ORIGINAL ARTICLE

Rapid Disc Diffusion Antibiotic Susceptibility Testing for Gram Negative Non- fermenters: *Pseudomonas* and *Acinetobacter*

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Key words:
Rapid Disc Diffusion; Antibiotic susceptibility, Acinetobacter; Pseudomonas

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ABSTRACT

Background: *Non fermentative Gram-negative bacilli are opportunistic pathogens associated with serious hospital infections. Antibiotic susceptibility pattern of these pathogens has become unpredictable and disc diffusion remains the preferred standardized, most used and cost-effective method according to the CLSI and EUCAST. However, the method is standardized for overnight incubation (16-18 hrs.), which delays result an extra day.* ***Objective:*** *The aim of our study was to examine the possibility and accuracy of manually interpreting disc diffusion zone diameter results for clinical isolates of Acinetobacter species and Pseudomonas species, after 6 hrs. and 8 hrs. incubation, in comparison to the standard overnight incubation.* ***Results:*** *This study showed that there was a good level of agreement for early zone measurement of Acinetobacter AST after 6 hrs., which improved with extending incubation to 8 hrs. As for Pseudomonas species, zone measurement at 8 hrs. resulted in minor errors of 10.4% and very major errors of 1.2%, which just exceeds the guideline allowed limits.* ***Conclusion:*** *Rapid antibiotic susceptibility testing and interpretation was manually possible. Both Acinetobacter and Pseudomonas were measurable at 8 hrs., but Acinetobacter measurements were more accurate with minimal errors, which makes it a promising cost-effective method for rapid delivery of AST results for Acinetobacter. These findings are worthy of further studies, to determine the best incubation time that would allow rapid results delivery with minimal errors for different bacterial species.*

INTRODUCTION

Antibiotic resistance has been named by the World Health Organization (WHO) as one of the major health issues threatening human well-being.[1] This danger is rising with the continued unmonitored abuse of antibiotics and the consequences of this abuse on cost and economy. It leads to loss of cheap alternatives and directs antimicrobial therapy towards new expensive precious antibiotics. Adding to this, the accompanied lengthy hospital stays, with the associated increase in morbidities and mortalities, as in the case of hospital acquired infections with multidrug resistant (MDR) strains with intrinsic resistance mechanisms as *Acinetobacter* and *Pseudomonas*. This is considered a major problem in our institute [2-4].

Multiple strategies are being applied to aid in the fight against the upsurge in antibiotic resistance, through the WHO global action plan to hinder resistance development. This plan focusses on multiple points, one of which is the indicated and well-timed use of antibiotics in therapy as a main pillar [5].

Empirical antibiotic therapy should be adjusted based on antibiotic susceptibility testing (AST) results as quickly as possible. Timely delivery of such data should be swift to re-evaluate, adjust and modify inappropriate therapy, deescalate the antimicrobial choice made empirically, or take decision to switch to orally administered antibiotic. This reduces duration of hospital stay with all its drawbacks [6,7].

Currently there are several AST automated systems that are widely used for reporting susceptibility results. However, the standardized Bauer Kirby disc diffusion technique remains the most broadly used method by microbiology labs. This is because of its reduced cost, accurate results, flexibility in changing the panel of antibiotics tested according to guidelines and availability. Moreover, heterogenous resistance might be detected, and determination of phenotypic resistance types as carbapenamase producers and extended spectrum beta lactamase (ESBL) producers [8-11].

The results for standardized disc diffusion method are reported after 18-20 hrs. incubation. Still some researchers demonstrated that it might be possible to reduce the incubation time, and issue results earlier without much effect on their accuracy. Weber et al suggested in their research, that overnight incubation was standardized to fit with the morning working schedules for microbiology laboratories. Currently most microbiology labs have extended working hours, which would make it more relevant to examine the possibility and accuracy of interpreting the zones at a shorter incubation time [12].

The aim of our study was to examine the possibility and accuracy of manually interpreting disc diffusion zone diameter results for clinical isolates of *Acinetobacter* species and *Pseudomonas* species, after 6 hrs. and 8 hrs. incubation, in comparison to the standard overnight incubation.

METHODOLOGY

Clinical isolates:

Thirty *Acinetobacter* clinical isolates and 20 *Pseudomonas* isolates were selected. Selection process aimed at including strains with different zone diameters and sensitivities to various antibiotics tested, reflecting strain population isolated in our laboratory. These strains were isolated from routine Microbiology laboratory of Alexandria Main University Hospital. Approval of the Ethical Committee was obtained from the Faculty of Medicine, Alexandria University.

