Comparative *in vitro* antimicrobial procedural efficacy for susceptibility of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas* species to chloramphenicol, ciprofloxacin and cefaclor

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ABSTRACT

The present study assesses the reliability of the *in vitro* susceptibility tests E-test, disc diffusion and Alamar blue-based microbroth dilution assays for evaluating the efficacy of chloramphenicol, ciprofloxacin and cefaclor against clinical and reference isolates of *Staphylococcus aureus, Escherichia coli* and *Pseudomonas* spp. for use in empirical therapy. Ciprofloxacin showed 82% agreement between E-test and microbroth dilution by the visible dye reduction method against all organisms. This figure was 67% for cefaclor and 60% for chloramphenicol. E-test and microbroth dilution showed excellent correlation against *S. aureus* with all three antibiotics; however, correlation was not observed in *Pseudomonas* spp. between E-test and microbroth dilution by the percentage dye reduction method. *E. coli* did not show significant correlation, indicating the presence of heteroresistance. Chloramphenicol, being bacteristatic in nature, did not show clear agreement between the susceptibility test methods used. This study indicates that E-test provides an indication of minimum inhibitory concentration (MIC) of a panel of antibiotics against clinical isolates; however, microbroth dilution (dye reduction) is the most sensitive method for the determination of MIC due to intracellular enzymatic reduction of the dye to formazan, which only occurs in viable organisms. The study highlights the need for harmonisation between E-test and microbroth dilution methods in clinical trials of new antibiotics and in monitoring the drug resistance patterns in community and healthcare settings.

KEY WORDS: Antimicrobial susceptibility testing.
Escherichia coli.
Pseudomonas.
Spectrophotometry.
Staphylococcus aureus.

Introduction

In vitro antimicrobial susceptibility testing (AST) procedures are not only used for evaluation of antimicrobial potential of antibiotics and phytochemicals but also serve as a diagnostic tool. The procedures provide a real-time picture of the clinical efficacy of empirical treatment in individual patients. It also has an impact on prescribing policies and formulary developments. The data generated through routine diagnostic testing has significance in tracking antimicrobial resistance in the community and across geographical areas.

In vitro AST methods comprise diffusion assays (disc assay and agar well dilution assay), dilution assay (microbroth dilution assay) and diffusion and dilution (E-test). The success or failure of antimicrobial therapy ideally is predicted by AST methodology adopted where microorganisms are divided into treatable and non-treatable categories on the basis of minimum inhibitory concentration (MIC) breakpoints. These breakpoints divide bacteria into three categories of susceptibility: susceptible (S), intermediate (I) and resistant (R).[1]

Minimum inhibitory concentration is considered to be the gold standard for susceptibility testing of organisms to antimicrobials.[2] The emergence and spread of multidrug-resistant microbes over the past decade demonstrates that the MIC breakpoints are a guide to *in vivo* antimicrobial therapy as well as to trends of emergence in resistant phenotypes. The method most commonly used for AST in clinical laboratories is the broth dilution method described by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]).[3]

Development of resistance to antimicrobial agents and the emergence of multidrug-resistant pathogens (e.g., *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Mycobacterium tuberculosis*) has resulted in worldwide concern.

Staphylococcus aureus is recognised as a major human pathogen responsible for skin and soft tissue,[4] respiratory,[5,6] bloodstream, breast and ocular infections. Methicillin-resistant *S. aureus* (MRSA) is responsible for chronic refractory infections[7] due to the acquisition of resistance towards current antimicrobials, and poses a serious problem for clinicians. Similarly, *Escherichia coli* poses serious health

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concerns as it accounts for 85% of urinary tract infections.[8]

Increasing resistance to third-generation cephalosporins among *E. coli* strains predominantly is due to the production of extended-spectrum β-lactamases (ESBLs) and has increased morbidity and mortality rates, especially among patients on intensive care and high-dependency units.

