Protocol

Broth Microdilution and Disk Diffusion

Test of Erythromycin and Clindamycin by Jorgensen et al. (2011)

Introduction

GoldBio utilizes two different laboratory standard tests to ensure that our antibiotics exceed standards for purity and effectiveness against bacteria. Broth microdilution method, the most popular method in the United States, is used because of its accuracy and clear results. In this method, wells are filled with broth containing different concentrations of the antibiotic. These are then inoculated with bacteria and incubated overnight. The next day, the minimal inhibitory concentration is determined. The use of the disk diffusion test, also called the Kirby-Bauer method, allows for the concurrence of results with the broth microdilution method, establishing a 'zone of inhibition' with which to gauge the efficacy of the antibiotic in question. In this method, agar plates are inoculated with the organism and small filter paper disks containing antibiotic are placed on the agar surface. The plate is then incubated and the zone of inhibition around each disk is determined. The use of these two methods of analysis allow GoldBio to ensure that our antibiotics are effective against the appropriate bacteria. Here, we describe a general protocol for these methods and the procedure that GoldBio follows when testing antibiotics.

Materials

- Mueller-Hinton broth, pH between 7.2-7.4
- Lysed horse blood
- Sheep blood
- pH meter
- Microdilution plates with conical bottom wells
- Distilled water
- Plastic bags
- Paper disks
- Antibiotics

Preparation of Mueller-Hinton agar:

- Prepare broth from dehydrated medium according to manufacturer's instructions.
- Autoclave and chill overnight at 2-8°C or in an ice bath.
- Ensure the pH falls between 7.2-7.4.
- Add additional cations if necessary.
- Add 2.5-5% (v/v) lysed horse blood.
- Check pH and confirm that it remains between 7.2-7.4.

Note: Do not add supplemental calcium or magnesium cations. Depending on the type of bacteria being used, additional cations might be necessary. Correct concentrations of divalent cations are: 20 to 25 mg of Ca^{++}/L for daptomycin and 10 to 12.5 mg of Mg^{++}/L.

Preparation of lysed horse blood

- Freeze and thaw defibrinated horse blood until the blood is lysed (5-7 freeze-thaw cycles).
- Asceptically mix equal volumes of lysed blood and sterile, distilled water.
- Centrifuge at 12, 000 g for 20 minutes.
- Decant the supernatant and recentrifuge if necessary.
- Add to broth for a final concentration of 2.5-5% lysed horse blood.

Method

Broth microdilution tests

This test involves the use of small volumes of broth dispensed in sterile microdilution plates with conical bottom wells. Each well should contain 0.1 ml of broth.

1. Add 0.1 (± 0.02) ml of broth containing antibiotic to each well. Include a growth control well and a sterility control (uninoculated well).

   Note: If inoculum is to be added by pipette. then prepare antibiotic solutions at twice the desired final concentration and fill wells to 0.05 ml.

   Note: Antibiotic dilutions are easily prepared in 10 ml of broth and dispensed into the wells.

   Note: GoldBio tested antibiotics using frozen panels that included Erythromycin (GoldBio Catalog # E-300) and Clindamycin (GoldBio Catalog # C-175), which were tested separately to define the minimum inhibitory concentration (MIC), and combinations of erythromycin and clindamycin of 1 µg/ml + 0.25 µg/ml and 1 µg/ml + 0.5 µg/ml in separate wells, based upon the results of a prior study. Each panel included a growth control well and a negative (medium only) control well.

2. Seal the plates in plastic bags and freeze at ≤ -20°C (≤ -60°C is best).

3. On the day of testing, inoculate panels with the standard density of 5 x 10^5 CFU/ml.

4. Seal plate in a plastic bag before incubating to prevent drying.

5. Incubate for 16-20 hours at 35 ± 2°C prior to visual determination of MICs.

Disk diffusion D-zone tests

1. Prepare agar as previously described. However, add 5% sheep blood instead of lysed horse blood.

2. Inoculate the plates with bacterial colonies that have reached 0.5 McFarland turbidity standard.

3. Place erythromycin (15 µg) and clindamycin (2 µg) disks 12 mm apart.

4. Incubate the plates at 35°C in 5% CO_2 for 20-24 hours.

5. Examine the plates. A positive D-zone test was noted by flattening of the clindamycin zone adjacent to the erythromycin disk with erythromycin-resistant isolates.

Associated Products

- Erythromycin (GoldBio Catalog # E-300)
- Clindamycin (GoldBio Catalog # C-175)

References

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