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Methods to Evaluate Colistin Heteroresistance in *Acinetobacter baumannii*

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Summary

II.

The nosocomial pathogen *Acinetobacter baumannii* is a growing threat to public health due to its increasing resistance to antibiotics including the last-line polymyxin, colistin. Heteroresistance to colistin has been described in *A. baumannii*, wherein a resistant subpopulation of cells co-existing with a majority susceptible subpopulation actively grows in the presence of antibiotic and can cause treatment failure. The shortcomings of diagnostic tests in detecting colistin heteroresistance are especially worrisome as they may lead to clinicians unknowingly prescribing an ineffective antibiotic, leading to increased patient morbidity and mortality.

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1. Lysogeny Broth agar plates containing colistin result in difficulty spot plating. If this becomes a problem during population analysis profile assays, we recommend pouring thinner plates than you may normally pour (15mL or less), and allowing plates to dry in the biosafety cabinet for 10-15 minutes prior to plating. While it is our recommendation to plate 10μL per dilution so as to conserve both time and plates, it is possible to use one LB plate per dilution.
2. For all assays, we recommend counting colonies at 24 hours after plating, and leaving plates at 37°C for an additional 24 hours to observe slow growing colonies within the bacterial population.
3. To control for the variance that appears by creating population analysis profile plates, it is advised to use a control strain where percent survival can be verified for each set of PAP plates that are prepared to ensure consistency with results.
4. Plates can be dried in a biosafety cabinet for 30 min with the lid removed, 20-25°C overnight, or at 35°C with the lid removed for 15 min.
5. Alternatively, a direct colony suspension method can be used in which morphologically similar colonies are picked directly from a plate and resuspended in saline and adjusted to a turbidity resulting in a $1-2 \times 10^8$ CFU/ml suspension.
6. Do not allow the suspension to sit for more than 60 min to prevent overgrowth and incorrect interpretation if MIC is also desired.
7. If inoculated plates are left sitting for prolonged periods of time before the disc is applied, the bacteria may begin growing in the absence of antibiotic, leading to erroneous results.
8. An empty sterile petri dish may be used to prepare discs.
9. If colonies are present.
10. It may require several passages before the subpopulation frequency begins to lower, if after several passages the percent resistance does not decrease then the increase may be due to conventionally resistant bacteria.

Several techniques can be used to detect heteroresistance and the purpose of this chapter is to outline effective methods for identifying, quantifying and analyzing heteroresistance to colistin in *A. baumannii*. We will highlight the advantages and disadvantages of techniques including population analysis profile (PAP), Etest, and disc diffusion, as well as additional methods to distinguish heteroresistance from other forms of resistance. While the scope of this chapter will focus on colistin heteroresistance in *A. baumannii*, these techniques can be adapted for the study of heteroresistance to other antibiotics and in other bacteria with slight modifications.

Keywords

Heteroresistance; resistant subpopulations; population analysis profile; antibiotic susceptibility testing; phenotypic antibiotic resistance; polymyxin; colistin

1. Introduction

Acinetobacter baumannii is a Gram-negative opportunistic nosocomial pathogen causing multiple types of infections including ventilator-associated pneumonia (VAP) and bacteremia, primarily in immunocompromised patients^{1, 2}. This pathogen is a growing global health concern due to its increasing resistance to antibiotics. The Centers for Disease Control and Prevention have categorized multidrug resistant *A. baumannii* as a serious threat to public health and the World Health Organization listed *A. baumannii* as the top priority antibiotic resistant pathogen for new drug development^{3, 4}. Unfortunately, isolates resistant to all available antibiotics have emerged, making this organism a significant obstacle to treat in the clinic.

To combat the growing threat of antibiotic resistance, we must address remaining gaps in our understanding of resistance mechanisms. Heteroresistance is an underappreciated and understudied phenomenon wherein a small subpopulation of bacterial cells resist an antibiotic, while the remainder of a clonal population is susceptible⁵. This is in contrast to “conventional” resistance in which the entire population is resistant to the antibiotic. During drug treatment of a heteroresistant isolate, the susceptible population is killed while the resistant cells survive and can rapidly expand. This rapid expansion makes this phenomenon distinct from persistence, in which a metabolically quiescent population of cells remains dormant and thereby resists antibiotics⁶. Heteroresistance was first described in 1947 and has since been identified in both Gram-negative and Gram-positive organisms against multiple classes of antibiotics^{7–14}.

