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Comparative Evaluation of Broth and Agar Dilution Techniques for Antimicrobial Susceptibility Testing against Multidrug Resistant *Pseudomonas aeruginosa*

Aamir Jamal Gondal,* Abdul Hannan, Sidrah Saleem and Muhammad Usman Arshad
Department of Microbiology, University of Health Sciences Lahore, Pakistan

ABSTRACT

Valuation of MIC is imperative and quite crucial for timely management of multiply drug resistant pathogens, surveillance epidemiology, comparative evaluation of novel drugs and their synergistic combinations. Availability of such different methods makes it difficult to compare diverse data and also invoke certain inherent errors attributed to the choice of methodology. However, routine clinical diagnostics warrants a robust and reliable routine method equally good on efficiency and efficacy. In this study agar dilution (AD) methodology, being less time consuming has been compared to more conventional broth microdilution (BMD) method using multiply drug resistant (n=12) and susceptible (n=12) clinical strains of *Pseudomonas aeruginosa*. The essential agreement (EA) in terms of plus minus one doubling dilution (±1 $\log^2$ dilution) was 92.3% for ceftazidime and 100% for ciprofloxacin. However, the EA with respect to all antimicrobials was equal to 90%. However, tazocin and meropenem each showed least EA of 84.6%. The correlation of BMD vs AD methodologies with respect to tested antimicrobials was statistically significant for all antimicrobials ($p < 0.001$). Hence, AD has been found to be a more reliable, rapid and economical method for antimicrobial susceptibility testing against multi-drug resistant bacteria.

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AJG and AH conceived and designed the study. AJG acquired and analyzed the data and wrote the article. AH, SS and MUA helped in analyzing the data and preparing the manuscript.

Key words
MIC, Extensively drug-resistant *Pseudomonas aeruginosa*, agar dilution method, broth dilution method

INTRODUCTION

Antimicrobial susceptibility testing (AST) plays vital role in therapeutic medicine. Quantitative methods for AST are especially beneficial in resistance surveillance, epidemiological studies of susceptibility, the comparative evaluation of new and existing agents, in vitro efficacy of drug combinations, to differentiate and establish equivocal disc results, and clinical management of those pathogens able to develop acquired resistance quite rapidly (Andrews, 2001). Currently, there are three methods *i.e*., agar dilution, broth microdilution and macrodilution, and gradient diffusion (E-test) which are employed to determine the minimum inhibitory concentration (MIC) of antimicrobial agents (Brown and Brown, 1991, Wiegand *et al.*, 2008).

Broth microdilution technique is reproducible to within one doubling dilution of the real end point in a dilution series. Bacteriostatic antimicrobials lead to trailing end points and interpretation can be subjective and variable. In addition, broth format also tends to affect the growth of anaerobic microorganisms. Broth microdilution, being time consuming and laborious is rarely performed in routine diagnostics setups (Gales *et al.*, 2001, Arroyo *et al.*, 2005). Agar dilution (AD) technique is considered more convenient due to the relative ease of procedure and interpretation of endpoints. However, biochemical properties of antimicrobial agents and unique organismal behavior may significantly influence the results of different techniques.

Pseudomonas aeruginosa is one of the commonest nosocomial pathogens and immunocompromised patients especially those on breathing machines, in-dwelling urinary catheters, intubated, with surgical or burn wounds and cystic fibrosis patients, cancer and AIDS patients are potentially at risk for serious, life-threatening infections. *P. aeruginosa* is notorious for its high tendency to develop resistance even during the treatment and has created new challenges in the area of antimicrobial drug therapy (Hannan *et al.*, 2014). The determination of MIC becomes necessary to detect early changes in resistance pattern.

In the present study agar dilution has been adopted as a comparatively rapid, reliable and economical routine method for *in vitro* susceptibility testing of anti-pseudomonal agents, and MIC of different bactericidal drugs was determined against domestic clinical strains of *P. aeruginosa* in order to compare the reciprocal advantage of agar dilution and broth microdilution techniques.

