ORIGINAL ARTICLE

A comparison of reduced and standard incubation time for antimicrobial susceptibility testing by the disk diffusion method

Nida Safdar, Aqib Sultan, Nasrullah Malik, Summiya Nizamuddin

Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore Pakistan

ABSTRACT

Background: Antimicrobial resistance is a major worldwide health problem, and early availability of antibiotic susceptibility testing results has become vital for the planning of treatment. Despite being commonly utilized, the disk diffusion method requires an 18 to 24 hours incubation, resulting in delays in clinical decision-making.

Material and Methods: This prospective study was performed in the Microbiology Department at Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, between October 2024 and March 2025. One hundred clinical isolates were tested against selected antibiotics using CLSI disk diffusion breakpoints. The zones of inhibition were measured at 6 hours, 10 hours, and 18–20 hours. Categorical agreement (CA) and error rates were calculated later.

Results: In total, 600 drug–organism combinations were analyzed. CA improved from 83.8% at 6 hours to 92.2% at 10 hours. Error rates declined markedly between 6 hours and 10 hours (mE: 7.0% → 3.2%; ME: 5.7% → 2.2%; VME: 3.8% → 1.1%). There was a consistent CA of >90% for meropenem, nitrofurantoin, vancomycin, and linezolid at early incubation periods; however, ciprofloxacin for Acinetobacter and Enterococcus species displayed a low accuracy initially, which improved at 10 hours.

Conclusion: An early measurement of zone of inhibition at 10 hours of incubation in disk diffusion testing has demonstrated notably reliable results for several therapeutically important drug-organism pairs. Particularly in resource-poor settings, AST reporting using shorter incubation times may enable timely initiation of targeted treatment and improvement in antimicrobial stewardship.

Keywords: Antibiotic susceptibility testing, Disk diffusion, Reduced incubation

BACKGROUND

Antimicrobial resistance (AMR) constitutes one of the major public health threats. The World Health Organization (WHO) reports that antibiotic resistance is reaching dangerously high levels all over the world, leading to increased mortality and morbidity. Six major pathogens, including *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa* were responsible for 3.57 million AMR-associated deaths in 2019, with projections estimating 10 million deaths by 2050.[1] To effectively treat bacterial infections, it is crucial to perform antimicrobial susceptibility testing (AST). The clinical microbiology labs help direct antibiotic treatment by providing local patterns of susceptibility.[2] Detection of resistance determinants through genotypic assays does not necessarily equate to phenotypic expression. Phenotypic methods, mainly broth microdilution (BMD) and disk diffusion (DD), remain the most widely used .[3]

The disk diffusion method, standardized in 1966 by Bauer *et al*., is simple, reliable, and cost-effective.[4] This method has remained the most widely used method in microbiological laboratories since its development, because it is inexpensive and allows flexibility in disk selection.[1] One significant constraint is the normal 18-24-hour incubation period, which is based on traditional laboratory practices rather than the dynamics of bacterial growth.[4] Getting faster AST results is crucial to prompt initiation of an appropriate therapy for improved outcomes.[5] Patients will benefit when the time taken for bacterial identification and AST is reduced, particularly in light of growing antibiotic resistance.[6]

Total Lab Automation (TLA) has enabled earlier plate imaging without requiring more effort from a laboratory worker.[5] Nevertheless, elevated costs, limited

Correspondence: Dr. Nida Safdar, Resident Microbiology, Department of Microbiology, Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, Pakistan

Email: drnidasafdar@gmail.com

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infrastructure, and a lack of established protocols hinder its implementation in low- and middle-income countries. Although research indicates that reducing the incubation time has no significant impact on susceptibility interpretation[5,7-10], these protocols need to be fully standardized and incorporated into the Clinical and Laboratory Standards Institute (CLSI) AST recommendations.[11] Currently, no research in South Asia has validated shortened incubation AST procedures, underscoring the study's geographical relevance.

In this study, our focus has been to investigate whether reducing the incubation time of disk diffusion can provide reliable results. We compared susceptibility results obtained at 6 and 10 hours with standard 24-hour incubation.