The frequency of the resistant subpopulation in heteroresistant strains can vary, and when it is exceedingly low (less than ~1 in 10,000 cells), this form of resistance is often undetected by current diagnostic tests. Recently, Band *et al* described strains of *Enterobacter cloacae* exhibiting heteroresistance (either detected or undetected) to the last-line polymyxin antibiotic colistin that caused colistin treatment failure and subsequent lethal infections in mice¹⁵. These results clearly demonstrated that heteroresistance can mediate failure of antibiotics *in vivo*. The shortcomings of diagnostic tests in detecting colistin heteroresistance is especially worrisome as this may lead to clinicians unknowingly prescribing ineffective antibiotics, causing increased patient morbidity and mortality.

Heteroresistance to colistin has been reported in *A. baumannii*^{16–21}. The purpose of this chapter is to detail effective methods of detecting, quantifying and analyzing heteroresistance to colistin in *A. baumannii*.

2. Materials

Lysogeny Broth (LB) - *Approximate formula per liter: Tryptone (10.0g), Yeast Extract (5.0g), Sodium Chloride (10.0g)*

Lysogeny Broth (LB) Agar - *Approximate formula per liter: Tryptone (10.0g), Yeast Extract (5.0g), Sodium Chloride (10.0g), Agar (15.0g)*

Sterile 100mm x 15mm polystyrene petri dishes

LB Agar plates

Sterile phosphate-buffered saline (PBS)

Sterile 5 mL polystyrene tubes

Sterile 96-well polystyrene plates

Blank 6 mm filter paper discs

Colistin Sulfate (*Sigma-Aldrich*)

Sterile 1 µL inoculating loops

Colistin Etest Strips (*Biomerieux*)

Bacterial strains

3. Methods

Unless otherwise mentioned, all steps are to be performed at room temperature and in a biosafety cabinet. Working stocks of colistin should be prepared fresh for each experiment and diluted in sterile water.

Population analysis profile (PAP)

Population analysis profile (PAP) is the gold standard method for detecting heteroresistance. Briefly, this technique involves the quantification of the proportion of resistant cells existing within a culture at a variety of antibiotic concentrations. PAP is the only method for identifying heteroresistance that also quantitatively determines the frequency of the resistant subpopulation of cells. While it is the most reproducible and reliable method for detecting heteroresistance, it is also more time-consuming and requires more materials than other methods²².

Day 1

1. Streak out isolates to be tested from freezer stocks on Lysogeny Broth (LB) agar plates and incubate overnight at 37°C.

Prepare Lysogeny Broth agar plates as described:

- a) Dissolve LB agar in sterile H₂O on a magnetic stir-plate.
- b) Autoclave LB agar.
- c) Cool LB agar in a water bath (60°C) for 1 hour.
- d) Remove agar from the water bath and place on a spinning magnetic stir plate.
- e) When agar has cooled (hot, but not uncomfortable to the touch), add colistin.
- f) Final concentrations of colistin in LB agar should be 0, 1, 2, 4, 8, 16, 32 or 128µg/mL.
- g) Pour agar into petri dishes and let stand overnight or until plates harden.
- h) Store plates at 4°C - Do not use plates that are more than 10 days old.
 - i. Agar plates should have a depth of 4mm (approximately 31 mL in a 100mm circular plate).
 - ii. Plates should be dried before use with no visible drops of water on the agar surface or lid⁵.
 - iii. Plates may be stored at 4°C but must be properly dried before use to remove excess moisture.

Day 2

1. Pick an isolated colony with a sterile loop from the LB plates and inoculate into 2mL sterile Lysogeny Broth and grow at 37°C shaking at 225 rpm overnight (~16 hours).

Day 3

1. Dilute 10µL of the overnight culture into 90µL sterile PBS in a sterile 96-well polystyrene plate. Make 10x serial dilutions ranging in concentration from 10⁻¹ to 10⁻⁷.
2. Plate dilutions ranging from 10⁻² to 10⁻⁸ in 10µL drops on agar plates of each colistin concentration¹ (0µg/mL-128µg/mL).
3. Allow plates to dry with covers removed for 15 minutes or until no liquid is visible on plates.
4. Incubate plates at 37°C for 16 hours.

Day 4—Analysis of data should be as follows:

Count the number of colonies at the highest dilution factor at which there is growth. For statistical interpretation, only count dilutions wherein the countable colonies are between 10 and 100. If it appears there are fewer than 10 colonies at your lowest dilution factor, we recommend plating a sample from this dilution in a higher volume on a single plate. With these data, the proportion of colistin resistant cells at each concentration can be quantified using the following formula:

$$\frac{(\text{number of colonies on colistin plate} \times \text{dilution factor})}{(\text{number of colonies on antibiotic free plate} \times \text{dilution factor})}$$

Interpret results as follows:

Based on our laboratory's experience performing this assay, as well as commentary in the literature, we recommend the following interpretation of population analysis profile results 5, 15:

Susceptible: Proportion of surviving bacteria at 2µg/mL-128µg/mL is below 10⁻⁷ (the limit of detection).