Another emerging pathogen is *Pseudomonas aeruginosa*, which is responsible for a variety of nosocomial infections including pneumonia, urinary tract infection, surgical wound and bloodstream infections. It is life-threatening in immunocompromised patients.[9,10]

A significant increase in the spread of MRSA, ESBLs and multidrug-resistant *P. aeruginosa* strains has extended the need for rapid and accurate AST methods. Over the past decade, E-test (AB Biodisk, Solna, Sweden) has emerged as a convenient test for the determination of MIC breakpoints. The E-test is an agar diffusion MIC method which uses a thin plastic strip coated with an antimicrobial gradient and a quantitative interpretive scale. The MIC is determined by reading the antimicrobial concentration printed on the test strip at its intersection with the growth inhibition zone.

Ease of performance and reports of excellent correlation of results with agar dilution techniques has led to the increased use of E-test for determining MICs. However, the presence of small resistant colonies or a haze of growth in the inhibition zone has contributed to inconsistent results.[11]

This study aims to compare the performance of conventional and current AST methods in determining the susceptibility of drug-resistant clinical isolates of *S. aureus, E. coli* and *Pseudomonas* species to ciprofloxacin, chloramphenicol and cefaclor in order to assess diagnostic reliability in developing countries.

Materials and methods

Test microorganisms included *S. aureus* (31 isolates), *E. coli* (21 isolates) and *Pseudomonas* spp. (eight isolates). *S. aureus* NCTC 6571, *E. coli* NCTC 10418 and *P. aeruginosa* NCTC 10662 were included as reference strains. The bacteria were maintained on trypticase soy agar slants and the cultures were activated in cation-adjusted Mueller Hinton (MH) broth 18–24 h prior to the test.

Antibacterial agents tested were cefaclor, chloramphenicol and ciprofloxacin. Cefaclor (pure salt) and antibiotic impregnated discs were obtained from Hi Media Laboratories, Mumbai. Ciprofloxacin (pure salt) was a gift from Dr. Reddy's laboratories (Hyderabad, India) and the E-test strips were provided by AB Biodisk.

Stock solutions (2.048 mg/mL) of ciprofloxacin and cefaclor were prepared, from which further dilutions were produced. A 2.275 mg amount of chloramphenicol salt (900 µg/mg) was used to prepare an initial stock solution (equivalent to 2.048 mg/mL). All three stock solutions were diluted to provide a suitable range of antibiotic concentrations[2] using standard sterile diluents as per the recommendations of EUCAST/BSAC. Although microbial contamination of antibiotic powder is rare, all stocks were filter sterilised using a 0.2 µm pore size membrane filter (Whatman).

Minimum inhibitory concentration breakpoints for the antibiotics against the bacterial strains for the different AST methods are shown in Table 1.

In the present study, the susceptibility of the test bacteria to the three antibiotics was tested by four methods: the Kirby Bauer disc-diffusion (DD) assay, E-test (ET), microbroth dilution (visual colour change method [MD{V}]) and microbroth dilution (spectrophotometric measurement of dye reduction [MD{R}]).

The Kirby Bauer DD assay was used to profile the resistance patterns of test isolates according to CLSI guidelines.[3] Muller Hinton agar plates (mean depth ± 4 mm) were inoculated with a 0.5 McFarland standard-adjusted test culture by swabbing each plate three times in order to determine the antibacterial resistance profiles. Sterile antibiotic discs (Hi Media) were applied to the agar surface using a sterile dispenser and gentle pressure with sterile forceps to ensure complete contact. The plates were incubated for 18–20 h at 35°C. Susceptibility was evaluated

Table 1. Minimum inhibitory concentration breakpoints of test antibiotics by different methods.

