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A Method for Antibiotic Susceptibility Testing: Applicable and Accurate

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ABSTRACT

Background: Emerging antibiotic resistance in pathogenic bacteria has driven the development of new assays for routine antibiotic testing.
Objectives: The purpose of this study was to evaluate the effects of different organic solvents in preparing two-fold decreases in serial penicillin concentration coated onto 96-well plates to design a method for antibiotic susceptibility testing.
Materials and Methods: Benzyl penicillin was dissolved in each solvent (sterile distilled water, PBS, diethyl alcohol, ethanol, butanol, chloroform, 2-propanol, and acetonitrile). Serial dilutions of each solution were loaded onto a 96-well microtiter plate and incubated at 37°C for 12 h. Next, 200 µL of sterilized Mueller-Hinton broth was added along with 50 µL of bacterial suspension at an adjusted concentration equivalent to 0.5 McFarland standards. The prepared plates were incubated at 37°C for 24 h. Optical density (OD) was measured at 540 nm.
Results: When comparing the ODs of each sample in 96-well microtiter plates with positive and negative controls, significant antibacterial activity was observed. Most activities ranged from 50 to 200 units of penicillin in samples that were diluted with distilled water, PBS, or isobutyl alcohol as a solvent. Analysis of the results suggested that, when using the aforementioned solvents, the minimum inhibitory concentration of penicillin against a sensitive strain of *Staphylococcus aureus* was ≥50 units of penicillin.
Conclusions: The results revealed that the accuracy and feasibility of this method can greatly reduce the waiting period of antibacterial sensitivity tests. Additionally, this method is low-cost and could benefit patients who urgently require proper antibiotic therapy.

Implication for health policy/practice/research/medical education:
Applying of this antibiotic sensitivity assay in the diagnostic microbiology laboratories is important. Because, precise determination of MIC and minimum bactericidal concentration can be a valuable guide for physicians and patients will be treated successfully.

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1. Background

The development and improvement of accurate and efficient methods of rapid antibiotic susceptibility testing is important for public health. Antimicrobial susceptibility information about pathogens may significantly reduce morbidity and mortality, cost of treatment, and duration of hospitalization if this information can be provided to clinicians in a rapid and timely fashion (1). To determine *in vitro* antimicrobial susceptibility, various methods are commercially available, and clinical microbiology laboratories choose a manual or instrument-based method for performing routine antimicrobial

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susceptibility testing (2, 3). The most commonly used methods include disk diffusion (4-6), broth microdilution (with or without use of an instrument for panel readings), and rapid automated instrument-based methods (7). The E-test may also be useful for some bacteria (8).

In many countries, the disk diffusion method is the most commonly used method in clinical laboratories. This test provides the greatest flexibility and cost-effectiveness; however, the test takes at least 24 h (9, 10) and there are limitations in its accuracy. Thus, several automated systems are now available that provide rapid antimicrobial susceptibility data (11). These include the Autobacs (General Diagnostics, Warner-Lambert Co., Morris Plains, NJ), the MS-2 (Abbott Laboratories, Dallas, TX), and the Auto Microbic system (AMS; Vitek, Inc., Hazelwood, MO). These systems provide interpretable results (susceptible or resistant) or an approximate or exact minimum inhibitory concentration (MIC) 3–10 h after inoculation. However, the cost of analysis, including materials and labor, show that these systems are expensive and not feasible for use in developing countries in which infectious diseases are relatively more common (12). Thus, it is necessary to develop a relatively simple, rapid, accurate, and less costly test for performing antimicrobial susceptibility testing to yield precise MICs and interpretation of bacterial isolate sensitivity within 6–12 h.

2. Objectives

The purpose of this study was to develop a rapid, simple, and cost-effective broth microdilution method for antibiotic susceptibility testing of pathogenic bacteria such as the methicillin-resistant *Staphylococcus aureus*.

3. Materials and Methods

3.1. Materials and Bacterial Strains

Benzyl penicillin ($C_{16}H_{17}N_2O_4Na$) with a potency of more than 1477 Unit/mg (Sigma, St. Louis, MO), distilled water, phosphate-buffered saline (PBS, 0.1 mM), diethyl ether (99.5%), ethanol (96%), isobutyl alcohol, propanol, chloroform, 96-well plates, and Mueller-Hinton broth (Merck, Germany) were purchased. The bacterial standard strains used in this study were as follows: *S. aureus* ATCC 25923 (as a quality control strain sensitive to ampicillin) and *S aureus* ATCC 43300 (resistant to methicillin and oxacillin), which were kindly provided by Dr. Mohammad Rahbar (from the Reference Laboratory of Iran).