Resistant: Proportion of surviving bacteria at a concentration above 2µg/mL is above 50%.

Heteroresistant: Proportion of surviving bacteria at a concentration at least four fold above the antibiotic's breakpoint is between 10⁻⁷ and 50%.

- a) Alternatively, dilutions can be plated with one sample per agar plate instead of the spot method. To do this, serial dilutions can be made by diluting 100 µL of overnight culture into 900µL sterile PBS in 5mL culture tubes, making 10x serial dilutions ranging in concentration from 10⁻¹ to 10⁻⁷. Once prepared, spread 100µL of each dilution onto an individual agar plate. To enumerate CFU, use the dilution that allows between 30-300 colonies to be counted and quantify using the methods mentioned above.

Etest

An alternative to PAP that can be utilized for heteroresistance testing are Etests. Etests are plastic strips manufactured to contain a gradient of antibiotic concentrations from top (highest concentration) to bottom (lowest concentration). These strips are applied to an agar plate on which bacteria have been streaked, and after incubation, a "zone of clearing" appears in which no bacterial growth is observed. The Minimum Inhibitory Concentration (MIC) for Etest is interpreted as the lowest concentration where the zone of clearing meets the Etest strip along the bottom edge. When some heteroresistant isolates are plated with an Etest strip, colonies can be observed within the zone of clearing. Compared to PAP, Etests are a non-quantitative method to observe heteroresistance as the appearance of colonies within the zone of clearing will depend on the frequency of the resistant subpopulation of cells (for heteroresistant isolates with a low frequency resistant subpopulation, colonies will not appear in the zone of clearing and thus these strains will not be classified as

heteroresistant using Etest). Another shortcoming is that Etest strips can be expensive and may not be ideal to screen numerous strains for heteroresistance. This method has been adapted and modified from the agar disc diffusion method standard described by CLSI²³ and is outlined below:

Day 1

1. Streak out bacterial strains from freezer stocks on Lysogeny Broth agar plates and grow overnight in a 37°C incubator.

Day 2

1. Pick an isolated colony with a sterile inoculating loop from the LB plates and transfer into 2mL sterile Lysogeny Broth. Subsequently grow at 37°C shaking at 225 rpm overnight (~16 hours).

Day 3

1. Prepare LB Agar plates for inoculation (see instructions and notes in the *population analysis profile* (PAP) section. LB agar plates used for Etest should not contain antibiotics).
2. Remove Etest strips from 4°C and allow them to reach room temperature before application (this takes about 30 minutes).
3. Dilute culture to be tested to between 1×10^8 - 2×10^8 CFU/ml (~0.5 McFarland Standard)⁶ and use the suspension within 15 min⁷.
4. Dip a sterile swab in the diluted culture tubes and rub off excess culture along the side of the tube. Swab along the surface of a dry petri dish (no visible water spots), rotate the plate 60 degrees, and then repeat these steps two more times. Allow excess moisture to dry before Etest strip application but do not exceed 15 min after inoculation⁸.
5. Apply Etest strip to the surface of the agar plate with the label side up. Make sure the strip does not shift during placement.
6. Within 15 min of Etest strip application, invert plates and incubate at 37°C for 16-20 hours.

Day 4—Examine plates for zones of clearing and then examine zones for any resistant colonies that may have appeared. Colonies can appear uniformly throughout the zone or close to the edge of the zone. Colonies may be potential spontaneous mutants rather than the resistant subpopulation of a heteroresistant strain and should thus be tested through the methods described in the “*Further distinction between heteroresistance and resistance*” section below.

Disc Diffusion

Disc diffusion is used to determine an organism’s MIC to an antibiotic using discs concentrated with the drug. Similar to Etest, this method is non-quantitative but can detect

heteroresistant isolates that harbor a resistant subpopulation present at a high frequency. Unlike Etest which contains a gradient of antibiotic concentrations, in disc diffusion, each disc is loaded with a specific concentration. The discs are placed on agar plates after bacteria have been spread out in a lawn. The antimicrobial diffuses radially from the disc creating gradients of antibiotic concentrations. After overnight incubation, the MIC for disc diffusion is interpreted by comparing the diameter of the zone of clearing and is drug and dose dependent. This method can be adapted to detect resistant subpopulations by observing colonies that may grow within the zone of clearing. While this method is not as sensitive as PAP, it can be useful to screen multiple strains for heteroresistance. Discs preloaded with antibiotic can be purchased commercially but can also be prepared in a laboratory. Preparing discs can be more cost effective when testing multiple isolates and can be used to work within a specific range of antibiotic concentrations for which commercial discs are not available. Results can vary if disc preparation or application is not carefully controlled. This method has been adapted and modified from the agar disc diffusion method standard described by EUCAST²⁴ and is outlined below:

Day 1

1. Streak out bacterial strains from freezer stocks on Lysogeny Broth agar plates (see instructions and notes in the *population analysis profile* (PAP) section. LB agar plates used for disc diffusion should not contain antibiotics) and grow overnight in a 37°C shaking incubator.