	E-test			Broth dilution (visual method)			Broth dilution (% reduction)		KB disk assay				Time kill	
	S	I	R	S	I	R	S	R	Disk content	S	I	R	S	R
Staphylococcus aureus														
Chloramphenicol	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤50 %	>50 %	30 µg	18.0 mm	13–17 mm	≤12.0 mm	≥3$\log_2$ reduction	>3$\log_2$ reduction
Ciprofloxacin	≤1 µg/mL	1–4 µg/mL	≥4 µg/mL	≤≤1 µg/mL	2 µg/mL	≥4 µg/mL	≤50 %	>50 %	5 µg	21.0 mm	15–20 mm	≤15.0 mm	≥3$\log_2$ reduction	>3$\log_2$ reduction
Cefaclor	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤50 %	>50 %	30 µg	>18.0 mm	15–17 mm	<14.0 mm	≥3$\log_2$ reduction	>3$\log_2$ reduction
Escherichia coli and *Pseudomonas* spp.														
Chloramphenico	≤1 µg/mL	1–4 µg/mL	≥4 µg/mL	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤50 %	>50 %	30 µg	18.0 mm	13–17 mm	≤12.0 mm	≥3$\log_2$ reduction	>3$\log_2$ reduction
Ciprofloxacin	≤1 µg/mL	1–4 µg/mL	≥4 µg/mL	≤1 µg/mL	2 µg/mL	≥4 µg/mL	≤50 %	>50 %	5 µg	21.0 mm	15–20 mm	≤15.0 mm	≥3$\log_2$ reduction	>3$\log_2$ reduction
Cefaclor	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤8 µg/mL	8–32 µg/mL	≥32 µg/mL	≤50 %	>50 %	30 µg	>18.0 mm	15–17 mm	<14.0 mm	≥3$\log_2$ reduction	>3$\log_2$ reduction

Table 2. Summary of interpretive errors for E-test on MH agar/broth.

			Discrepancies		
		Agreement	Very major	Major	Minor
KB disc assay	*Staphylococcus aureus*[*]				
	Chloramphenicol	28/31 (90)	0/31 (0)	0/31 (0)	3/31 (10)
	Ciprofloxacin	29/31 (94)	0/31 (0)	2/31 (6)	0/31 (0)
	Cefaclor	24/31 (77)	1/31 (3)	0/31 (0)	6/31 (19)
	Escherichia coli[†]				
	Chloramphenicol	7/21 (33)	0/21 (0)	12/21 (57)	2/21 (10)
	Ciprofloxacin	18/21 (86)	0/21 (0)	3/21 (14)	0/21 (0)
	Cefaclor	16/21 (76)	0/21 (0)	4/21 (19)	1/21 (5)
	Pseudomonas spp.[‡]				
	Chloramphenicol	3/8 (38)	0/8 (0)	5/8 (62)	0/8 (0)
	Ciprofloxacin	7/8 (88)	0/8 (0)	1/8 (12)	0/8 (0)
	Cefaclor	6/8 (75)	0/8 (0)	1/8 (13)	1/8 (13)
Microbroth dilution (visible)	*Staphylococcus aureus*				
	Chloramphenicol	13/31 (42)	18/31 (58)	0/31 (0)	0/31 (0)
	Ciprofloxacin	24/31 (77)	7/31 (23)	0/31 (0)	0/31 (0)
	Cefaclor	16/31 (52)	6/31 (19)	3/31 (10)	6/31 (19)
	Escherichia coli				
	Chloramphenicol	13/21 (62)	1/21 (5)	0/21 (0)	7/21 (33)
	Ciprofloxacin	17/21 (81)	3/21 (14)	1/21 (5)	0/21 (0)
	Cefaclor	18/21 (86)	1/21 (5)	0/21 (0)	2/21 (10)
	Pseudomonas spp.				
	Chloramphenicol	6/8 (75)	0/8 (0)	1/8 (13)	1/8 (13)
	Ciprofloxacin	7/8 (88)	1/8 (13)	0/8 (0)	0/8 (0)
	Cefaclor	5/8 (63)	1/8 (13)	0/8 (0)	2/8 (25)
Microbroth dilution (% dye reduction)	*Staphylococcus aureus*				
	Chloramphenicol	17/31 (55)	10/31 (32)	4/31 (13)	0/31 (0)
	Ciprofloxacin	17/31 (55)	8/31 (26)	6/31 (19)	0/31 (0)
	Cefaclor	8/31 (26)	9/31 (29)	8/31 (26)	6/31 (19)
	Escherichia coli				
	Chloramphenicol	10/21 (48)	2/21(10)	4/21 (19)	5/21 (24)
	Ciprofloxacin	8/21 (38)	8/21(38)	5/21 (24)	0/21 (0)
	Cefaclor	7/21 (33)	8/21(38)	6/21 (29)	0/21 (0)
	Pseudomonas spp.				
	Chloramphenicol	2/8 (25)	1/8 (13)	2/8 (25)	3/8 (38)
	Ciprofloxacin	4/8 (50)	3/8 (38)	1/8 (13)	0/8 (0)
	Cefaclor	3/8 (38)	3/8 (38)	0/8 (0)	2/8 (25)