E. coli (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were included in the study as internal quality control for the standard disc diffusion procedure.

The above selected clinical strains were subjected to species identification using VITEK-2 GN card, VITEK-2 compact system (bioMérieux, Marcy l'Étoile, France). Of the 30 *Acinetobacter* species included in the study, 24 were identified as *Acinetobacter baumanii*, four as *Acinetobacter calcoaceticus* and two as *Acinetobacter lwoffi*. As for *Pseudomonas* species, all were identified as *Pseudomonas aeruginosa*. Antibiotic susceptibility was also performed via VITEK-2 AST card N222 and any strains that showed discrepancy between the disc diffusion and VITEK result were excluded from the study (as this discrepancy is not within the scope of our study) [13-15].

Antibiotic susceptibility testing (AST):

Overnight growth on blood agar was used to prepare 0.5 McFarland suspension from each isolate by touching 3-5 separate colonies by a sterile loop and suspending them in 2ml sterile saline, the 0.5 McFarland was adjusted using the Densichek Plus (bioMérieux) turbidimeter. Disc diffusion was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16].

Disc diffusion tests were performed and read during laboratory working hours. Initially, the AST by disc diffusion was done and zone measurements were registered after 6 hrs. of incubation at 37°C. The measurement was taken by 2 different trained laboratory members and each registered 6-hour zone diameter independently (6ZD). Then plates were reincubated and read again after another 2 hrs. giving the 8-hour zone diameter (8ZD). Early on the following day, the final standard zone measurement was registered after overnight incubation giving the (SZD) [17].

The average reading was recorded, and results interpreted according to CLSI cutoffs. The discs choice depended on the availability of supply and sensitivity pattern of our isolates [16].

Acinetobacter species were tested against Ampicillin-sulbactam 10/10 ug, Piperacillin tazobactam 100/10 ug, Meropenem10 ug, Gentamycin 10 ug, Tobramycin 10 ug, Amikacin 30 ug, Doxycycline 30 ug, Minocycline 30 ug, Tetracycline 30 ug, Levofloxacin 5 ug and Trimethoprim- sulfamethoxazole 1.25/23.75 ug [16].

Pseudomonas species were tested against Piperacillin tazobactam 100/10 ug, Cefepime 30 ug, Aztreonam 30ug, Imipenem 10ug, Meropenem10 ug, Gentamycin 10 ug, Tobramycin 10 ug, Amikacin 30 ug, Ciprofloxacin ug, Levofloxacin 5 ug and Ofloxacin 5 ug [16].

Statistical analysis:

For each antibiotic tested zone diameters (6ZD and 8ZD) were compared with the final standard zone measurement (SZD). The difference in zone diameters was calculated and mean difference and standard deviation for each antibiotic was estimated. Categorical agreement and errors in result interpretation were calculated according to guidelines [18].

Errors are presented as either minor errors (mE), Major errors (ME) or Very Major Errors (VME) where the accepted limit is < 10% for mEs, < 3% for MEs and <1% for VMEs. Simple linear regression was used to calculate coefficient of determination (R^2) (95% CI) to test if 6ZD/8ZD significantly predicted the final result. (R^2 range is from 0 to 1). Where a value of 1 suggests an optimum fit, i.e: the model used is a 100% reliable predictor.

RESULTS

Susceptibility results of the tested *Acinetobacter* and *Pseudomonas* clinical isolates after 6, 8 and overnight incubation

This work was carried on 30 strains of *Acinetobacter* species and 20 strains of *Pseudomonas* species. Sensitivities to various antibiotics tested are shown in table 1 and table 2.

The total zones measured for 30 *Acinetobacter* species against 11 antibiotics include 330 zone readings after 6 hrs., 8 hrs and overnight incubation, giving a total of 990 measurement.

As for the 20 *Pseudomonas* isolates tested against 12 antibiotics, growth visibility was difficult at 6 hrs., and our personnel failed to read 80% of pseudomonas AST plates at 6 hrs. Therefore, 240 zone diameters were registered after 8 hrs. and overnight of incubation of *Pseudomonas,* giving a total of 480 zone measurements.

