Cation Concentration Variability of Four Distinct Mueller-Hinton Agar Brands Influences Polymyxin B Susceptibility Results

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Polymyxins have been the only alternative therapeutic option for the treatment of serious infections caused by multidrug-resistant *Acinetobacter baumannii* or *Pseudomonas aeruginosa* isolates. For this reason, it is of crucial importance that susceptibility tests provide accurate results when testing these drug-pathogen combinations. In this study, the effect of cation concentration variability found on different commercial brands of Mueller-Hinton agar (MHA) for testing polymyxin B susceptibility was evaluated. The polymyxin B susceptibilities determined using Etest and disk diffusion were compared to those determined by the CLSI reference broth microdilution method. In general, the polymyxin B MIC values were higher when determined by Etest than when determined by broth microdilution against both *A. baumannii* and *P. aeruginosa* isolates. A high very major error rate (10%) was observed, as well as a trend toward lower MICs, compared to those determined by broth microdilution when the Merck MHA was tested by Etest. Poor essential agreement rates (10 to 70%) were observed for *P. aeruginosa* when all MHA brands were tested by Etest. Although an excellent categorical agreement rate (100%) was seen between the disk diffusion and broth microdilution methods for *P. aeruginosa*, larger zones of inhibition were shown obtained using the Merck MHA. The high cation concentration variability found for the MHA brands tested correlated to the low accuracy, and discrepancies in the polymyxin B MICs were determined by Etest method, particularly for *P. aeruginosa* isolates.

Acinetobacter baumannii and *Pseudomonas aeruginosa* are important nosocomial pathogens worldwide. These microorganisms are noted for their intrinsic resistance to antibiotics and for their ability to acquire genes encoding resistance determinants. Unfortunately, the accumulation of distinct mechanisms of resistance leads to the development of multidrug-resistant (MDR) isolates. The emergence of MDR isolates, including those resistant to polymyxins is a major concern. Association of multiple resistance mechanisms limits significantly the choices for treating most of the hospital-acquired infections caused by nonfermenting Gram-negative bacilli (19).

Polymyxins are polycationic peptides that act in the Gram-negative bacterium cell wall, promoting disruption of the membrane and loss of genetic material, leading to cellular death. In the 1960s, polymyxins were the major therapeutic option available for treating *A. baumannii* and *P. aeruginosa* infections. However, their use was replaced by more-active and less-toxic antimicrobial agents such as cephalosporins and aminoglycosides (10, 18) in the following two decades. In the 1990s, the clinical use of polymyxins was reestablished due to the emergence of MDR *A. baumannii* and *P. aeruginosa* isolates, especially those resistant to carbapenems. In the meantime, polymyxins have been considered the only reasonable option for treating most serious infections caused by MDR *A. baumannii* or *P. aeruginosa* isolates (3, 19, 27). As the need for polymyxin usage increases in the clinical setting, routine microbiology laboratories must perform the polymyxin susceptibility testing under high-quality standard conditions. Since the cation composition of Mueller-Hinton agar (MHA) may vary among manufacturers and even among distinct lots of the same manufacturer (13), the aim of the present study was to determine the influence of different commercial brands of MHA for testing the polymyxin B susceptibility of *A. baumannii* and *P. aeruginosa* clinical isolates.

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MATERIALS AND METHODS

Polymyxin B susceptibility testing. A total of 10 clinical isolates of *A. baumannii* and 10 *P. aeruginosa*, recovered from bloodstream and respiratory tract infections, were selected for the present study. Isolates were collected from February to August 2007 as part of multicenter study which enrolled eight different hospitals located in the São Paulo metropolitan area. To represent distinct hospitals, at least one isolate per hospital was selected. Nonduplicate isolates per patient were included in the study. The susceptibility to polymyxin B was determined by Etest method according to the manufacturer's instructions (AB bioMérieux, Marcy l'Étoile, France) and by the disk diffusion (Oxoid, Basingstoke, United Kingdom) method, except for *A. baumannii*, according to the methods of the Clinical and Laboratory Standards Institute (CLSI) (6). The Etest and disk diffusion techniques were performed using four distinct commercial brands of MHA. The medium powder was obtained from Oxoid (Basingstoke, United Kingdom), lot 579019; Difco (BD Diagnostic Systems, Sparks, MD), lot 7093809; Merck (Darmstadt, Germany), lot VL698237; and Himedia (Mumbai, India), lot 26206. Broth microdilution was selected as the reference method using the four commercial brands of Mueller-Hinton broth (MHB): Oxoid, lot 724245; Difco, lot 9106707; Merck, lot VL136293; and Himedia, lot 89292. The MHB was prepared from the powder and supplemented with Ca²⁺ and Mg²⁺ according to CLSI guidelines (6). The polymyxin B sulfate was acquired from Sigma-Aldrich (Germany). The percent essential agreement $\pm 1\text{-log}_2$ variation in the poly-