MATERIALS AND METHODS

Bacterial strains

Sixty-one clinical isolates of *P. aeruginosa* obtained from different tertiary care hospitals in Lahore, Pakistan were investigated. The isolates were identified by their

* Corresponding author: ajgondal119@gmail.com
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colony morphology, cultural characteristics and biochemical profile using API 20NE (bioMerieux, France). The identified strains were then preserved in Microbank™ vials (Pro-Lab Diagnostics, UK) at –80±5°C. *P. aeruginosa* (ATCC® 27853) and *Escherichia coli* (ATCC® 25922) were included as reference strains for quality control.

Antimicrobial agents and stock solutions

Antimicrobial discs of piperacillin/tazobactam (TZP; 100/10 µg/ml), amikacin (AK; 30 µg/ml), ciprofloxacin (CIP; 5 µg/ml), ceftazidime (CAZ; 30 µg/ml), meropenem (MEM; 10 µg/ml) and aztreonam (ATM; 30 µg/ml) were obtained from Oxoid. The base material of antimicrobials was obtained from GlaxoSmithKline and Searle Company limited Pakistan. The stock solutions of antimicrobials were prepared in recommended solvents and diluents according to CLSI and stored at –80 ±5°C.

Antimicrobial susceptibility testing (AST)

AST was performed by standard Kirby-Bauer disc diffusion method. Antimicrobial discs were applied on the semi-confluent lawn of pure organism on Mueller-Hinton agar plates (Oxoid, UK) aseptically according to the "Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Eleventh Edition" (M02-A11; 2012) published by Clinical and Laboratory Standards Institute (CLSI, USA). The inoculated plates were read for zones of inhibition after 18 h of incubation at 35±2°C. The zones of inhibition were interpreted as per the breakpoints given in the guidelines of "Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement" (M100-S24; CLSI 2014).

Minimum inhibitory concentration (MIC)

MICs of only twenty-four clinical strains were determined by broth microdilution and agar dilution methods due to financial constraints. The strains were selected such a way that they represented the maximum spectrum of MIC range. The concentration ranges evaluated were 1-1024 µg/ml, 0.25-64 µg/ml, 2-512 µg/ml, 0.0625-32 µg/ml and 2-512 µg/ml for ceftazidime, meropenem, amikacin, ciprofloxacin and piperacillin/ tazobactam, respectively. The initial bacterial inoculum was prepared by direct colony suspension method in sterile normal saline (0.85% w/v). The turbidity of the inoculum was adjusted to achieve a density equivalent to 0.5 McFarland (approx. 1.5 x 10^8 CFU/ml of suspension).

Agar dilution (AD) method

AD technique was carried on using Mueller Hinton agar plates according to the CLSI document "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition" (M07-A9; 2012). Series of agar plates were incorporated with serial dilutions of antimicrobial agents within a specified range. The initial inoculum was further diluted 1:10 by adding 0.1 ml of suspension in 0.9 ml of sterile normal saline to achieve the final inoculum containing approx. 1.5 x 10^7 CFU/ml of suspension. The inoculum was used within 15 min of preparation. The agar plates were spot inoculated with the multipoint inoculator using 3-mm pins to deliver 2.0 µl of inoculum to produce 5 to 7 mm spots (MAST Diagnostics, UK). In each series of plate, growth control and sterility plates were also included. The reference strains of *P. aeruginosa* (ATCC® 27853) and *E. coli* (ATCC® 25922) were also inoculated for quality control. After the inoculated spots had dried, the agar plates were incubated aerobically at 35±2°C for 18 h. The lowest concentration of antimicrobial agent that inhibited the visible growth of the selected strain was taken as MIC.

Broth microdilution (BMD) method

BMD was carried out in 96-well microtitre plates. The initial inoculum was further diluted 1:100 by cation-adjusted Mueller-Hinton broth (CA-MHB) to achieve the final inoculum containing approx. 1.5 x 10^6 CFU/ml. In each row, 100 µl of drug was added serially to achieve desired concentration range for that drug. Then 10 µl of diluted bacterial inoculum was added in each well of specified row to achieve the final inoculum dilution containing approx. 1.5 x 10^5 CFU/ml. No drug was added in growth control well and no inoculum was added in sterility control. The plates were incubated at 35±2°C for 18 h aerobically as described by "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition" (M07-A9; 2012). The MIC was taken as the minimum amount of drug that resulted in the absence of turbidity in well and was interpreted according to the criteria given by CLSI supplement 2014.

