at 25 C. At 37 C, 32, 34, and 41 positive cultures were obtained with the same media, respectively. The same specimens, inoculated onto BHI with and without gentamicin, yielded 23 and 39 positive cultures, respectively. Of 90 specimens that were either urine, cutaneous, or mucocutaneous, the predominant flora again were yeasts, although on nine occasions dermatophytes were isolated. Positive cultures, 32, 34, and 41, were obtained with CC, SDA, and SDA containing gentamicin, respectively. Bacterial contamination was significantly reduced by the gentamicin, especially on BHI incubated at 37 C. None of the specimens was positive for systemically pathogenic fungi, other than species of *Candida*, *Torulopsis*, and *Aspergillus*. The effectiveness of varying concentrations of gentamicin was investigated by comparing growth of recently isolated bacteria. Of the bacterial isolates, 33% grew on CC, 16% grew on SDA containing gentamicin, 50 µg/ml, and 3% grew on SDA with a gentamicin concentration at 100 µg/ml. With BHI, 3% grew in the presence of 50 µg of gentamicin/ml and <1% grew at 100 µg of gentamicin/ml.

Gentamicin is one of the newer antibiotics of the aminoglycoside group (20). It possesses a broad spectrum of activity against many gram-positive bacteria and most gram-negative bacteria as well, including relatively resistant species such as *Proteus* and *Pseudomonas* (19). It has found wide acceptance in tissue culture and virology laboratories as a preventive of bacterial contamination since there is no interference with virus replication (4, 12-14). In addition, gentamicin is stable over a wide pH range (14) and when autoclaved. Both of these factors are obvious advantages for the clinical laboratory.

A number of antimicrobial agents, such as chloramphenicol, tetracycline, neomycin, polymixin B, penicillin, and streptomycin, have been used to suppress bacterial contaminants in mycological media (5-7). However, some are not satisfactory, either because of instability or

because they inhibit some isolates of certain fungi (8). Furthermore, most are not effective against many of the gram-negative bacteria, especially *Pseudomonas* and *Proteus* species (13, 14). Taplin was the first to add gentamicin to media for the isolation of fungi (17). He later developed a medium, dermatophyte test medium (DTM), for isolation of dermatophytes that contained gentamicin as well as tetracycline and cycloheximide (18). Since then, a number of reports have shown that gentamicin is a valuable aid in the isolation of fungi (8, 10, 15, 17).

Many mycologists purchase commercially prepared media, but gentamicin-containing media (except DTM) are not available commercially. Therefore, a method for adding gentamicin to previously prepared agar media was investigated.

MATERIALS AND METHODS

Gentamicin was obtained as gentamicin reagent solution (GRS, Schering Corp.), which is a sterile

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aqueous solution of gentamicin sulfate equivalent to 10 mg of gentamicin base per ml, and added to agar slants as follows. A 0.1-ml amount of gentamicin was deposited onto the surface of slants of Sabouraud dextrose agar (SDA, BBL, glucose [40 g/liter], pH 5.6) and BHI agar prepared from dehydrated medium (BBL) to give a concentration of 100 μ g/ml of medium. Each tube contained 10 ml of solidified agar medium. The tubes were then placed in a slanted position for a minimum of 18 h at room temperature. This allowed the gentamicin and synesis fluid to spread over the slant surface and diffuse throughout the medium. Specimens were then streaked onto the surface of the slants. SDA slants, with or without gentamicin, were incubated at 25 C whereas BHI slants, with or without gentamicin, were incubated at 37 C. In addition, a tube of a modified SDA containing cycloheximide and chloramphenicol, but no gentamicin (Mycosel, BBL), was also inoculated and incubated at 25 C. Cultures inoculated with specimens obtained from the respiratory tract were incubated at both 25 and 37 C, whereas other cultures were incubated only at 25 C.

All cultures were examined for fungi and bacteria three times a week for 3 weeks. Gram stains were done on all bacterial colonies and all fungi were identified by using standard procedures.

The efficacy of these same media to inhibit bacteria was evaluated also in the Agar Dilution Susceptibility Laboratory of the Microbiology Laboratory, Johns Hopkins Hospital. Media were prepared by starting with a dehydrated product and adding gentamicin in a final concentration of 50 or 100 μ g/ml to the cooled, molten agar before dispensing into gridded petri plates (10 by 10 cm), approximately 35 ml per plate. Plates were inoculated with recently isolated bacteria that had been selected for antibiotic susceptibility testing. Each isolate was inoculated into 2 ml of Mueller-Hinton broth (BBL) to produce a noticeable turbidity, incubated for 2 h in a 37 C water bath, and then adjusted with saline to the turbidity of a 0.5 McFarland standard, representing approximately 10^8 organisms per ml. Using a Steers replicating device (16), 0.01 ml of a 1:10 dilution of the standardized suspension of each isolate was spotted onto the surface of the media, with the final inoculum size being 10^5 organisms. The diluted bacterial suspension was subcultured onto Trypticase soy agar with 5% sheep blood and incubated at 37 C for 12 to 18 h to confirm purity of the culture.