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TABLE 1 Polymyxin B susceptibility results for *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as determined by Etest and disk diffusion testing of four distinct commercial MHA products and comparison of the results to those of the reference method, broth microdilution^a

Strain	Broth microdilution MIC (μg/ml)	Etest MIC (μg/ml)				Disk diffusion zone diam (mm)			
		Oxoid	Difco	Merck	Himedia	Oxoid	Difco	Merck	Himedia
<i>A. baumannii</i>									
A009	4 (R)	16 (R)	6 (R)	1.5 (S)	3 (R)	—	—	—	—
A026	4 (R)	4 (R)	4 (R)	3 (R)	4 (R)	—	—	—	—
A028	0.25 (S)	0.5 (S)	0.5 (S)	1.5 (S)	0.38 (S)	—	—	—	—
A041	0.5 (S)	0.5 (S)	0.5 (S)	0.19 (S)	0.5 (S)	—	—	—	—
A049	0.25 (S)	0.5 (S)	0.5 (S)	0.19 (S)	0.5 (S)	—	—	—	—
A210	0.5 (S)	0.5 (S)	0.75 (S)	0.125 (S)	0.5 (S)	—	—	—	—
A220	0.5 (S)	0.5 (S)	0.5 (S)	0.19 (S)	0.125 (S)	—	—	—	—
A228	0.5 (S)	1.5 (S)	0.5 (S)	0.25 (S)	0.5 (S)	—	—	—	—
A256	0.5 (S)	0.5 (S)	0.5 (S)	0.25 (S)	0.38 (S)	—	—	—	—
A263	0.5 (S)	1 (S)	0.5 (S)	0.19 (S)	0.5 (S)	—	—	—	—
% EA ^b (±1 log ₂)		80	100	80	90	—	—	—	—
<i>P. aeruginosa</i>									
P008	0.5 (S)	2 (S)	0.75 (S)	0.125 (S)	2 (S)	16 (S)	17 (S)	18 (S)	17 (S)
P097	0.5 (S)	3 (I)	1.5 (S)	0.38 (S)	2 (S)	16 (S)	16 (S)	18 (S)	17 (S)
P098	1 (S)	4 (I)	1.5 (S)	0.38 (S)	3 (I)	15 (S)	16 (S)	17 (S)	15 (S)
P101	0.5 (S)	0.5 (S)	1 (S)	≤0.064 (S)	0.75 (S)	16 (S)	21 (S)	27 (S)	20 (S)
P196	0.5 (S)	1.5 (S)	1.5 (S)	0.25 (S)	1.5 (S)	16 (S)	17 (S)	18 (S)	16 (S)
P231	0.25 (S)	1.5 (S)	2 (S)	0.25 (S)	3 (I)	15 (S)	16 (S)	18 (S)	16 (S)
P316	0.5 (S)	1.5 (S)	1.5 (S)	0.38 (S)	1.5 (S)	16 (S)	16 (S)	19 (S)	16 (S)
P327	0.5 (S)	2 (S)	1.5 (S)	0.5 (S)	3 (I)	14 (S)	15 (S)	16 (S)	15 (S)
P337	≤0.25 (S)	1.5 (S)	1.5 (S)	0.5 (S)	1.5 (S)	16 (S)	16 (S)	17 (S)	16 (S)
P342	1 (S)	1.5 (S)	1.5 (S)	0.19 (S)	4 (I)	14 (S)	16 (S)	17 (S)	16 (S)
% EA (±1 log ₂)		20	40	70	10	—	—	—	—

^a Means of triplicate reader results are presented. Interpretations are indicated in parentheses: I, intermediate; R, resistant; and S, susceptible (according to CLSI standards, 2011). —, Not applicable.