MATERIAL AND METHODS

This prospective study was conducted from October 2024 to March 2025, in the Microbiology Department, Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, after approval of the Institutional Review Board Committee (IRB number: IRB-23-32). A waiver for informed consent was also approved.

A total of 100 positive blood culture bottles were selected. The selection of microorganisms was based on their prevalence to ensure representation of the clinically relevant pathogens. The samples with monomicrobial bacterial growth were included in the study. Samples showing yeast, polymicrobial growth, or repetitive samples from a patient were eliminated from the study for an accurate comparative analysis and avoidance of duplication.

Clinical isolates, commonly encountered in a clinical microbiology laboratory, were chosen, including 10 isolates each of *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Salmonella* Typhi*, Salmonella paratyphi A*, methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*. The list of antimicrobials tested on each isolate is shown in Table-I. Although nitrofurantoin is not the recommended therapeutic option for sepsis, it was included in this research to enable methodological comparability and maintain consistency with previous research on early disk diffusion protocols.[4]

Quality control organisms used in this study are mentioned in Table 01. These were sub cultured, and AST was performed by the disk diffusion method. Mean inhibitory zones were measured and compared with the quality control ranges of CLSI (version 2024).

AST was performed according to CLSI guidelines. A suspension equivalent to 0.5 McFarland was prepared using fresh bacterial subcultures. After preparing a confluent lawn on Mueller-Hinton agar plates using this suspension, antibiotic disks were applied within 15 minutes of inoculation. The plates were incubated at 35-37 °C in ambient air. Zones of inhibition were measured after 6 and 10 hours of incubation. Susceptibility or resistance was interpreted according to established zone sizes, and standard 18 to 24-hour readings served as the reference for comparison.

The data were analyzed utilizing the Statistical Package for the Social Sciences (SPSS), version 24.0 (IBM Corp., Armonk, NY, USA). Frequencies and percentages were used to present descriptive statistics. A p-value of <0.05 was categorized as statistically significant. Any discrepancy from the reference method was categorized as very major errors (VME), major errors (ME), or minor errors (mE). A VME was referred to a resistant organism that had been incorrectly reported as susceptible, and a susceptible isolate misclassified as resistant was reported as ME. Errors were reported as minor (mE) when any misclassifications included the intermediate category. The calculation of the mE rate was performed using the total number of isolates tested as the denominator, whereas the ME and VME rates were calculated using the numbers of susceptible and resistant isolates, respectively.

Table-I: Bacteria and antibiotics evaluated during the study.

Staphylococcus species	Enterococcus species	Enterobacterales	Salmonella species	Acinetobacter species	Pseudomonas aeruginosa
		QC organisms			
S. aureus ATCC 29213	E. faecalis ATCC 29212	K. pneumoniae ATCC 13883 E. coli ATCC 25922		A. baumannii ATCC 747	P. aeruginosa ATCC 27853

100 clinical isolates					
MRSA (10) MSSA (10)	E. faecium (10) E. faecalis (10)	K. pneumoniae (10) E. coli (10)	S. Typhi (10) S. paratyphi A (10)	A. baumannii (10)	P. aeruginosa (10)
Antibiotics					
Nitrofurantoin 300 µg	Ciprofloxacin 5 µg	Ciprofloxacin 5 µg	Ciprofloxacin 5 µg	Ciprofloxacin 5 µg	Ciprofloxacin 5 µg
Erythromycin 15 µg	Nitrofurantoin 300 µg	Nitrofurantoin 300 µg	Ceftriaxone 30 µg	Ceftriaxone 30 µg	Piperacillin-tazobactam 100/10 µg
Clindamycin 2 µg	Vancomycin 30 µg	Co-trimoxazole 25 µg	Ampicillin 10 µg	Piperacillin-tazobactam 100/10 µg	Cefipime 30 µg
Cefoxitin 30 µg	Linezolid 30 µg	Cefazolin 30 µg	Meropenem 10 µg	Cefipime 30 µg	Ceftazidime 20 µg
Co-trimoxazole 25 µg	Ampicillin 10µg	Ceftriaxone 30 µg		Ceftazidime 20 µg	Meropenem 10 µg
		Piperacillin-tazobactam 100/10 µg		Gentamicin 10 µg	
		Cefipime 30 µg		Meropenem 10 µg	
		Ceftazidime 20 µg			
		Gentamicin 10 µg			
		Meropenem 10 µg			