For a part of the study, seven kinds of media were inoculated: SDA; SDA with gentamicin, 50 μ g/ml; SDA with gentamicin, 100 μ g/ml; the cycloheximide-chloramphenicol agar; BHI; BHI with gentamicin, 50 μ g/ml; BHI with gentamicin, 100 μ g/ml. The first four were inoculated in duplicate to allow incubation at both 37 and 25 C. Only single plates of the latter three were inoculated inasmuch as incubation was at 37 C only. For some of the studies, not all the media were used because, for example, it soon became apparent that virtually all of the isolates would grow on SDA without gentamicin. All plates were incubated overnight in an upright position and then read on a flat dark surface with the aid

of indirect lighting. Thick confluent growth, large granular growth, light areas with distinctly thick edges, or groups of colonies greater than three in number were considered positive (resistant to the antibiotic).

The studies using tubes of media with and without gentamicin were conducted on 165 routine clinical specimens received in the Mycology Laboratory of Johns Hopkins Hospital. These included 75 from the respiratory tract (sputa, bronchial washings, and transtracheal aspirations) and 90 that were either urine, skin, or mucocutaneous. Specimens from usually sterile sites, such as bone marrow and cerebrospinal fluid, were not included.

RESULTS

Table 1 shows the recovery rates and distribution of fungi isolated from the 75 specimens originating from the respiratory tract. The recovery of yeasts at 25 C was approximately equal on the three media incubated at that temperature. Filamentous fungi of the genus *Aspergillus* were inhibited on the cycloheximide-chloramphenicol agar but not on the SDA or SDA plus gentamicin. At 37 C, recovery of fungi, especially *Candida albicans*, was significantly better on BHI plus gentamicin than on plain BHI, and the gentamicin significantly reduced contamination due to bacteria. In four of the specimens, nonpigmented septate hyphae compatible with the morphology of *Aspergillus* and other *Hyphomycetes* had been seen with the microscope before culture; in 18 others, yeasts were observed, with and without hyphal fragments.

The bacterial contamination encountered in respiratory specimens is shown also in Table 1. At 25 C, contamination was significantly reduced on both the cycloheximide-chloramphenicol medium and the SDA containing gentamicin, with the latter being the more effective, especially against gram-negative rods.

Table 2 presents the results with the 90 specimens of urine, skin, or mucocutaneous tissue, of which 9 were microscopically positive for hyphae and 11 were positive for yeast, with or without hyphal fragments. The recovery of nine species (all yeasts and dermatophytes) was superior on the tubes of SDA plus gentamicin compared to the recovery on plain SDA or the cycloheximide-chloramphenicol agar, and the bacterial contamination was reduced maximally on the agar containing the gentamicin. Both the SDA plus gentamicin and the cycloheximide-chloramphenicol agar inhibited most of the gram-positive cocci, but the SDA plus gentamicin was the more inhibitory to gram-negative rods.

The efficacy of gentamicin in SDA and BHI to

TABLE 1. Recovery of fungi and incidence of bacterial contamination on tubed agar slants, with and without gentamicin, and inoculated with 75 clinical specimens from the respiratory tract

Organism	Media and temp of incubation				
	25 C			37 C	
	CC	SDA	SDA+G ^a	BHI	BHI+G ^b
Fungi					
<i>Candida albicans</i>	33	32	35	17	33
<i>C. tropicalis</i>	1	2	3	1	1
<i>C. krusei</i>	1	1	1	1	1
<i>Aspergillus niger</i>	0	1	1	1	1
<i>A. flavus</i>	0	3	3	3	3
Bacteria					
Gram-positive cocci	3	18	3	26	7
Gram-positive rods	0	0	0	0	0
Gram-negative rods	8	17	5	14	4

^a CC, Cycloheximide-chloramphenicol medium (Mycosel, BBL).^b SDA+G, Sabouraud dextrose agar containing 100 µg of gentamicin per ml.^c BHI+G, Brain heart infusion containing 10 µg of gentamicin per ml.

TABLE 2. Recovery of fungi and incidence of bacterial contamination on tubed agar slants, with and without gentamicin, inoculated with 90 specimens of urine, cutaneous or mucocutaneous tissues and incubated at 25 C

Organism	Media		
	CC	SDA	SDA+G ^a
Fungi			
<i>Candida albicans</i>	22	23	25
<i>C. tropicalis</i>	1	1	1
<i>C. parapsilosis</i>	1	2	2
<i>Torulopsis glabrata</i>	1	2	2
<i>Trichophyton rubrum</i>	1	1	2
<i>T. mentagrophytes</i>	1	1	1
<i>T. tonsurans</i>	3	1	2
<i>Epidermophyton floccosum</i>	1	1	2
<i>Microsporum gypseum</i>	1	0	1
<i>Aspergillus</i> sp.	0	2	3
Bacteria			
Gram-positive cocci	2	21	1
Gram-positive rods	0	1	0
Gram-negative rods	8	16	5