[*]VRSA (n=2), VISA (n=3), MRSA (n=12), MARSA (n=20) [†]ESBLs (n=6), [‡]MARP (n=1).

Table 3. Comparison of AST methodologies using Pearson's correlation coefficient.

	Chloramphenicol		Ciprofloxacin		Cefaclor	
	E-test *vs.* MD(V)	E-test *vs.* MD(R)	E-test *vs.* MD(V)	E-test *vs.* MD(R)	E-test *vs.* MD(V)	E-test *vs.* MD(R)
Staphylococcus aureus (n=31)	0.99	0.7	0.99	0.61	0.99	0.57
Escherichia coli (n=29)	0.69	0.74	0.76	0.69	0.18	0.5
Pseudomonas spp. (n=8)	0.99	0.97	0.99	0.3	0.97	0.97

by measuring the diameter of the clear inhibition zone around the antibiotic disc. Plates were examined visually for isolated colonies within the inhibition zone that may have represented resistance. All the experiments were performed in triplicate, and the cultures were classified as sensitive, intermediate or resistant.

Muller Hinton agar plates (mean depth ± 4 mm) were inoculated by confluent swabbing of the surface with 0.5 McFarland-adjusted inoculum suspensions. Inoculated plates were allowed to dry and then E-test strips were applied to each plate. The plates were incubated for 18–20 h at 35°C. E-test results were interpreted by recording the point of intersection of growth inhibition with the MIC scale on the strip.[12,13]

Minimal inhibitory concentration of the antibiotics used was calculated by an Alamar blue-based microbroth dilution assay.[14,15] The range of antibiotic concentrations evaluated was 0.032–512 µg/mL (chloramphenicol); 0.001–64 µg/mL (ciprofloxacin) and 0.008–512 µg/mL (cefaclor). Included were a negative (no growth) control, a positive growth control and increasing concentrations of the test antibiotic. The turbidity of the test inoculum was adjusted visually by comparing it to a 0.5 McFarland standard. Then, 50 µL of the 0.5 McFarland-adjusted bacterial suspension in saline was added to 125 µL MH broth to achieve a final bacterial cell concentration of 10^6 cells. Plates were incubated at 35°C for 2.5 h, and then 25 µL of stock drug was added. After 24 h, 10 µL 0.1% Alamar blue was added to each well.

The MIC was interpreted visually as the minimum concentration of the antibiotic that inhibited microbial growth after overnight incubation, indicated by no colour change of the Alamar blue (from blue to red due to reduction of the dye).

Measuring absorbance spectrophotometrically,[16] the MIC can be interpreted as the minimal concentration of the drug that results in <50% dye reduction. The absorption spectra of the oxidised and reduced forms of Alamar blue was measured at 570 nm and 600 nm on a PowerWave 340 (BIOTEK, USA) microtitre plate reader. The percentage of Alamar blue reduced after 60 min incubation was calculated.