^b % EA, percent essential agreement.

myxin B MICs determined by broth microdilution using distinct MHB brands was observed (data not shown). In this manner, the reference polymyxin B MIC was set as the calculated mean using the MIC values obtained by testing the four brands of MHB in triplicate.

The Etest and disk diffusion tests were performed in triplicate and read by three observers blinded to the commercial brand of MHA. The final Etest MICs and diameter inhibition zones for polymyxin B were calculated as the mean of all individual results that included the three observers on three distinct occasions. The results were interpreted according to CLSI parameters, which are as follows for zone diameters: ≥ 12 mm = susceptible (S) and ≤ 11 mm = resistant (R) for *P. aeruginosa* and MICs of ≤ 2 $\mu\text{g/ml}$ (S), 4 $\mu\text{g/ml}$ (intermediate [I]), and ≥ 8 $\mu\text{g/ml}$ (R) for *P. aeruginosa* and MICs of ≤ 2 $\mu\text{g/ml}$ (S) and ≥ 4 $\mu\text{g/ml}$ (R) for *Acinetobacter* spp. (7). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were tested as the quality control strains.

Essential agreement was defined as when the Etest results agreed within ± 1 -log₂ dilution compared to the reference broth microdilution test. A result was determined to be discrepant if there was ± 2 -log₂ dilution difference between test results. Categorical agreement was defined if the test results were within the same susceptibility category, and errors were ranked as follows: very major error, false-susceptible result by Etest; major error, false-resistant result by Etest; and minor error, intermediate result by Etest method and resistant or susceptible category by broth microdilution test (25).

Determination of ions concentration in the MHA and MHB of distinct commercial brands. An aliquot of 100 mg of each culture medium brand was diluted in 3 ml of HNO₃ and 2 ml of H₂O₂ and then subjected to digestion in a microwave oven for 30 min. The Mueller-Hinton medium solutions were transferred to a volumetric flask, and deionized water was added to a final volume of 25 ml. The concentrations of calcium,

magnesium, manganese, iron, and zinc contained in distinct commercial brands of Mueller-Hinton agar and broth were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES) in a Perkin-Elmer 3000 DV at the Laboratório de Química, Universidade Estadual de Campinas, UNICAMP, São Paulo, Brazil (20).

RESULTS

Polymyxin B susceptibility tests. Considering the results achieved by the reference method (broth microdilution) using the four MHB tested, 2 of 10 *A. baumannii* isolates were determined to be resistant to polymyxin B (MIC = 4 $\mu\text{g/ml}$). All *P. aeruginosa* isolates (MICs ranging from ≤ 0.25 to 1 $\mu\text{g/ml}$) were susceptible to polymyxin B (Table 1). In general, polymyxin B MIC values were higher as determined by Etest than as determined by broth microdilution against both *A. baumannii* ($\pm 2 \log_2$) and *P. aeruginosa* ($\pm 3 \log_2$) isolates. An essential agreement of 100% was found for Difco media when used to test *A. baumannii* isolates. In contrast, the essential agreement rates between the broth microdilution and the Etest were 80% for the Merck and Oxoid MHAs and 90% for Himedia. A slight trend toward lower MICs was observed using Merck MHA, leading to the miscategorization of a polymyxin-resistant isolate as susceptible (very major error).

Poor essential agreement was observed between the Etest and broth microdilution polymyxin B MICs using all MHA brands for testing against *P. aeruginosa*. Although no very major and major errors were observed, the minor errors rates were determined to be 20 and 40% when testing Oxoid and Himedia media, respectively. The disk diffusion method presented 100% categorical

TABLE 2 Ion concentrations in several distinct commercial brands of Mueller-Hinton broth and agar

Culture medium	Commercial brand	Ion concn (mg/liter) ^a				
		Calcium	Magnesium	Manganese	Iron	Zinc
MHB	Oxoid	3.1	3.9	<0.04*	0.6	0.6
	Difco	2.4	2.5	<0.04*	0.8	0.2
	Merck	2.1	0.6	<0.04*	<0.5†	<0.2†
	Himedia	2.2	1.1	<0.04*	<0.5†	0.3
MHA	Oxoid	20.9	13.3	<0.09*	<2.2†	<0.7†
	Difco	16.1	4.9	<0.09*	<0.6*	<0.7†
	Merck	7.5	6.2	19.3	<2.2†	1.1
	Himedia	25.3	31.2	<0.09*	<2.2†	<0.7†
CLSI parameters ^b		20–25	10–12.5	–	–	–

^a *, The concentration was lower than the minimal value for detection; †, the concentration was lower than the minimal value for quantification; –, no specific CLSI recommendation.