Table 1: Susceptibility results for 30 *Acinetobacter* isolates

Antibiotic	Sensitive			Intermediate			Resistant			Total
	6hrs	8hrs	overnight	6hrs	8hrs	overnight	6hrs	8hrs	overnight	
Amikacin	6	6	6	5	5	6	19	19	18	90
Ampicillin-sulbactam	7	8	6	6	6	7	17	16	17	90
Doxycycline	12	12	11	3	3	4	15	15	15	90
Gentamycin	3	6	6	7	4	4	20	20	20	90
Levofloxacin	5	7	7	7	5	4	18	18	19	90
Meropenem	7	8	8	5	4	5	18	18	17	90
Minocycline	21	21	22	2	3	2	7	6	6	90
Piperacillin-tazobactam	6	7	7	7	6	6	17	17	17	90
Sulphamethoxazol-trimethoprim	9	10	11	11	9	6	10	11	13	90
Tetracycline	7	7	8	10	10	7	13	13	15	90
Tobramycin	8	9	8	7	6	7	15	15	15	90
Total	91	101	100	70	61	58 16.4%	169	168	172	990
Percentage /330	27.6%	30.6%	30.3%	21.2%	18.5%		51.5%	51.2%	52.7%	

Table 2: Susceptibility results for 20 *Pseudomonas* isolates

Antibiotic	Sensitive		Intermediate		Resistant		Total
	8hrs	overnight	8hrs	overnight	8hrs	overnight	
Amikacin	14	16	2	0	4	4	40
Aztreonam	3	3	3	7	14	10	40
Cefepime	4	6	6	7	10	7	40
Ciprofloxacin	7	6	6	7	7	7	40
Gentamycin	13	14	1	0	6	6	40
Imepenem	14	14	1	0	5	6	40
levofloxacin	10	11	5	4	5	5	40
Meropenem	12	13	1	1	7	6	40
Norfloxacin	9	11	2	1	9	8	40
Ofloxacin	12	13	1	1	7	6	40
Piperacillin/ tazobactam	11	14	5	2	4	4	40
Tobramycin	15	15			5	5	40
Total	125	136	32	30	83	74	480
Percentage /240	52%	56%	13.3%	13%	34.7%	31%	

Mean values of difference in zone diameters of AST after increased incubation time of the tested *Acinetobacter* isolates:

As for *Acinetobacter,* the average (mean) difference in zone diameter measurements of 6ZD and 8ZD versus SZD for each antibiotic tested are registered in table 3 and variations represented by figure 1. A mean represented by a negative value indicates an increase in the zone size as incubation time increased.

Table 3: Mean in zone diameter difference between 6ZD, 8 ZD versus SZD measured in mm

Antibiotic	Difference 6ZD & SZD				Difference 8ZD & SZD			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Amikacin	0.0667	1.388	-3	4	0.1333	1.008	-3	3
Ampicillin-sulbactam	0.2	1.3995	-4	3	0.3333	0.8841	-1	3
Co-trimoxazole	-0.0667	1.7604	-3	4	0.1333	1.2794	-2	3
Doxycycline	-0.0333	1.3515	-3	3	0.0333	0.9279	-2	2
Gentamycin	0.3333	1.583	-4	4	0.3333	0.9589	-1	3
levofloxacin	0.1	1.3734	-2	3	0.2667	1.0807	-2	3
Meropenem	-0.1333	1.4559	-5	3	-0.0333	1.1592	-3	3
Minocyclin	-0.3333	1.2954	-2	2	-0.1333	1.0743	-2	2
Pipracillin tazobactam	-0.5	1.3326	-4	2	0.1	0.9229	-2	2
Tetracyclin	0.1667	1.6206	-3	4	0.0667	1.1427	-2	3
Tobramycin	0	1.4856	-3	4	0.1667	1.3412	-2	4