3.2. Preparation of Serial Dilutions

One row of a 96-well microplate was marked for each solvent. A stock antibiotic solution of benzyl penicillin ($C_{16}H_{17}N_2O_4Na$) with a potency of 1477 Unit/mg (Lot number 023H189) was prepared. Next, a 1mL microtube was selected for each solvent. Following this step, 400 µL of the selected solvents was added to each tube, and an adequate amount of benzyl penicillin powder was added. Thus, stock solutions of antibiotics containing 1 unit/µL were prepared. Different concentrations of antibiotic (200, 100, 50, 25, 12.5, and 6.25 unit/well) were then prepared from the base antibiotic stock solution. Plates were incubated at 35°C for 24 h for evaporation and finalization of the process of loading the antibiotic into the plate. Plates were stored in a refrigerator set at 4°C until required.

3.3. Antibiotic Sensitivity Assay

Prepared plates were removed from the refrigerator and 200 µL of Mueller-Hinton broth was added to each well containing antibiotics. As described above, each row of the plate corresponded to a specific solvent and one well in each row served as a negative control (blank) and another as a positive control. The negative control well or blank did not contain bacterial inoculum, whereas the positive control well was free from antibiotics. Two bacterial strains were used, one that is sensitive to penicillin and the other that is resistant to penicillin.

3.4. Inoculum Standardization

A McFarland 0.5 turbidity standard was prepared by adding 99.5 mL of 1% sulfuric acid to 0.5 mL of 1.175% barium chloride solution. This solution was dispensed into tubes comparable to those used for inoculum preparation. The tubes were sealed and stored under dark conditions at room temperature. The McFarland 0.5 standard provides turbidity (OD = 7) comparable to that of a bacterial suspension containing 1.5×10^8 colony-forming units (CFU)/mL or 1.5×10^5 CFU/µL. Turbidity of the prepared bacterial suspensions was compared by observing the black lines through the suspension. A 50-µL volume of this suspension was added to each well to obtain a final concentration of approximately $50 \times 1.5 \times 10^5$ CFU/wells. These bacteria were tested in separate plates. Inoculated plates were incubated at 37°C, and the optical density (OD) of each well was measured at 0, 12, 18, and 24 h after initiation of incubation using an ELISA reader device set at 540 Nanometer. The mean of the OD of different concentrations of each bacterium and solvent was compared and analyzed using unilateral variance analysis (ANOVA). In our statistical analyses, $\alpha = 0.005$ was considered acceptable significant variation and the results were analyzed using SPSS Ver. 16.

4. Results

The results of this study revealed that antibiotic sensitivity assays can be performed using this modified method of manual broth microdilution, which does not require expensive devices. Loading various antibiotic concentrations in different rows of a 96-well plate allows the use of single plates for evaluating the sensitivity of different antibiotics at the same time. In this study, six different concentrations of penicillin were prepared in different solvents. *Figure 1* shows the prepared serial dilution in one row of a plate. OD data from measurements

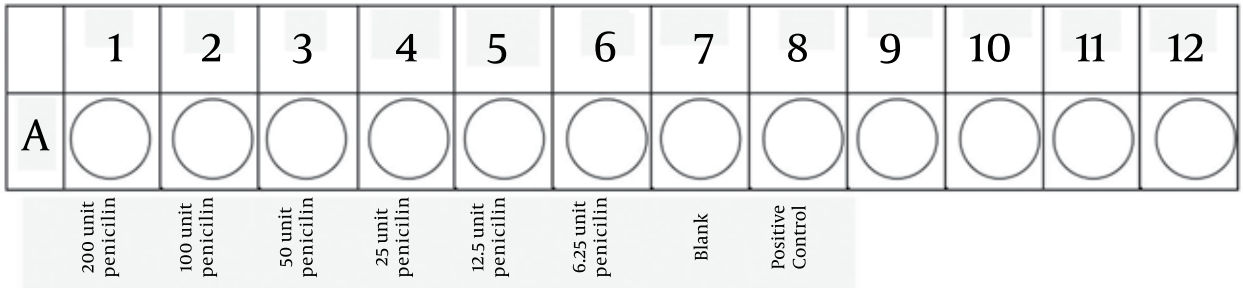

Figure 1. Example of a Row of a 96-Wells Plate Used for the evaluating Antibiotic Sensitivity Using the broth Microdilution Method