Day 2

1. Pick an isolated colony with a sterile inoculating loop from the LB plates and transfer into 2mL sterile Lysogeny Broth, and then grow at 37°C shaking at 225 rpm overnight (~16 hours).

Day 3

1. Prepare antibiotic discs (or antibiotic discs can be purchased from a manufacturer).
 - a. Prepare colistin sulfate stock solution (concentrations are based on total µg/disc so calculate accordingly, ex. 128µg disc would require 12.8µL of 10mg/mL stock solution).
 - b. Add 5-20µL of antibiotic/solution to blank filter paper discs⁹.
 - c. Allow discs to air-dry for at least 15-20 minutes before application to plates.
2. Dilute culture to be tested to between 1×10^8 - 2×10^8 CFU/ml (~0.5 McFarland Standard)⁶ and use the suspension within 15 min⁷.
3. Dip a sterile swab into the diluted culture tubes, rub off excess culture along the side of the tube, and swab along the surface of a dry petri dish (no visible water spots). Rotate the plate 60 degrees and repeat, and then rotate the plate an

additional 60 degrees and repeat. Allow excess moisture to dry before application of the disc, but do not exceed 15 min after inoculation.⁸

4. Apply disc to the surface of the agar plate with the label side up. Make sure the strip does not shift during placement.
5. Within 15 min. of disc application, invert plates and incubate at 37°C for 16-20 hours.

Day 4

1. Examine plates for zones of clearing and then examine zones for any resistant colonies. Colonies can appear uniformly throughout the zone or close to the edge of the zone. These colonies can be examined for potential heteroresistance with the methods described below.

Further distinction between heteroresistance and resistance

To verify that heteroresistance observed by PAP, Etest, or disc diffusion is not due to a stable mutation in a small proportion of the total population or due to persister cells, further examination is necessary. Two methods can be utilized to distinguish between heteroresistance and these other forms of resistance¹⁵.

Time-Kill Assay

The purpose of this assay is to distinguish the resistant subpopulation in heteroresistance from persisters. Canonically, persisters are a subset of the population of a strain of bacteria that resist antibiotics by entering a state of quiescence with no or very limited growth. Heteroresistance differs from persistence in that the resistant subpopulation of a heteroresistant strain is able to grow and rapidly expand in the presence of antibiotics.

Day 1

2. Streak out isolates from freezer stocks on Lysogeny Broth agar plates and grow overnight in a 37°C incubator.

Day 2

1. Inoculate 2mL LB with a single colony from overnight plates and grow at 37°C shaking at 225rpm.

Day 3

2. Dilute 10μL overnight culture into 10mL LB without colistin, and 10mL LB with colistin (10μg/mL).
3. Serially dilute and plate these cultures on LB agar plates with (10μg/mL) and without colistin. This is time zero.
4. Every hour for eight hours, serially dilute and plate both cultures on LB agar plates with (10μg/mL) and without colistin overnight (~16 hours).

Day 4—Analysis of data should be as follows

Colony forming units counted from LB agar plates without colistin (Total CFU) and Colony forming units counted from LB agar plates with colistin (Resistant CFU) should be plotted on a graph over the eight-hour duration of this experiment. An increase of Resistant CFU is expected to be observed over time in colistin heteroresistant strains of *Acinetobacter baumannii*.

Resistant Colony Restreak

A single colony picked from a population analysis profile plate or from within the zone of clearing on an Etest or disc diffusion assay plate¹⁰ can be grown overnight in broth in the absence of colistin (several days of subculture if necessary) and the methods used to detect heteroresistance can be repeated to determine whether the cells maintain or lose their resistance phenotype¹¹. If the strains are heteroresistant, there should be a decrease in the frequency of the resistant subpopulation after culture in the absence of colistin. If the colonies now exhibit stable resistance, the frequency of the resistant subpopulation should not decrease after just a few days of subculture in media without colistin.

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Figure 1. Sample population analysis profile (PAP) procedure.
Note- while the authors prefer to plate eight serial dilutions in 10µL drops per plate, there are multiple ways to achieve these results.

Figure 2.
Example graph of a typical population analysis profile with three strains of varying susceptibility.

Figure 3. Example of Etest and disc diffusion assay results showing three different susceptibility profiles.

The Minimum Inhibitory Concentration (MIC) for Etest is interpreted as the lowest concentration where the zone of clearing meets the Etest strip along the bottom edge. The MIC for disc diffusion is interpreted by comparing the diameter of the zone of clearing and is drug and dose dependent and can be accessed through EUCAST guidelines²⁴.