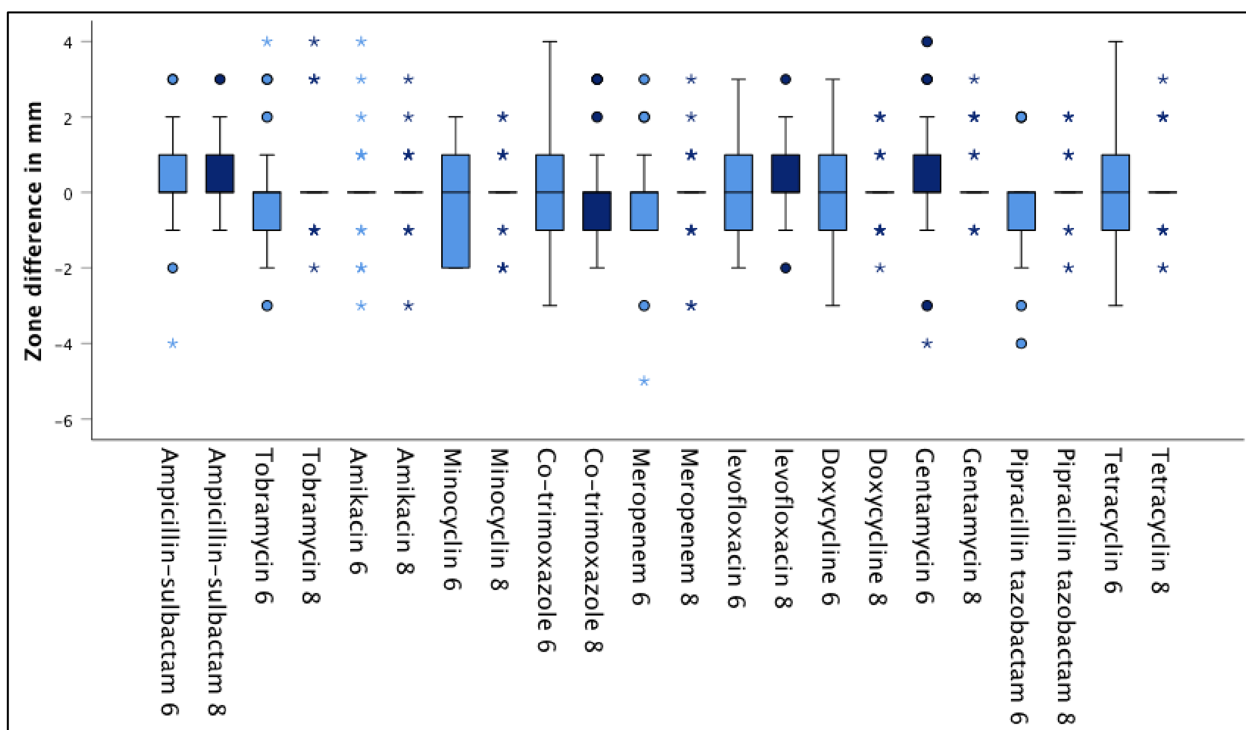

Fig. 1: Box and Whisker plot represents differences in zone measurements between 6 ZD /8 ZD and SZD for *Acinetobacter* species. The bold line within the box represents the median, the box represents 50% of results (Interquartile interval), while the whiskers extend to the nonoutliers, and separate data points represent outliers.

This difference in zone diameters resulted in minor errors (mEs) in 30 measurements representing 9.1 % of reading at 6 hrs. which decreased to 4.5% at 8 hrs., while very major errors were 1.1 % for 6 hrs. measurements and further decreased to 0.5% after 8hrs incubation. The categorical agreement was >98% for antibiotics tested. (Table 4)

Agreement and Errors for early zone measurements in comparison to standard zones:

Table 4 shows the categorical agreement between the zone diameters of AST values after 6- and 8-hours incubation of the tested *Acinetobacter* clinical isolates, as well as the error detection rates.

Table 4: Categorical agreement and errors detection for *Acinetobacter* spp

Antibiotic	Errors detected						Categorical agreement [d] %		Coefficient of determination (r^2)	
	mE[a]		ME[b]		VME[c]					
	6ZD	8ZD	6ZD	8ZD	6ZD	8ZD	6ZD	8ZD	6ZD	8ZD
Amikacin	1	1	-	-	-	-	99.7%	99.7%	$R^2 = 0.9257$	$R^2 = 0.9599$
Ampicillin-sulbactam	5	3	-	-	-	-	98.5%	99.1%	$R^2 = 0.8841$	$R^2 = 0.963$
Doxycycline	1	1	-	-	-	-	99.7%	99.7%	$R^2 = 0.9337$	$R^2 = 0.9661$
Gentamycin	3		-	-	-	-	99.1%	100%	$R^2 = 0.9706$	$R^2 = 0.9802$
Levofloxacin	5	1	-	-	-	-	98.5%	99.7%	$R^2 = 0.8973$	$R^2 = 0.9614$
Meropenem	2	1	-	-	-	-	99.4%	99.7%	$R^2 = 0.9089$	$R^2 = 0.942$
Minocycline	2	1	-	-	-	-	99.4%	99.7%	$R^2 = 0.9643$	$R^2 = 0.9643$
Piperacillin-tazobactam	1		-	-	-	-	99.7%	100%	$R^2 = 0.9381$	$R^2 = 0.9573$
Sulphamethoxazole-trimethoprim	5	3	-	-	-	-	98.5%	99.1%	$R^2 = 0.9282$	$R^2 = 0.9734$
Tetracycline	3	3	-	-	1		99.1%	99.1%	$R^2 = 0.8855$	$R^2 = 0.9439$
Tobramycin	2	1	-	-	1	1	99.4%	99.7%	$R^2 = 0.9037$	$R^2 = 0.9286$
Total	30	15	-	-	2	1				
Error percent	9.1%	4.5%	-	-	1.1%	0.5%				