are shown in *Figure 2*. This figure is divided into six parts, which is arranged into three columns. Column A shows sterile distilled water used as a solvent. In column B, benzyl penicillin was dissolved using 1x PBS. Finally, diethyl alcohol ether was used as a solvent in column C. Each column is related to a particular solvent and different antibiotic concentration against sensitive bacteria (upper) and resistance bacteria (lower). Difference in OD after a 24 h incubation is shown in this figure. Positive controls of resistant and sensitive bacteria showed active growth, whereas for sensitive bacteria, different concentrations of penicillin in each solvent decreased bacterial growth. The amount decrease was, however, not significant. Increasing concentrations of penicillin can repress the growth of sensitive bacteria. No solvent completely eliminated the antimicrobial effect of penicillin.

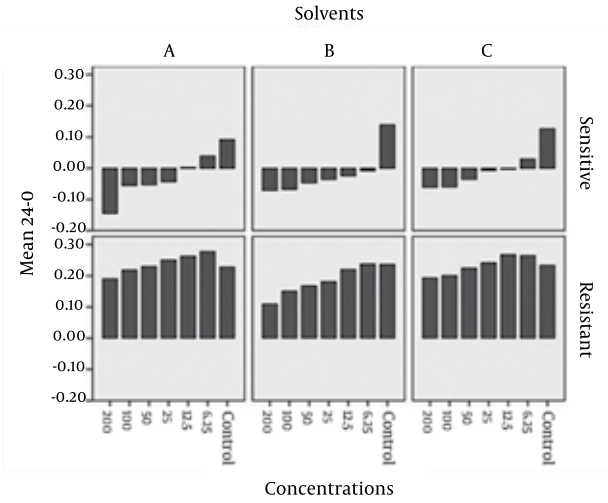

Figure 2. Effect of Penicillin Concentrations Prepared Using Different Solvents on the Sensitivity and Resistance of *S. aureus*

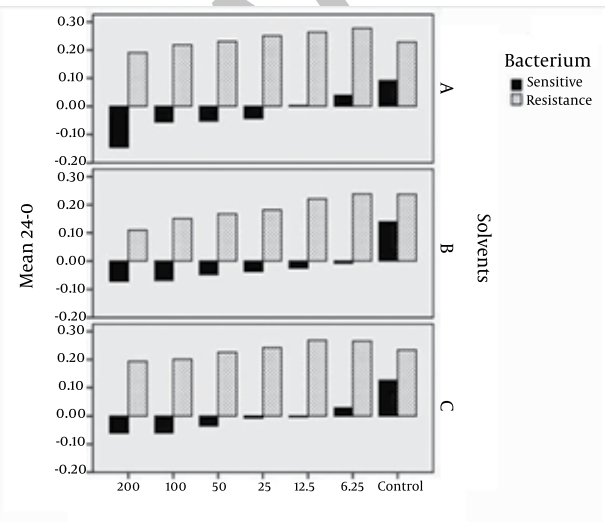

Figure 3. Double Comparison of the Effects of Penicillin Concentrations Prepared Using Different Solvents on Sensitive and Resistant Bacteria

Figure 2 shows the results of the antibiotic susceptibility test from six microtiter plates. Parts A, B, and C represent various concentrations of penicillin (200, 100, 50, 25, 12.5, and 6.25 unit/well) prepared using sterile distilled water, PBS, and diethyl alcohol as solvents, respectively. In this procedure, 50 μL of penicillin-sensitive and penicillin-resistant *S. aureus* suspension equivalent to 0.5 McFarland standards were inoculated into the upper and lower sides of the plate, respectively. Next, 200 μL of sterile Muller-Hinton broth was added to each well and the plate was incubated for 24 h. Finally, the OD at 540 Nanometer was measured. The x-axis represents penicillin concentrations and the y-axis indicates the average absorbance of 24 h cultures. There was no significant variance in the effect of different penicillin concentrations on the growth rate of resistant bacteria. However, when PBS was used as a solvent, decreased bacterial growth was observed. A comparison of different concentrations of penicillin and their effects on sensitive and resistant bacteria is shown in *Figure 3*. Test plates loaded with varying concentrations of penicillin were maintained in a 4°C refrigerator for 6 months. As shown in *Figure 3*, the antibacterial activity was not diminished by penicillin.