Pearson correlation coefficients were calculated for each antibiotic to compare the association between MIC results of the E-test to microbroth dilution and time-kill methods. Further correlation was studied by calculating the percentage of agreement that gave identical MIC results within the accuracy limits of standard tests ($\pm 1 \text{Log}_2$ dilution). Finally, to check whether one testing method produced significantly lower or higher results compared to another method, Wilcoxon signed rank test was performed using GraphPad Prism (version 4) software. Discrepancies were interpreted as very major (when the reference method misinterpreted a resistant or intermediate strain as susceptible), major (when a susceptible strain was misinterpreted as resistant or intermediate) and minor (when a resistant strain was misinterpreted as intermediate by the reference method).

Results

A summary of interpretive errors for each of the sensitivity methods against the strains tested is presented in Table 2. With the exception of the DD assay, all methods produced unacceptably high frequencies of very major errors for the three antibiotics tested.; the highest being for the susceptibility testing of *S. aureus* against chloramphenicol by the MD (V) method. Comparison of AST methodologies using Pearson's correlation coefficient is shown in Table 3.

The distribution of differences in $\log_2$ MICs, the percentage agreement and *P* values for all antibiotics and various AST methods are given in Tables 4–7. The E-test tended to give lower MICs with Gram-negative organisms than did the microbroth dilution assay (visible MIC). A comparison of the overall results for Gram-positive and Gram-negative organisms indicates that E-test is more suitable for AST of *S. aureus* against bacteriostatic drugs.

Discussion

Current empirical antibiotic therapy to a large extent is based on the clinician's knowledge of new antimicrobial agents in the majority of developing south Asian countries, including India. Thus, the majority of clinical investigators are not following good clinical practices, and this contributes to the increase in antimicrobial drug resistance. Accurate *in vitro* clinical AST and interpretation are a must for a successful clinical outcome.

Many workers have evaluated the efficiency of commercial and reference susceptibility methods to detect antibiotic resistance in human pathogenic microorganisms.[16–18] Researchers from across the world have demonstrated moderate efficiency of E-test and good correlation between E-test, microbroth dilution, agar dilution and disc-diffusion methods for susceptibility testing of Gram-positive and Gram-negative human pathogens.[19,20]

Tigecycline has shown 23% disagreement in results between DD and microbroth dilution methods,[21] while 48% disagreement has been reported between E-test and agar dilution assays for both aerobic and anaerobic microbes.[22] Imipenum has shown 67% disagreement between E-test and broth microdilution procedures against *Pseudomonas*,[23] and 33% disagreement has been reported for levofloxacin against *S. aureus* using E-test and agar dilution methods.[24]

Trovofloxacin exhibits reduced MIC by E-test against *P. aeruginosa* and MRSA when compared to agar dilution and microbroth dilution assay. It is recommended that disc diffusion, agar and microdilution, but not E-test, be used for routine trovafloxacin susceptibility testing of *P. aeruginosa* and MRSA.[12] Furthermore, the cost and limited availability of E-test must be taken into account in developing countries such as India.[25] In routine diagnostic laboratories, the KB disc assay can be adopted for primary screening of isolates, followed by a reference agar dilution method, on which the correct drug dosage can be calculated.

The present study was undertaken in order to identify the most reliable AST method for use in healthcare settings for detection of resistance. The results support the need for the careful monitoring of susceptibility breakpoints and the emergence of rapid changes in resistance to antibiotics.

When susceptibility testing is performed, broth microdilution and time kill studies should be adopted as gold standards for MIC determination. Tentative MIC breakpoints can be inferred from E-test in clinical/pathology laboratories. However, if more than two antibiotics exhibit the same MIC concentration against clinical isolates of

Table 4. Distribution of differences in MICs with three antibacterial agents for Gram-negative bacilli (E-test *vs*. microbroth dilution [visual] Alamar blue method).