^b That is, the cation concentrations recommended for testing according to CLSI standards, 2009.

agreement with those for broth microdilution for all of the *P. aeruginosa* isolates tested. However, Merck MHA produced larger zones of inhibition than those of other MHA brands. Upon comparing the results of polymyxin B MIC as determined by Etest, we found no disagreement among the three readers for all of the MHA brands except for the Merck MHA (20% within ± 2 -log₂ dilutions).

Dosage of ion concentration in the MHA and MHB. The ion concentrations for the MHB and MHA commercial brands tested are presented in Table 2. The concentrations of the evaluated cations, such as calcium, magnesium, iron, and zinc, differed for each brand of MHB. Manganese concentrations were below of the detection limit for all MHB brands. Although the highest concentrations of calcium and magnesium were detected in the Oxoid MHB, they were far below those recommended by the CLSI. The zinc content in the Oxoid MHB was 2- and 3-fold higher than that seen in the Himedia and Difco MHBs, respectively. Iron was detected but was not quantified in the Merck and Himedia MHBs. However, the iron concentration was quite similar when we compared the Oxoid and Difco MHBs (Table 2).

A higher variation in ion concentrations was observed among the distinct MHA brands, as shown in the Table 2. The calcium and magnesium concentrations in the Oxoid MHA were 20.9 and 13.3 mg/liter, respectively. The Merck MHA had the lowest calcium concentration (7.5 mg/liter), while Difco and Merck MHA showed the lowest magnesium concentrations (4.9 and 6.2 mg/liter, respectively). Oxoid and Himedia MHAs showed more elevated calcium concentrations than the other two commercial brands. Only the Merck MHA presented a detectable zinc concentration in its composition. The Difco and Himedia MHA brands presented insufficient iron concentration to be detected and quantified, respectively, by ICP-OES.

DISCUSSION

The increasing incidence of hospital-acquired infections caused by MDR *P. aeruginosa* and *A. baumannii* worldwide has placed polymyxin agents as one of the last therapeutic options for treatment of these infections (15). However, accurate susceptibility testing for these compounds, which is known to be essential for therapeutic management, is still problematic in routine clinical microbiology laboratories. It has been reported that polymyxin

MICs may not be reproducible and that many factors influence its determination, such as the inoculum and the cation concentration content of the Mueller-Hinton medium (2, 14, 22, 24). In addition, it has been observed that MICs obtained by agar dilution and Etest methods are higher than those obtained by the reference broth microdilution technique (17). Moreover, the disk diffusion technique has been considered an unreliable technique for predicting the susceptibility to polymyxins among *A. baumannii* isolates. Regardless of the antimicrobial susceptibility technique used for determining the antimicrobial category of susceptibility, the cation concentrations of the Mueller-Hinton medium should be adjusted for reliable test results since high or low cation concentrations may result in false resistance (major error) or false susceptibility (very major error), respectively (6).

CLSI guidelines recommend a calcium concentration ranging from 20 to 25 mg/liter and a magnesium concentration ranging from 10 to 12.5 mg/liter to assure reliable antimicrobial susceptibility results (6). The calcium and magnesium concentrations measured for each of the MHB brands evaluated in our study were far below the recommendations, confirming that the cation concentration must be adjusted before testing, as recommended by CLSI guidelines. Each batch of MHB used in the present study was cation adjusted before use according to the initial concentration measured. For this reason, no essential agreement discrepancies were achieved by broth microdilution among the MHB brands tested. The four distinct commercial brands of MHA presented higher concentrations of calcium and magnesium compared to those of MHB from the same manufacturer. However, none of them displayed the correct concentrations of calcium and magnesium as recommended by the CLSI. The higher polymyxin B MICs exhibited by Etest and the smaller-diameter zones of inhibition determined by disk diffusion were probably due to distinct cation concentrations in the MHA brands tested. The polymyxin resistance is controlled by two-component regulatory systems, including PmrA/B, PhoP/Q, and also the more recently described ParR/S. These systems respond to environmental variations such as an elevated calcium or a low magnesium concentration, distinct pHs, and the presence of iron in the medium. The environmental signals are detected by the sensor kinase gene that modifies the expression of the second gene, a response regulator, which in turn promotes modifications in the Gram-negative lipopolysaccha-