5. Discussion

Medical intervention to fight infection primarily involves selecting appropriate antibiotics to actively inhibit or kill the causative infectious agent (13, 14). Several different methods are commercially available for determining the quantitative susceptibility of pathogenic bacteria (15, 16). In developing countries, the disk diffusion test is still among the most commonly used antimicrobial susceptibility assays. However, the major disadvantages of this method include lack of interpretive criteria for some organisms and inability to provide pre-

cise data regarding the level of an organism's resistance or susceptibility that can be provided by other MIC methods (17). Recently, these problems have forced scientists to consider alternative methods. Thus, methods based on serial dilutions have been developed to overcome these problems (18). While these methods avoid the drawbacks of disk diffusion methods, they require other factors (preparing different concentrations of antibiotics in rows of a 96-well plate and reader devices such as scanners and plate analyzers), increasing the cost of the assay (19). Therefore, a number of broth dilution systems using dried or frozen drug dilutions in microwell plates are recommended to decrease costs (20, 21). The primary advantages of these newly developed tests include quantitative data and cost-effectiveness. However, proper techniques for preparing microwell plates loaded with different antibiotics and practical training to conduct the tests are essential.

This study was carried out based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI), for dissolving the powder of penicillin in the recommended dilutions (22, 23). The preparation of different antibiotic concentrations was carried out at room temperature and the plates were dried in a 35°C incubator for 24 h or in a 4°C refrigerator for 1 week or more. We tested various solvents for preparing different penicillin concentrations in a microwell plate and found that PBS is the best solvent. For comparison, positive control and blank wells were used to determine the potential activity of penicillin in each well. In this study, penicillin was selected as the sample antibiotic.

After incubation for 18–24 h, results can be read manually using an ELISA reader. Growth appears as turbidity or as a deposit of cells at the bottom of a well. Negative-growth control wells should be read first. If the positive control wells do not exhibit growth, the results are invalid. To ensure detection of penicillin-resistant staphylococci, results should be interpreted only after 12 h of incubation. Prepared plates can be stored in a refrigerator at 4°C for more than 6 months without loss of activity and are used by adding an adequate volume of medium and bacterial suspension. The advantage of this method compared with other methods is that it is cost-effective, feasible, and uses basic instruments. Because of the low cost, this method may be suitable for antibiotic susceptibility testing worldwide, particularly in developing countries.

The accuracy and feasibility of this method can greatly reduce the waiting time involved in standard laboratory antibacterial sensitivity assays. This method can be utilized without costly devices and specialized manpower in the clinical laboratory and provides different benefits to the patients who require proper antibiotic treatment to control bacterial infections. In the present study, various available solvents were used to prepare penicillin solutions. Each solvent was tested for the effectiveness of penicillin against a bacterial strain. Solutions of penicillin prepared using distilled water, ethyl alcohol, and PBS as solvents showed antibacterial activity.