[a]mE (minor errors): number of Intermediately susceptible results reported as sensitive or resistant and vice versa / total number of results
[b]ME (major errors): number of false resistant results/ total number of susceptible results
[c]VME (very major errors): number of false sensitive results / total number of resistant results
[d]Categorical agreement ; percentage of number of results at 6 and 8 hrs. reading falling in the same category as the final results.

Mean values of difference in zone diameters after increased incubation time of the tested *Pseudomonas* clinical isolates:

Regarding the tested *Pseudomonas* strains, table 5 shows the average (mean) difference between 8 ZD versus SZD for each antibiotic tested, variations represented by figure 2.

Table 5: Mean in zone diameter difference between, 8ZD versus SZD measured in mm

Antibiotic	Difference 8 ZD & SZD			
	Mean	SD	Min	Max
Pipracillin-Tazobactam	-1.15	2.059	-5	3
Gentamycin	-0.4	1.5009	-3	4
Tobramycin	-0.45	1.1459	-3	2
Amikacin	-0.9	1.619	-5	1
Ciprofloxacin	-0.6	0.9403	-2	1
Levofloxacin	-0.25	1.4824	-3	3
Aztreonam	-0.7	1.4903	-4	2
Cefepime	-1	1.7168	-5	1
Imepenem	-0.4	2.1374	-6	4
Meropenem	-0.8	1.5079	-4	2
Ofloxacin	-0.65	1.0894	-3	1
Norfloxacin	-0.2	1.3992	-2	3

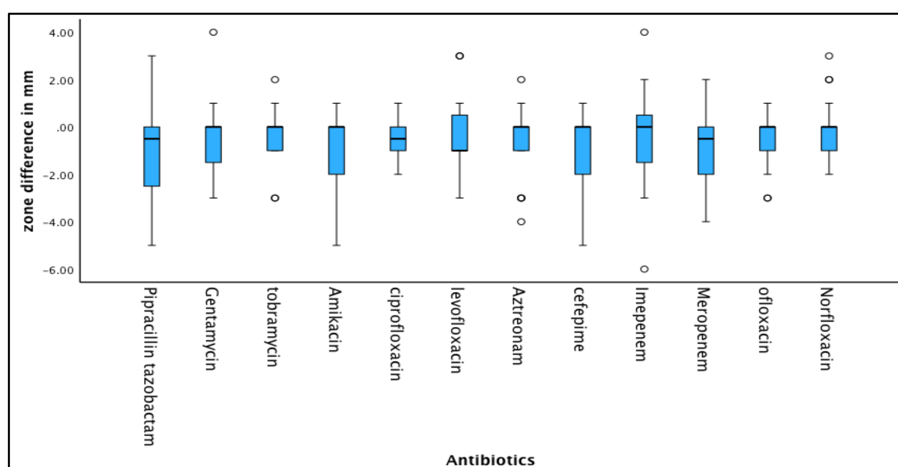

Fig. 2: Box plot represents differences in zone measurements between 8 ZD and standard disk diffusion zone for *Pseudomonas* species. The bold line within the box represents the median, the box represents 50% of results (Interquartile interval), while the whiskers extend to the nonoutliers, and separate data points represent outliers.

This difference in zone diameters resulted in very minor errors in 25 measurements representing 10.4 % of reading at 8 hrs., while major errors were 0.8 % and very major errors represented 1.2% after 8hrs incubation. The categorical agreement was >97% for antibiotics tested. (Table 6)

Agreement and Errors for early zone measurements in comparison to standard zones of *Pseudomonas* isolates:

Table 6 shows the categorical agreement between the zone diameters of AST values after 8-hours incubation of the tested *Pseudomonas* clinical isolates, as well as the error detection rates.