Statistical analysis and comparative evaluation

Essential agreement (EA) was defined as an endpoint by AD being plus or minus one doubling dilution (log2 dilution series) to BMD for a particular antimicrobial and expressed in percentage of strains (Rennie *et al.*, 2012). For comparison of MICs, the doubling dilution difference in individual MICs of the selected antimicrobial agents for the AD and BMD methods was calculated by formula *i.e.*, log2 (MIC by AD) -log2 (MIC by BMD) = ±log2 dilution difference (Krishnan *et al.*, 2009).

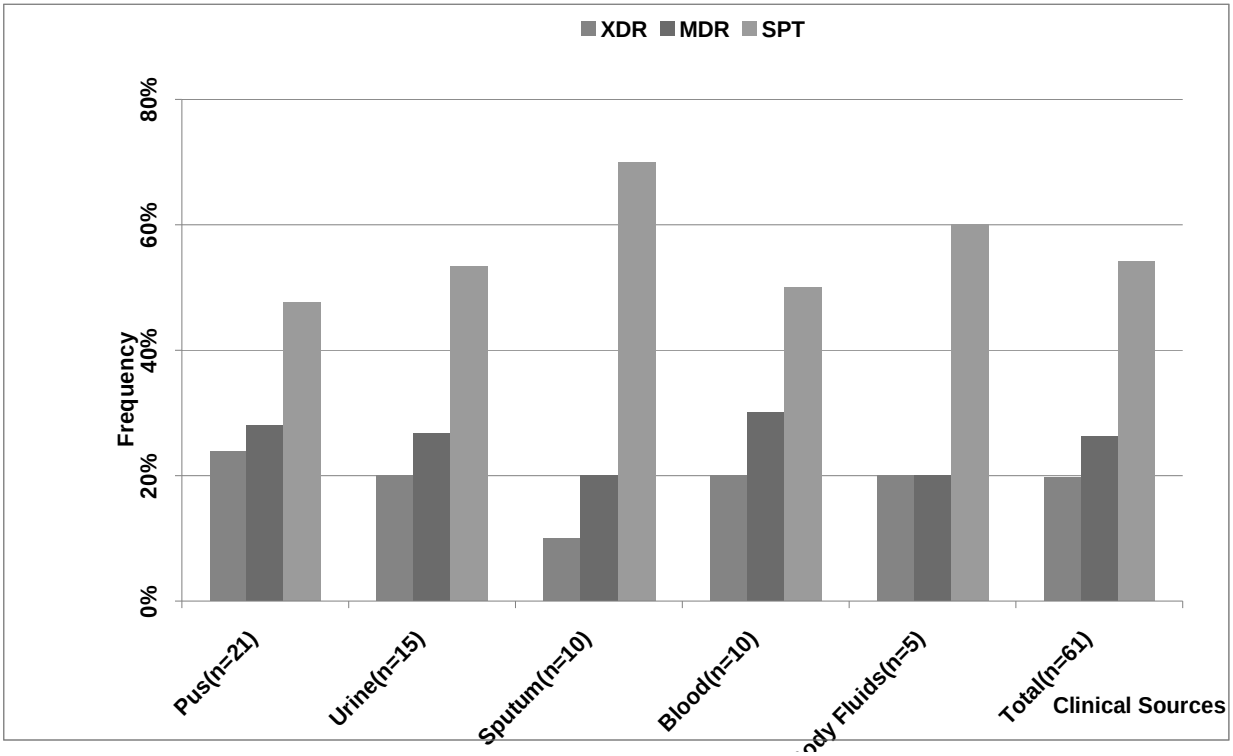

Fig. 1. Clinical sources and distribution of extensively drug resistant (XDR), multiple resistant (MDR) and susceptibility (SPT) *P. aeruginosa.*

The frequency of clinical strains with MICs measured by the two methods agreeing to within ±1 doubling dilutions was calculated; essential agreement was reported as the percentage of strains for AD MIC was the same or one doubling dilution apart from the BMD MIC (Reynolds *et al*., 2003). Pearson Correlation Coefficient was used to determine the association of the two methodologies. The data was analysed using IMB SPSS 22 statistical software.