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References

1. Backes BA, Cavalieri SJ, Rudrik JT, Britt EM. Rapid antimicrobial susceptibility testing of Gram-negative clinical isolates with the AutoMicrobic system. *J Clin Microbiol*. 1984;**19**(6):744-7.
2. Chiang YL, Lin CH, Yen MY, Su YD, Chen SJ, Chen HF. Innovative antimicrobial susceptibility testing method using surface plasmon resonance. *Biosens Bioelectron*. 2009;**24**(7):1905-10.
3. Murray PR, Niles AC, Heeren RL. Comparison of a highly automated 5-h susceptibility testing system, the Cobas-Bact, with two reference methods: Kirby-Bauer disk diffusion and broth microdilution. *J Clin Microbiol*. 1987;**25**(12):2372-7.
4. Bauer AW. Current status of antibiotic susceptibility testing with single high potency discs. *Am J Med Technol*. 1966;**32**(2):97-102.
5. Barry AL, Garcia F, Thrupp LD. An improved single-disk method for testing the antibiotic susceptibility of rapidly-growing pathogens. *Am J Clin Pathol*. 1970;**53**(2):149-58.
6. Hubert SK, Nguyen PD, Walker RD. Evaluation of a computerized antimicrobial susceptibility system with bacteria isolated from animals. *J Vet Diagn Invest*. 1998;**10**(2):164-8.
7. Wayne PA. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard M7-A7. 7th ed. *Clinical and laboratory standards institute*; 2006.
8. Woods GL, Bergmann JS, Witebsky FG, Fahle GA, Boulet B, Plaunt M, et al. Multisite reproducibility of E test for susceptibility testing of Mycobacterium abscessus, Mycobacterium chelonae, and Mycobacterium fortuitum. *J Clin Microbiol*. 2000;**38**(2):656-61.
9. Jones RN, Biedenbach DJ, Marshall SA, Pfaller MA, Doern GV. Evaluation of the Vitek system to accurately test the susceptibility of Pseudomonas aeruginosa clinical isolates against cefepime. *Diagn Microbiol Infect Dis*. 1998;**32**(2):107-10.
10. Liao CH, Kung HC, Hsu GJ, Lu PL, Liu YC, Chen CM, et al. In-vitro activity of tigecycline against clinical isolates of Acinetobacter baumannii in Taiwan determined by the broth microdilution and disk diffusion methods. *Int J Antimicrob Agents*. 2008;**32** Suppl 3:S192-6.
11. Baker CN, Stocker SA, Rhoden DL, Thornsberry C. Evaluation of the MicroScan antimicrobial susceptibility system with the auto SCAN-4 automated reader. *J Clin Microbiol*. 1986;**23**(1):143-8.
12. Valdivieso-Garcia A, Imgrund R, Deckert A, Varughese BM, Harris K, Bunimov N, et al. Cost analysis and antimicrobial susceptibility testing comparing the E test and the agar dilution method in Campylobacter jejuni and Campylobacter coli. *Diagn Microbiol Infect Dis*. 2009;**65**(2):168-74.
13. Lagarde M, Palmer N. The impact of contracting out on health outcomes and use of health services in low and middle-income countries. *Cochrane Database Syst Rev*. 2009(4):CD008133.
14. Lee SS, Chen YS, Tsai HC, Wann SR, Lin HH, Huang CK, et al. Pre-

dictors of septic metastatic infection and mortality among patients with Klebsiella pneumoniae liver abscess. *Clin Infect Dis*. 2008;**47**(5):642-50.
15. Torres E, Villanueva R, Bou G. Comparison of different methods of determining beta-lactam susceptibility in clinical strains of Pseudomonas aeruginosa. *J Med Microbiol*. 2009;**58**(Pt 5):625-9.
16. Martinez-Lamas L, Trevino M, Romero-Jung PA, Regueiro BJ. [Comparison between Phoenix system and agar-based methods for antimicrobial susceptibility testing of Streptococcus spp]. *Rev Esp Quimioter*. 2008;**21**(3):184-8.
17. Forbes BA, Sahm DF, Weissfeld AS. *Bailey & Scott's diagnostic microbiology*. United State of America: Mosby; 2007:194-198.
18. Mittman SA, Huard RC, Della-Latta P, Whittier S. Comparison of BD phoenix to vitek 2, microscan MICroSTREP, and Etest for antimicrobial susceptibility testing of Streptococcus pneumoniae. *J Clin Microbiol*. 2009;**47**(11):3557-61.
19. Clavier B, Perrier-Gros-Claude JD, Foissaud V, Dumas M, Aubert G, Barbe G, et al. [Comparison of the results of the E test and the method for determining minimum inhibitory concentrations in solid media for penicillin G, amoxicillin, and cefotaxime for S. pneumoniae. A multicenter study]. *Pathol Biol (Paris)*. 1998;**46**(6):369-74.
20. Hansen SL, Freedy PK. Concurrent comparability of automated systems and commercially prepared microdilution trays for susceptibility testing. *J Clin Microbiol*. 1983;**17**(5):878-86.
21. Jones RN, Thornsberry C, Barry AL, Gavan TL. Evaluation of the Sceptor microdilution antibiotic susceptibility testing system: a collaborative investigation. *J Clin Microbiol*. 1981;**13**(1):184-94.
22. Schwalbe R, Steele-Moore L, Goodwin AC. *Antimicrobial susceptibility testing protocols*. New York: CRC Press; 2007.
23. Wayne PA. Clinical and Laboratory standards institute. Document M100-S16.