Table 6: Categorical agreement and errors determination for *Pseudomonas* spp

Antibiotic	Errors detected			Categorical agreement [d] %	Coefficient of determination (r^2)
	mE[a]	ME[b]	VME[c]		
Amikacin	2			99.2%	$R^2 = 0.9184$
Aztreonam	4			98.3%	$R^2 = 0.926$
cefepime	5			97.9%	$R^2 = 0.8623$
Ciprofloxacin	2			99.2%	$R^2 = 0.9829$
Gentamycin	1			99.6%	$R^2 = 0.8985$
Imepenem	1		1	99.2%	$R^2 = 0.8863$
levofloxacin	1			99.6%	$R^2 = 0.9494$
Meropenem		1		99.6%	$R^2 = 0.9613$
Norfloxacin	3			98.6%	$R^2 = 0.9298$
Ofloxacin	2			99.2%	$R^2 = 0.9623$
Pipracillin tazobactam	3			98.6%	$R^2 = 0.7092$
tobramycin	0			100%	
Total	25	1	1		
Error percent	10.4%	0.8%	1.2%		

[a]mE (minor errors): number of Intermediately susceptible results reported as sensitive or resistant and vice versa / total number of results
[b]ME (major errors): number of false resistant results/ total number of susceptible results
[c]VME (very major errors): number of false sensitive results / total number of resistant results
[d]Categorical agreement ; percentage of number of results at 6 and 8 hrs. reading falling in the same category as the final results.

Regression analysis of measurements after 6 and 8-hours incubation versus overnight incubation for the tested *Acinetobacter* and *Pseudomonas* clinical isolates:

Regression analysis was calculated for each antibiotic 6 hrs. measurement, to estimate strength of accurate result prediction versus final zone readings for *Acinetobacter* isolates. All readings showed a positive correlation with r^2 coefficient ranging from (R^2 0.8841 - $R^2 = 0.9706$) with all antibiotic measurement giving a r^2 coefficient above 0.9, except for ampicillin-sulbactam, levofloxacin and tetracycline. (Figure 3)

The 8 hrs ZD reading versus the final reading showed a positive correlation with r^2 coefficient ranging from ($R^2 = 0.9286$ - $R^2 = 0.9802$) with all antibiotics giving a r^2 coefficient above 0.9. (Figure 4)

As for the tested *Pseudomonas* isolates, the linear regression also showed a positive correlation but the r^2 coefficient ranged from ($R^2 = 0.7$ - $R^2 = 0.96$). (Figure 5)