ride, leading to polymyxin resistance (1, 4, 12, 21, 24). Previous studies have also reported increased polymyxin B and colistin MICs against *P. aeruginosa* and *A. baumannii* associated with high calcium or magnesium concentration in culture media (9, 11, 26). It is also reported that high concentrations of magnesium can interfere with the activity of other antimicrobials, such as fluoroquinolones and carbapenems (5, 8).

Our results corroborate the need for cation supplementation in the culture medium as recommended by the CLSI (6). To know the right amount to be supplemented, we must know the exact cation content of the Mueller-Hinton medium, especially for the MHA. Due to MHA's solid nature, cation quantification cannot be usually carried out at the biochemistry section of the clinical laboratory as we could perform for MHB. In our opinion, the cation concentrations of each Mueller-Hinton lot should be stated on the labels of the Mueller-Hinton bottles since this information is not available even on the medium Quality Assurance Certificate of most of the manufacturers. In our study, the cation concentration was described in just one Mueller-Hinton bottle from one out of four brands tested. The Mueller-Hinton bottles from other manufacturers simply stated that the calcium and magnesium concentrations were in accordance with CLSI guidelines. Manufacturers were asked about the calcium and magnesium content of the respective lots tested in our study. The cation concentration provided by one of the manufacturers was in accordance with our measurement. For the remaining media, the information was either not given after contact (two brands) or the informed concentrations were not in accordance with our measurement (one brand).

Our results demonstrate that the polymyxin B susceptibility phenotype varied according to the MHA brand tested, probably due to the two-component regulatory system response. Clinical microbiology laboratories should be aware of this in order to avoid reporting erroneous susceptibility results for polymyxin B. While cation supplementation is not readily available since most of the manufacturers do not state the exact cation content on their MHA bottles, laboratory technicians should attempt to gentamicin and tetracycline results tested against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains to detect unsatisfactory cation content as recommended by the CLSI (6). If the results are inadequate for quality control strains, the use of a new medium lot is recommended. Moreover, if the problem persists, even when using a new lot, the manufacturer should be contacted. Although there is no CLSI recommendation for manganese, iron, or zinc supplementation in the culture medium, diverse studies have shown that these ions can also influence the antimicrobial susceptibility tests results for other antimicrobial agents than polymyxin (13, 16).

Polymyxin susceptibility rates remain quite elevated against nonfermenting Gram-negative bacilli. Differences in the cation composition of the MHA can generate categorical errors in the polymyxin B susceptibility tests. Although the occurrence of such errors is currently low, it is expected to increase as polymyxin becomes more frequently prescribed for the treatment of infections caused by MDR pathogens and as MICs increase toward the resistance breakpoint. Clinical microbiology laboratories must be aware of polymyxin susceptibility methods discrepancies to avoid reporting erroneous results. Moreover, resistant isolates detected by Etest must be sent to a reference laboratory to confirm this phenotype by reference broth microdilution. Also, we suggest that

manufacturers should state the exact cation content of each MHA lot on the bottle label since the measurement is not easily accomplished by the routine clinical microbiology laboratory. Meanwhile, laboratory technicians should pay attention to the gentamicin and tetracycline results for ATCC strains.

