Comparison of Charcoal- and Starch-Based Media for Testing Susceptibilities of *Legionella* Species to Macrolides, Azalides, and Fluoroquinolones

SUSAN L. PENDLAND,1 STEVEN J. MARTIN,1 CONNIE CHEN,1 PAUL C. SCHRECKENBERGER,2 AND LARRY H. DANZIGER1*

Department of Pharmacy Practice1 and Department of Pathology,2 University of Illinois, Chicago, Illinois

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We compared growth characteristics of 46 *Legionella* strains grown on buffered charcoal yeast extract α (BCYEα) agar and buffered starch yeast extract (BSYE) agar and MICs of macrolides, azalides, and fluoroquinolones for these organisms. Growth was poor and not reproducible on BSYE agar. Growth was excellent on BCYEα, and MICs were easy to interpret. BCYEα is superior to BSYE for testing susceptibilities of *Legionella* species by agar dilution.

Currently, there is no standardized method for testing susceptibilities of *Legionella* species. Some investigators advocate using tissue culture models to more accurately reflect the activities of agents against these intracellular pathogens (10). Neither the intracellular models nor labor-intensive agar or broth dilution methods are feasible for most hospital laboratories. The E-test appears to be a viable alternative for determining susceptibilities of these fastidious organisms (16). However, it does not resolve the problem of which medium to use for testing the synergy of antimicrobial agents. Buffered charcoal yeast extract (BCYE) agar is the medium of choice for isolation of *Legionella* species (14). Because of the excellent growth obtained on BCYE agar, initial susceptibility studies were also conducted with this medium (15). When the charcoal in BCYE was shown to inhibit the activities of some antimicrobial agents, studies were conducted to determine a better medium for testing susceptibility (1, 2, 11). The most widely studied include buffered yeast extract (2, 5, 12) and buffered starch yeast extract (BSYE) broths and agar media (3, 4, 6–9, 11, 17, 18). Our studies of *Legionella* susceptibility on BSYE agar were plagued by poor growth, particularly with non-*Legionella pneumophila* species. This resulted in our reevaluation of media for susceptibility and synergy testing. The purpose of this study was to compare BCYEα and BSYE media for testing by agar dilution the susceptibilities of *Legionella* species to macrolides, azalides, and fluoroquinolones.

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Organisms were obtained from the University of Illinois Hospital (Chicago, Ill.), Northwestern University Hospital (Chicago, Ill.), Abbott Laboratories (Chicago, Ill.), the Centers for Disease Control and Prevention (Atlanta, Ga.), and the American Type Culture Collection (Rockville, Md.). The *Legionella* organisms tested included 41 clinical isolates and 5 American Type Culture Collection (ATCC) strains grown on BCYE agar. The 11 remaining species included *L. bozemanii* (n = 3), *L. longbeachae* (n = 3), *L. dumoffii* (n = 2), *L. gormanii*, *L. erythra*, and *L. micdadei*.

The *Legionella* organisms were maintained on BCYE agar (Micro Diagnostics, Inc., Lombard, Ill.). Susceptibility testing was performed with both BCYEα agar (Oxoid-Unipath, Ogdensburg, N.Y.) and BSYE agar. The BSYE agar was prepared according to the directions of Sawatari et al. (19). Test tubes containing the antibiotics and molten agar medium (50°C) were mixed three times by gently inverting the test tubes prior to dispensing their contents into petri plates. The total volume in each petri plate was 20 ml, which consisted of 1.0 ml of antibiotic and 19 ml of BCYEα or BSYE agar. The BCYEα and BSYE media were prepared 1 day prior to testing and stored overnight in plastic bags at 4 to 6°C.

Antibiotics were prepared according to National Committee for Clinical Laboratory Standards guidelines (13) or per the manufacturer’s recommendations. Final antibiotic concentrations (in micrograms per milliliter) tested on BCYEα agar were as follows: 0.12 to 2 for erythromycin (Abbott Laboratories), 0.06 to 1.0 for azithromycin (Pfizer Laboratories, New York, N.Y.), 0.06 to 1.0 for clarithromycin and 14-hydroxy clarithromycin (Abbott Laboratories), and 0.12 to 2 for ciprofloxacin (United States Pharmacopeia, Rockville, Md.) and levofloxacin (R. W. Johnson Pharmaceutical Research Institute, Raritan, N.J.). Final antibiotic concentrations (in micrograms per milliliter) tested on BSYE agar were as follows: 0.004 to 0.12 for erythromycin and azithromycin and 0.001 to 0.03 for clarithromycin, 14-hydroxy clarithromycin, ciprofloxacin, and levofloxacin.