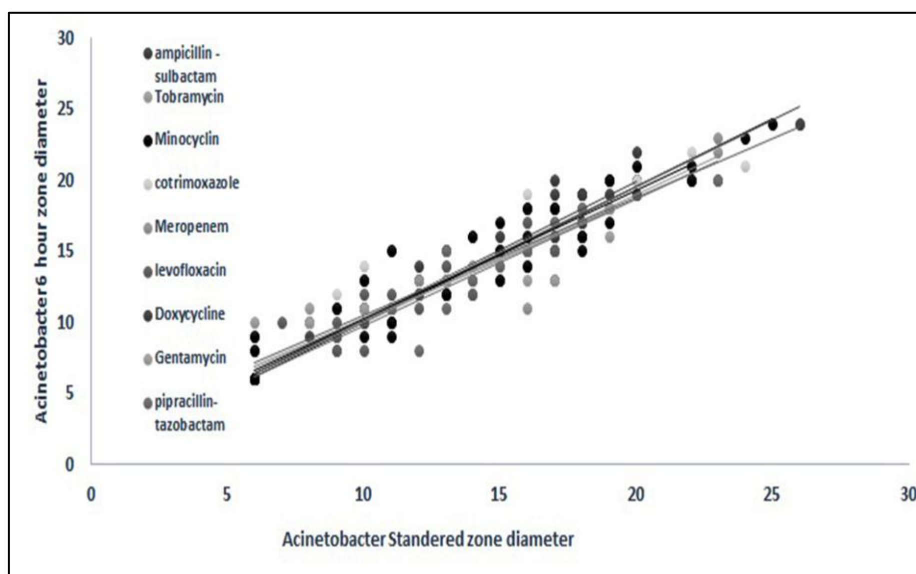

Fig. 3: Regression Analysis for 6 hrs ZD measurements of *Acinetobacter* spp

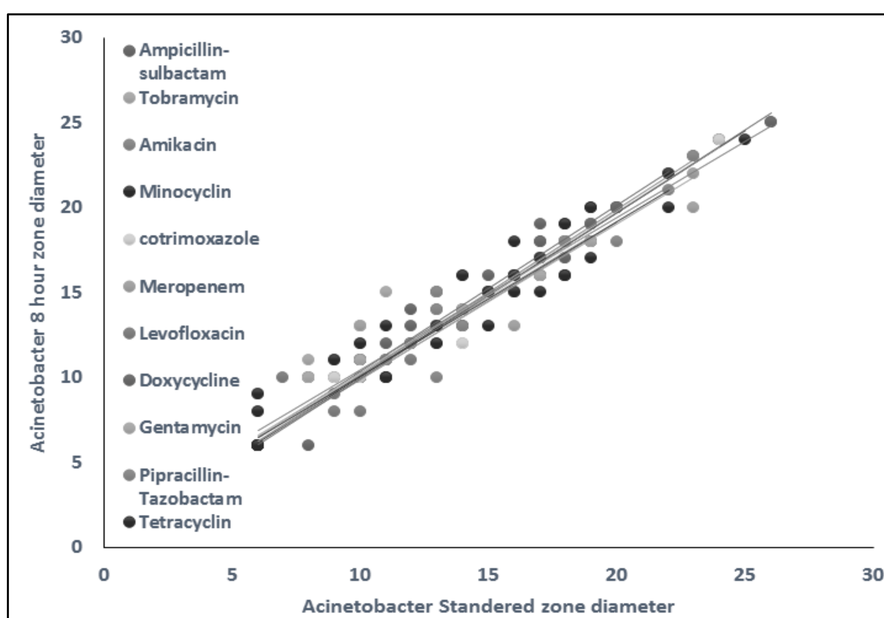

Fig. 4: Regression Analysis for 8 hrs ZD measurements of *Acinetobacter* spp

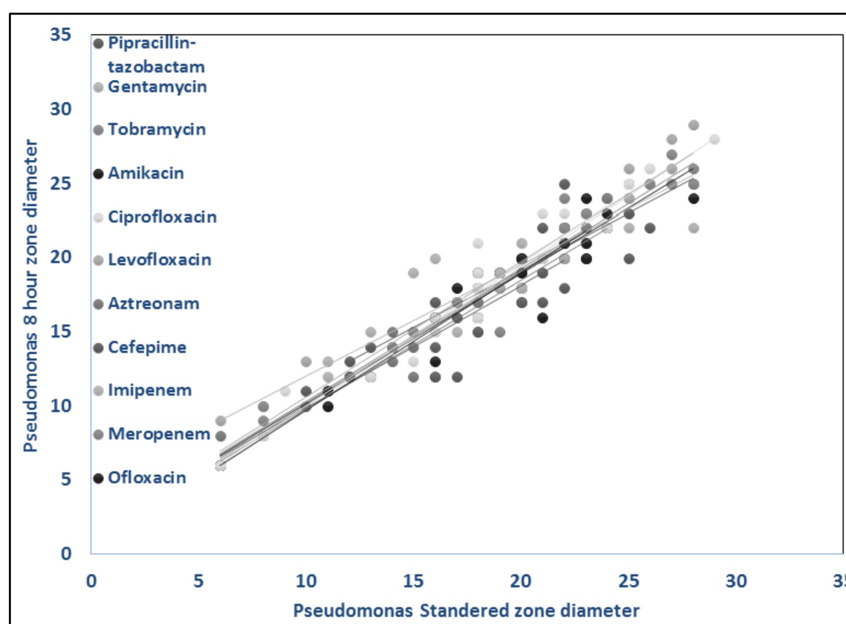

Fig. 5: Regression Analysis for 8 hrs ZD measurements of *Pseudomonas* spp

DISCUSSION

The current study aimed to examine the value of early manual interpretation of AST by disc diffusion for *Acinetobacter* and *Pseudomonas* species using CLSI standard breakpoints and its feasibility and accuracy [16]. The results of this study would provide a better use of the popular, simple and cost-effective disc diffusion technique for prompt result delivery, for proper patient management.

Multiple recently published studies attempted to standardize early measurement of disc diffusion zones using automated reading systems [12,19] based on the fact that automated zone measurements are objective, accurate and consistent. However, these automated systems are not available in most laboratories of developing countries. Therefore, it was in our interest to check if early manual measurement and interpretation of zone diameters, will give similar concordant results as standard zone measurements.

It was noticed in the current study that the mean of zone diameter differences between early measurements and standard zones was higher than those reported by others [12, 20], with more outliers reported in the current work, although two independent measurements were taken to decrease bias. However, this is probably attributable to the subjectivity of manual measurement which would be less accurate than the automated systems that utilize sensitive high resolution magnifying cameras for measuring the zones.