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REFERENCES

- Adams MD, et al. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob. Agents Chemother.* 53:3628–3634.
- Andrews J, Walker R, King A. 2002. Evaluation of media available for testing the susceptibility of *Pseudomonas aeruginosa* by BSAC methodology. *J. Antimicrob. Chemother.* 50:479–486.
- Appleman MD, et al. 2000. *In vitro* activities of nontraditional antimicrobials against multiresistant *Acinetobacter baumannii* strains isolated in an intensive care unit outbreak. *Antimicrob. Agents Chemother.* 44:1035–1040.
- Arroyo LA, et al. 2011. The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob. Agents Chemother.* 55:3743–3751.
- Atamaca S. 1998. Effect of zinc concentration in Mueller-Hinton agar on susceptibility of *Pseudomonas aeruginosa* to meropenem. *J. Med. Microbiol.* 47:653.
- CLSI. 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—8th ed. Document M07–A8, vol. 29, no 2. Clinical Laboratory Standard Institute, Wayne, PA.
- CLSI. 2011. Performance standards for antimicrobial susceptibility testing; 21st informational supplement. Document M100–S21. Clinical Laboratory Standard Institute, Wayne, PA.
- Daly JL, et al. 1997. Effect of zinc concentration in Mueller-Hinton agar on susceptibility of *Pseudomonas aeruginosa* to imipenem. *J. Clin. Microbiol.* 35:1027–1029.
- Davis SD, Iannetta A, Wedgwood RJ. 1971. Activity of colistin against *Pseudomonas aeruginosa*: inhibition by calcium. *J. Infect. Dis.* 124:610–612.
- Drabick JJ, et al. 1998. Covalent polymyxin B conjugate with human immunoglobulin G as an antiendotoxin reagent. *Antimicrob. Agents Chemother.* 42:583–588.
- Felegie TP, Yu VL, Rumans LW, Yee RB. 1979. Susceptibility of *Pseudomonas maltophilia* to antimicrobial agents, singly and in combination. *Antimicrob. Agents Chemother.* 16:833–837.
- Fernández L, et al. 2010. Adaptive resistance to the “last hope” antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR–ParS. *Antimicrob. Agents Chemother.* 54:3372–3382.
- Fernández-Mazarrasa C, Mazarrasa O, Calvo J, del Arco A, Martínez-Martínez L. 2009. High concentrations of manganese in Mueller-Hinton agar increase MICs of tigecycline determined by Etest. *J. Clin. Microbiol.* 47:827–829.
- Gales AC, Reis AO, Jones RN. 2001. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J. Clin. Microbiol.* 39:183–190.
- Gales AC, Jones RN, Sader HS. 2011. Contemporary activity of colistin and polymyxin B against a worldwide collection of Gram-negative pathogens: results from the SENTRY Antimicrobial Surveillance Program (2006–09). *J. Antimicrob. Chemother.* 66:2070–2074.
- Garrod LP, Waterworth PM. 1969. Effect of medium composition on the

- apparent sensitivity of *Pseudomonas aeruginosa* to gentamicin. J. Clinical Pathol. 22:534–538.
17. Heijden IMvan der, et al. 2007. Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant *Pseudomonas aeruginosa* to polymyxins. Annals Clin. Microbiol. Antimicrob. 6:1–8.
 18. Horton J, Pankey GA. 1982. Polymyxin B, colistin, and sodium colistimethate. Med. Clin. N. Am. 66:135–142.
 19. Levin AS, et al. 1999. Intravenous colistin as therapy for nosocomial infections caused by multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Clin. Infect. Dis. 28:1008–1011.
 20. Matoso E, Cadore S. 2011. Determination of inorganic contaminants in glue by inductively coupled argon plasma optical emission spectrometry. Talanta 84:335–340.
 21. Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA. 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. PLoS Genet. 4:e1000233. c18 doi:10.1371/journal.pgen.1000233.
 22. Miyazaki H, et al. 2009. Effect of the inoculum size on carbapenem susceptibilities of β -lactamase-negative, ampicillin-resistant *Haemophilus influenza*. Curr. Microbiol. 58:18–24.
 23. Moskowitz SM, Ernst RK, Miller SI. 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bacteriol. 186:575–579.
 24. Moskowitz SM, et al. 2010. Colistin susceptibility testing: evaluation of reliability for cystic fibrosis isolates of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. J. Antimicrob. Chemother. 65:1416–1423.
 25. NCCLS. 2000. Development of *in vitro* susceptibility testing criteria and quality control parameters. Document M23-42. National Committee for Clinical Laboratory Standards, Wayne, PA.
 26. Ward PB, Palladino S, Looker JC, Feddema P. 1993. *p*-Nitrophenyl-glycerol in susceptibility testing media alters the MICs of antimicrobials for *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 31:489–496.
 27. Young ML, Bains M, Bell A, Hancock RW. 1992. Role of *Pseudomonas aeruginosa* outer membrane protein OprH in polymyxin and gentamicin resistance: isolation of an OprH-deficient mutant by gene replacement techniques. Antimicrob. Agents Chemother. 36:2566–2568.