^a SDA+G, Sabouraud dextrose agar containing 100 µg of gentamicin per ml.

inhibit bacteria is further presented in Table 3. The SDA without gentamicin, used as a control, showed growth of most of the recent clinical bacterial isolates, whereas the gentamicin suppressed bacterial growth significantly, and the concentration of 100 µg/ml was superior to 50 µg/ml under certain of the conditions. The gentamicin at either concentration was superior to the cycloheximide-chloramphenicol agar. These results were similar irrespective of the temperature of incubation. The efficacy of gentamicin added to BHI, and incubated at 37 C, was even more striking than with the SDA. Of the recent clinical isolates of bacteria, 97% were capable of growth on the BHI without

TABLE 3. Comparative inhibition of bacteria, recently isolated from clinical specimens and cultivated on petri plates of media, with or without gentamicin

Media	No. of isolates that grew/no. plated	%
SDA ^a	127/141	90
SDA plus gentamicin (50 µg/ml)	68 ^b /432	16
SDA plus gentamicin (100 µg/ml)	19 ^b /573	3
CC ^c	70 ^b	33
BHI ^d	328/338	97
BHI plus gentamicin (50 µg/ml)	9 ^b /338	3
BHI plus gentamicin (100 µg/ml)	2 ^b /338	<1

^a Incubated at 25 and 37 C.^b Predominantly gram-negative rods.^c Incubated at 25 and 37 C.^d Incubated only at 37 C.

gentamicin but were inhibited by either 50 µg or 100 µg of gentamicin per ml; growth was only 3 and <1%, respectively.

DISCUSSION

This study was undertaken to determine whether gentamicin, an agent of known value for selective isolation of fungi, could effectively be added to a previously prepared agar slant. This was accomplished by simply adding an aqueous solution of the antibiotic to the agar surface and allowing the slant to remain at ambient temperature for at least 18 h. The method was also found satisfactory for agar plates, but we have emphasized the results with slants because of their greater clinical usefulness, at least in our experience. The advantage of this procedure is that one can

purchase commercially prepared nonselective media and render them selective for fungi as required.

Although Taplin (17), Dolan (9), and Larsh and Goodman (10) have shown gentamicin to be useful for the isolation of fungi, they used gentamicin in lower concentrations than we found effective, or in combination with other antibiotics. In an earlier preliminary study, the results of which are not reported here, we tried 50 μ g of gentamicin per ml in all media, but, while this proved effective in BHI agar, the results with SDA agar were disappointing. This is not surprising when it is considered that aminoglycoside antibiotics are much more active at an alkaline pH. Smith et al. (15) found that 40 μ g of gentamicin per ml added to Littman oxgall medium was not effective in inhibiting gram-negative bacteria from the intestinal tract. They attributed this to the frequent presence of gentamicin-resistant strains in their hospital. Our results with pure cultures of recently isolated bacteria showed that 16% of the organisms could grow on SDA with 50 μ g of gentamicin per ml, but only 3% grew at a concentration of 100 μ g/ml; and 33% of the isolates were not inhibited by chloramphenicol in an SDA containing cycloheximide as well as the antibacterial agent. Also apparent was the fact that the low pH (5.6) of SDA does not prevent the growth of the vast majority of bacteria found in clinical specimens.

The improved isolation of fungi on the media containing gentamicin is attributed to the suppression of contaminating bacteria. Although it is difficult to provide graphic evidence for this, the technologists reading the cultures were impressed by the ease of detecting fungal growth on the gentamicin-treated media and the ease of obtaining pure cultures for further identification.

In agreement with the experience of others (e.g., reference 1), the presence of cycloheximide appeared to inhibit some yeasts, such as *C. tropicalis*, and would have prevented diagnosis of the two cases of pulmonary aspergillosis (*Aspergillus niger* and *A. flavus*), reemphasizing the need to use more than one isolation medium. The presence of cycloheximide did not appear to offer a significant advantage in the isolation of the seven dermatophytes recovered in this study, contrary to the experience of others (e.g., reference 1).

The effect of gentamicin was especially notable in BHI incubated at 37 C. This combination should prove advantageous in the rapid isolation of yeasts and may prove to be advantageous in the diagnosis of systemic mycoses. It should be noted, however, that the present

study did not include *Histoplasma*, *Blastomyces*, *Coccidioides*, *Cryptococcus*, or other of the important fungal pathogens; thus no conclusion can be drawn regarding the efficacy of these media for isolation of these organisms. It should be further noted that gentamicin or other aminoglycosides are inhibitory to *Nocardia* species, with about three-fourths of isolates susceptible to 50 μ g of gentamicin per ml (2, 3, 11). Therefore, a medium without antibacterial antibiotics should be included when these organisms are suspected.

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