Despite this, our results showed that 6ZD for *Acinetobacter* species were in good agreement both categorical and quantitative (r^2) with the SZD, and that agreement improved by extending incubation to 8 hrs. and percentage of errors decreased to an acceptable limit. Changes in *Acinetobacter* measured zone diameters varied with time; some antibiotic zones showed an increase in size over time, this was more evident with susceptible isolates, and others showed a decrease in zone size, which mainly occurred with resistant isolates. Although this difference in zone diameters represents the bias between the two readings (early and final readings), but still good agreement was detected between these two readings. This is because the change in zone diameter was not enough to change the category of interpretation (sensitive, intermediate, resistant), or produce significant errors (especially major and VME) with the subsequent good categorical agreement between the early and final measurements.

On the contrary *Pseudomonas* growth was not visible enough at 6 hrs. of taking zone measurements. Similarly, Hombach et al [20] reported that *Pseudomonas* was not readable at 6 hrs. incubation and that readability improved starting from 8 hrs. onwards. *Pseudomonas* measurement showed good categorical agreement at 8 hrs., with most zone measurements showing a minor increase in size with increased incubation time (ranging from 1 to 0.2 mm increase) taking into consideration that zone edges became sharper and clearer cut with increased incubation time. Homach et al proceeded to measure zone sizes up to 12 hr. incubation and stated that this measurement reflected the best agreement (99.9%) with standard zone. Also, during their work they observed that twelve of 24 species/antibiotic combinations measured zone, showed minor zone increase over time [20].

Pseudomonas tends to have a relatively longer doubling time ranging from 35 min up to 1.5 hrs. depending on the type of media used and was reported to need extended incubation times in automated sensitivity systems for accurate results [21, 22]. This explains the inability to read the plates at 6 hrs. which improved at 8 hrs. incubation with good categorical agreement, but $\mathbf{r^2}$ for antibiotics as cefepime, gentamycin, imipenem and piperacillin-tazobactam ranged from (0.7-0.89). This indicates that early zone readings predicted the standard zone measurement in 70%-89% only of the measured zones, which was also reflected by rate of mEs of 10.4% just exceeding the acceptable limit of 10%. Also, VMEs were 1.2%, exceeding the acceptable limit of 1%, and suggesting that zone reading after further incubation of 10-12 hours would improve the accuracy and decrease errors [20].

The rate of errors was higher in the mE category for both *Acinetobacter* and *Pseudomonas*. This occurred due to initial zone reading falling in approximation with the intermediate category values in which a minor change in zone diameter will cause a change in category from I to S/R and vice versa. This finding was similarly observed by Stefano Mancini et al [23], who stated that categorical agreement was lowest for strains categorized as intermediate.

The decrease in size of zone diameters overtime may be due to mutations and/or induction of resistance followed by degradation of the antibiotics [24,25]. It may also be attributable to the presence of mixed susceptibility phenotypes resulting in heterogenous populations, in which a portion is sensitive and the other resistant. Also, zone diameter would be affected by other non-bacterial factors, including the antibiotic diffusion gradient and its molecular weight [26]. All these factors would lead to variation in zone diameter over incubation time. Therefore, focused specific species/antibiotic studies are required to set the optimum time for early interpretation of zone measurements.

Limitation factors for our study include not extending zone monitoring after 8 hrs. incubation since this work was carried out during the laboratory working hours which doesn't extend to cover a period of more than 8 hrs. incubation. Also, a study of a larger number of strains with different susceptibility phenotypes from different settings are needed to confirm the findings and study pattern of change in zone diameter based on the susceptibility phenotype.

CONCLUSION

The rapid antibiotic susceptibility testing and interpretation was manually possible at 6 hrs for *Acinetobacter* spp but not for *Pseudomonas* spp. Both *Acinetobacter* and *Pseudomonas* were measurable at 8 hrs. but *Acinetobacter* measurements were more accurate with minimal errors, which makes it a promising cost-effective method for rapid delivery of AST results for *Acinetobacter* spp after 8 hrs. incubation using the established CLSI guidelines and methodology. On the other hand, *Pseudomonas* spp needs further studies on larger number of strains, to determine the best incubation time that would allow manual measurement of zones with most accurate results and minimal errors.

We declare that there are no commercial or other interests or associations, that might be perceived as posing a conflict of interest or bias in connection with the submitted article.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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