RESULTS

The detail of clinical sources and the distribution of XDR, MDR and SPT categories of clinical strains of *P. aeruginosa* are given in Figure 1. The highest number of strains were isolated from pus (n=21, 34.4%) followed by urine (n=15, 24.6%), sputum (n=10, 16.4%), blood (n=10, 16.4%), and different body fluids (n=5, 8.2%).

For resistant (XDR and MDR) clinical strains of *P. aeruginosa* the values of MIC obtained by agar dilution were in range of 64-1024, 32-128, 16-256, 16-256, and 16-32 µg/ml for ceftazidime, amikacin, meropenem, tazocin and ciprofloxacin, respectively versus the MIC values obtained by broth microdilution as 32-1024, 16-128, 8-256, 16-256, and 16-32 µg/ml for ceftazidime, amikacin, meropenem, tazocin and ciprofloxacin, respectively. For SPT clinical strains of *P. aeruginosa* the values of MIC obtained by agar dilution were in range of 2-8, 4-16, 0.25-1, 4-8, and 0.25-1 µg/ml for ceftazidime, amikacin, meropenem, tazocin and ciprofloxacin, respectively versus the MIC values obtained by broth microdilution as 1-4, 2-8, 0.125-1, 2-8, and 0.125-1 µg/ml for ceftazidime, amikacin, meropenem, tazocin and ciprofloxacin, respectively.

The frequency of similar MICs by two methods was 58.3% for ciprofloxacin and 23.1% for amikacin. The frequency of plus one doubling dilution was 57.7, 57.7, 53.8, 38.5 and 30.7 for ceftazidime, amikacin, meropenem, ciprofloxacin and tazocin respectively, as shown in Table I.

The EA in terms of plus minus one doubling dilution (±1 $\log_2$ dilution) was 92.3% for CAZ and 100% for CIP. However, the EA with respect to all antimicrobials was equal to 90% as given in Table II. The correlation of BMD vs AD with respect to tested antimicrobials was statistically significant for all antimicrobials ($p < 0.001$) as shown in Table III.

Table I.- Comparison of agar dilution method and broth microdilution methods for determination of minimum inhibitory concentration (µg/ml) of antimicrobial drugs against clinical strains of multidrug resistant *P. aeruginosa* (n= 12).

Strain I.D.	Minimum inhibitory concentrations (µg/ml)									
	Agar dilution method					Broth microdilution method				
	CAZ	AK	MEM	TZP	CIP	CAZ	AK	MEM	TZP	CIP
P-1	256	128	256	64	32	128	64	128	32	32
P-2	128	64	256	64	32	64	32	128	32	32
P-3	256	128	16	32	32	128	64	16	16	32
P-4	512	64	16	32	32	256	32	8	32	16
P-5	128	64	32	16	32	64	32	16	16	16
P-6	512	64	128	32	32	128	32	32	32	16
P-7	256	128	128	128	32	128	64	32	128	32
P-8	1024	32	256	256	16	1024	16	256	256	16
P-9	256	128	128	128	32	64	128	256	128	16
P-10	128	64	16	32	32	128	128	16	64	32
P-11	64	128	16	32	32	32	64	16	32	32
P-12	1024	32	16	64	32	1024	64	16	128	32

AK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; µg/ml, microgram per milliliter.

Table II.- Comparison of agar dilution method and broth microdilution methods for determination of minimum inhibitory concentration (µg/ml) of antimicrobial drugs against clinical strains of susceptible *P. aeruginosa* (n= 12).

Strain I.D.	Minimum inhibitory concentrations (µg/ml)									
	Agar dilution method					Broth microdilution method				
	CAZ	AK	MEM	TZP	CIP	CAZ	AK	MEM	TZP	CIP
S-1	8	4	0.25	4	0.25	2	4	0.125	8	0.125
S-2	4	8	0.25	4	0.5	4	2	0.125	4	0.50
S-3	2	8	2	8	0.25	1	2	1	2	0.25
S-4	2	4	1	4	0.5	2	2	0.5	2	0.25
S-5	2	8	0.5	8	0.5	1	4	0.25	4	0.50
S-6	2	16	1	4	0.5	1	8	0.25	2	0.25
S-7	8	4	1	8	0.5	4	4	0.5	2	0.50
S-8	8	8	1	4	1	4	4	0.5	8	1
S-9	8	8	2	8	1	2	2	0.5	8	1
S-10	8	4	2	4	1	4	4	1	2	0.50
S-11	4	8	1	4	0.25	2	4	0.5	2	0.125
S-12	8	8	0.5	8	1	4	4	0.25	8	0.50

For abbreviations see Table I.