The agar dilution method was used to determine the MIC of each antibiotic (13). MICs were determined on the two media with a low (10⁴ CFU/spot) inoculum and a high (10⁶ CFU/spot) inoculum. For the high inoculum, a direct suspension of the organisms was prepared in sterile water to match the turbidity of a 0.5 McFarland standard with a spectrophotometer at 625 nm. A replicator device (Craft Machine Inc., Chester, Pa.) was used to inoculate approximately 8 μl of each bacterial suspension onto the BCYEα and BSYE agar plates, resulting in a final inoculum of approximately 10⁶ CFU/spot. For the low inoculum, a portion of each bacterial suspension was then further diluted 1:100. With the replicator device, the final bacterial inoculum yielded approximately 10⁴ CFU/spot. All tests were performed in duplicate, and all plates were incubated at
35°C in humidified room air for 72 h. BCYEα and BSYE agar plates without antibiotics were used as a growth control, while blood agar plates (Micro Diagnostics, Inc.) were included as a control to ensure the purity of the bacterial inoculum. The MIC was read as the lowest concentration of an antimicrobial agent showing no visible growth or only a faint haze. MICs were read at 48 and 72 h of incubation.

Growth was excellent and MICs were easy to read for all Legionella strains on BCYEα medium when we used an inoculum of 10⁴ CFU/spot and an incubation time of 48 h. Growth characteristics and MICs obtained on BCYEα medium with an inoculum of 10⁶ CFU/spot or incubation period of 72 h did not differ significantly from those obtained with the lower inoculum or shorter incubation time. Similar growth could not be duplicated on BSYE agar (Fig. 1). Although some growth of L. pneumophila strains was supported on BSYE medium, growth was poor and not reproducible at an inoculum of 10⁴ CFU/spot and enhanced growth was seldom observed when the incubation time was increased from 48 to 72 h. We observed an increase in growth quantity and quality (i.e., more luxuriant growth) when the inoculum size was increased to 10⁶ CFU/spot. The non-L. pneumophila strains did not grow at all on BSYE medium with the low inoculum. At the high inoculum most of the organisms grew, but growth was sparse and not reproducible. Figure 1 clearly demonstrates the difference in growth characteristics that we observed with the different media.

The MICs (MICs at which 90% of the isolates are inhibited [MIC₉₀s] and ranges) of all antibiotics on BCYEα and BSYE media for all organisms are listed in Table 1. The MICs obtained on BCYEα agar were approximately 5 twofold dilutions higher than those obtained on the BSYE medium. MICs determined on BCYEα agar were easy to interpret, and results were reproducible. Several investigators (3, 4, 7–9, 11, 17, 18) have recommended BSYE over BCYEα medium, which precluded accurate determination of MICs with BSYE media. Our findings support the conclusions of Edelstein and Edelstein in that BSYE agar did not support the growth of our non-L. pneumophila strains as well as that of some of the L. pneumophila strains. In addition, we studied the effects of different inoculum sizes and different incubation times. We found that the MICs with BCYEα were 4 to 6 dilutions higher or approximately 32-fold greater than with BSYE media.

Based on the results obtained in our laboratory, we chose BCYEα agar for in vitro synergy testing of macrolides and fluoroquinolones against Legionella species and for determining the susceptibilities of these organisms. Excellent growth characteristics, easily readable endpoints, and consistent reproducibility were the primary reasons for our choice of BCYEα medium. While we acknowledge that charcoal does result in some inactivation of antimicrobial activity, we believe...
that the falsely elevated MIC results are preferable to potentially erroneous low results obtained because of poor growth. Buffered yeast extract broth medium is an alternative to BCYE agar in that it supports the growth of almost all Legionella species and is much less inhibitory for antibiotics (6).

Based on our experience, we advocate the use of BCYE over BSYE medium for in vitro testing by agar dilution of susceptibilities of Legionella species and synergy testing of drugs against them.

REFERENCES