		% isolates with respective MIC difference*							
	No. of strains	<-2	-2	-1	0	1	2	>2	*P* value†
Chloramphenicol	29	0	3.57	7.14	32.15	10.71	21.43	25	0.0054
Ciprofloxacin	29	0	3.57	14.29	21.43	21.43	25	14.28	0.1174
Cefaclor	29	10.71	10.71	7.14	32.14	3.57	10.71	25	0.5195
All agents		3.57	5.95	9.52	28.57	11.9	19.05	21.43	

Overall agreement within accuracy limits: 49.99%

*Zero indicates the percentage of isolates for which MICs are identical; –1, +1 indicate ±1Log_2 dilution difference, etc.

†*P* values obtained from Wilcoxon rank test.

Table 5. Distribution of differences in MICs with three antibacterial agents for Gram-negative bacilli (E-test *vs*. microbroth dilution [% dye reduction) Alamar blue method).

		% isolates with respective MIC difference*							
	No. of strains	<-2	-2	-1	0	1	2	>2	*P* value†
Chloramphenicol	29	0	17.86	10.71	0	10.71	57.14	3.57	0.1921
Ciprofloxacin	29	0	0	32.14	25	14.29	28.57	0	0.3933
Cefaclor	29	0	21.43	7.14	14.29	17.86	35.71	3.57	0.837
All agents		0	13.1	16.66	13.1	14.29	40.47	2.38	

Overall agreement within accuracy limits: 44.05%

*Zero indicates the percentage of isolates for which MICs are identical; –1, +1 indicate ±1Log_2 dilution difference, etc.

†*P* values obtained from Wilcoxon rank test.

Table 6. Distribution of differences in MICs with three antibacterial agents for *Staphylococcus aureus* strains (E-test *vs*. microbroth dilution [visual] Alamar blue method).

		% isolates with respective MIC difference*							
	No. of strains	<-2	-2	-1	0	1	2	>2	*P* value†
Chloramphenicol	31	0	0	0	41.94	3.22	54.84	0	0.001
Ciprofloxacin	31	0	0	3.26	70.96	3.2	22.58	0	0.0195
Cefaclor	31	0	25.81	22.58	45.16	6.45	0	0	0.0049
All agents		0	8.61	8.61	52.69	4.29	25.81	0	

Overall agreement within accuracy limits: 69.96%

*Zero indicates the percentage of isolates for which MICs are identical; –1, +1 indicate ±1Log_2 dilution difference, etc.

†*P* values obtained from Wilcoxon rank test.

Table 7. Distribution of differences in MICs with three antibacterial agents for *Staphylococcus aureus* strains (E-test *vs*. microbroth dilution [% dye reduction] Alamar blue method).

		% isolates with respective MIC difference*							
	No. of strains	<-2	-2	-1	0	1	2	>2	*P* value†
Chloramphenicol	31	0	16.13	0	6.45	67.74	9.68	0	0.1951
Ciprofloxacin	31	0	0	32.27	48.38	0	19.35	0	0.1504
Cefaclor	31	0	19.35	9.68	35.48	3.23	32.26	0	0.4595
All agents		0	11.83	13.98	30.1	23.66	20.43	0	

Overall agreement within accuracy limits: 67.74%

*Zero indicates the percentage of isolates for which MICs are identical; –1, +1 indicate ±1Log_2 dilution difference, etc.

†*P* values obtained from Wilcoxon rank test.

S. aureus, E. coli and P. aeruginosa, their MIC_{50} should be reconfirmed using an indicator-based broth microdilution method to rule out heteroresistant isolates.

Thus, careful standardisation of methodologies is required to ensure accurate results and there should be development of integrative MIC breakpoint harmonisation between E-test and microbroth dilution methods. □

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