Table III.- Essential agreement for AD vs BMD against *P. aeruginosa.*

Antimicrobial agents	% of strains with log_2 dilution difference*					Essential Agreement (EA, %) (±1 dilution)	Correlation AD vs BMD	P value
	-2	-1	0	+1	+2			
Ceftazidime		7.7	26.9	57.7	7.7	92.3	0.943	<0.001
Amikacin		7.7	23.1	57.7	11.5	88.5	0.780	<0.001
Meropenem		3.9	26.9	53.8	15.4	84.6	0.809	<0.001
Tazocin	3.8	15.4	38.5	30.7	11.6	84.6	0.956	<0.001
Ciprofloxacin		7.7	53.8	38.5		100	0.923	<0.001
Total						90.0		

(*)AD vs BMD

DISCUSSION

MIC values obtained by AD method exhibited a general trend of plus one dilution of MIC with respect to BMD method for the tested antimicrobial agents. This is in contrast to another study which reported higher frequency of similar MICs by both techniques (Amsler *et al.*, 2010). The agar depth and moister content on the surface, cation concentration and the diffusion properties of the drugs may contribute to such effects; in the case of broth microdilution such factors are better controlled. Moreover, the dynamics of organismal growth and the interaction with drugs tend to play role in discrepancies including, but not limited to, the growth in a spot form on the surface of agar and the uniform distribution of drug and bacteria in the liquid broth (Lorian, 2005).

EA is a quick and dependable tool for comparative analysis. An EA of >90% is considered significant by Federal Drug Agency, USA (Rennie *et al.*, 2012). EA between the methods was considered excellent if the MICs were within ±1 doubling dilution for 90% of strains; good when 80% of the MICs were within ±1 doubling dilution and poor if <80% of MICs were within ±1 doubling dilution.

Overall the two methods showed excellent concordance with an EA of 90%. Overall EAs of 78.7% and 86.7% for AD versus BMD having been reported against *Campylobacter* species using different drugs (Luber *et al.*, 2003, Halbert *et al.*, 2005, Reynolds *et al.*, 2003). This difference is somehow related to the slower growth trends of *Campylobacter* species and usage of bacteriostatic drugs thereby giving trailing end points.

In the present study EAs for ceftazidime and tazocin were 92.3% and 84.6%, respectively. One $\log^2$ dilution of the breakpoints was 62% for ceftazidime, and 59% for piperacillin-tazobactam (Sader *et al.*, 2006). The lower EA results may be attributed to the determination of categorical end points for drugs in contrast to our study where individual values of MIC were considered for comparison purpose.

Ciprofloxacin achieved highest level of 100% EA for the clinical strains and is comparable to other studies which reported 91.5% and 96.3% (Halbert *et al.*, 2005, Luber *et al.*, 2003). In our study meropenem showed 84.6% EA which is lower than as reported by 96.3% for doripenem against *Pseudomonas* species (Amsler *et al.*, 2010).

Statistically significant correlation rates of 94.3%, 78.0%, 80.9%, 95.6% and 92.3% were observed for ceftazidime, amikacin, meropenem, tazocin and ciprofloxacin (p value <0.001). The least correlation for amikacin may be attributed to the involvement of cation content role in different types of Mueller Hilton recipes especially against *P. aeruginosa* (Lorian, 2005). However it is comparable with another study wherein EAs of 91, 92, 88, 88 and 86% were reported for ceftazidime, amikacin, meropenem, tazocin and ciprofloxacin, respectively (Saiman *et al.*, 1999).

It is concluded that the agar dilution technique has exhibited comparable and reliable results for the drugs tested and can be adopted quite conveniently for routine antimicrobial susceptibility testing with the added advantages of efficiency, convenience and economy.

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Conflict of interest statement

There is no conflict of interest.

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