RESULTS

A total of 600 drug-microorganism combinations were evaluated. One hundred positive blood culture bottles with a mean age of 33.24 years ±20.9 SD were included in the study. Of them, 53% were males and 47% were females. After 6 hours, 94% of the plates had an obvious bacterial growth, and at 10 hours, 100% of the plates showed growth. The categorical agreement (CA) and error rates were calculated for all different drug-organism combinations. Prolonging the incubation timings from 6 hours to 10 hours considerably improved the CA. At 6 hours, the mean CA was 84.5% (SD ±5.2%), which rose significantly to 90.5% (SD ±3.5%) by 10 hours ($p < 0.01$). Certain combinations, like meropenem for *Salmonella species* and nitrofurantoin for MSSA/MRSA, achieved a perfect agreement even at 6 hours. These findings truly signify how beneficial it can be to get early AST results for specific drugs, if not all of them.

Analysis showed that error rates also improved significantly over time. The minor errors (mE) declined from 6.0% (SD ±3.0%) at 6 hours to 2.5% (SD ±2.6%) at 10 hours ($p < 0.01$). Similarly, major errors (ME) and very major errors (VME) reduced from 4.0% (SD ±2.7%) and 5.5% (SD ±2.9%) at 6 hours to 1.5% (SD ±2.0%) and 0.5% (SD ±1.5%) at 10 hours, respectively ($p < 0.05$ for ME; $p < 0.01$ for VME). The reduction in error rates at 10 hours signifies the reliability of early susceptibility reporting and resistance assessments with modified incubation periods.

Figures 1 and 2 show the analysis between organisms-drug combinations and time intervals. The highest CA (>90%) was observed in *E. coli* and *K. pneumoniae* with meropenem and nitrofurantoin; however, CA was moderate for ciprofloxacin and ceftriaxone, which improved significantly by 10 hours, decreasing the error rates. Meropenem had a perfect CA (100%) for *S.* Typhi and *S. paratyphi* A, even at 6 hours, and CA for ceftriaxone improved from 95% at 6 hours to 100% at 10 hours. There were significant major and minor errors with ciprofloxacin and ampicillin at 6 hours, but the CA exceeded 90% at 10 hours. *P. aeruginosa* persistently displayed a high CA (>90%) with piperacillin–tazobactam and cefepime, and ciprofloxacin improved from 80% to 90% with time. *A. baumannii* maintained a high CA (>90%) for gentamicin and meropenem, but it was only 70% for ciprofloxacin at 6 hours, improving to 85% at 10 hours. No errors were detected in MSSA/MRSA for nitrofurantoin, exhibiting perfect CA (100%), while an improvement from 70–85% at 6 hours to >90% at 10 hours was seen for erythromycin and clindamycin. Enterococcal isolates also had a 100% CA with vancomycin and linezolid, with a slight improvement for ciprofloxacin, which increased from 70% to 80%. Overall, meropenem, nitrofurantoin, vancomycin, and linezolid proved to be the most reliable for early reporting, with a rising CA and a reduction in errors by increasing incubation time from 6 to 10 hours. However, ciprofloxacin for *Enterococcus* and *Acinetobacter* revealed less reliable results at the early

time of 6 hours, but the results improved after incubation at 10 hours.

A positive correlation between CA and incubation time was observed, which showed that prolonging the incubation time from 6 hours to 10 hours improved the agreement with the reference standard. A negative association between error rates and incubation time was observed, where the errors decreased with extending incubation times. These findings highlight two important aspects: one, AST results are reliable at an earlier incubation time for specific drug and organism pairs; two, optimization of incubation periods will minimize the unnecessary delays in diagnostic microbiology workup. The figure shows categorical agreement for every organism-drug combination tested, at 10 hours. The intensity of the color depicts CA% with exact values written inside the boxes. A clear improvement in CA and a drop in error rates was noted between 6 and 10 hours.

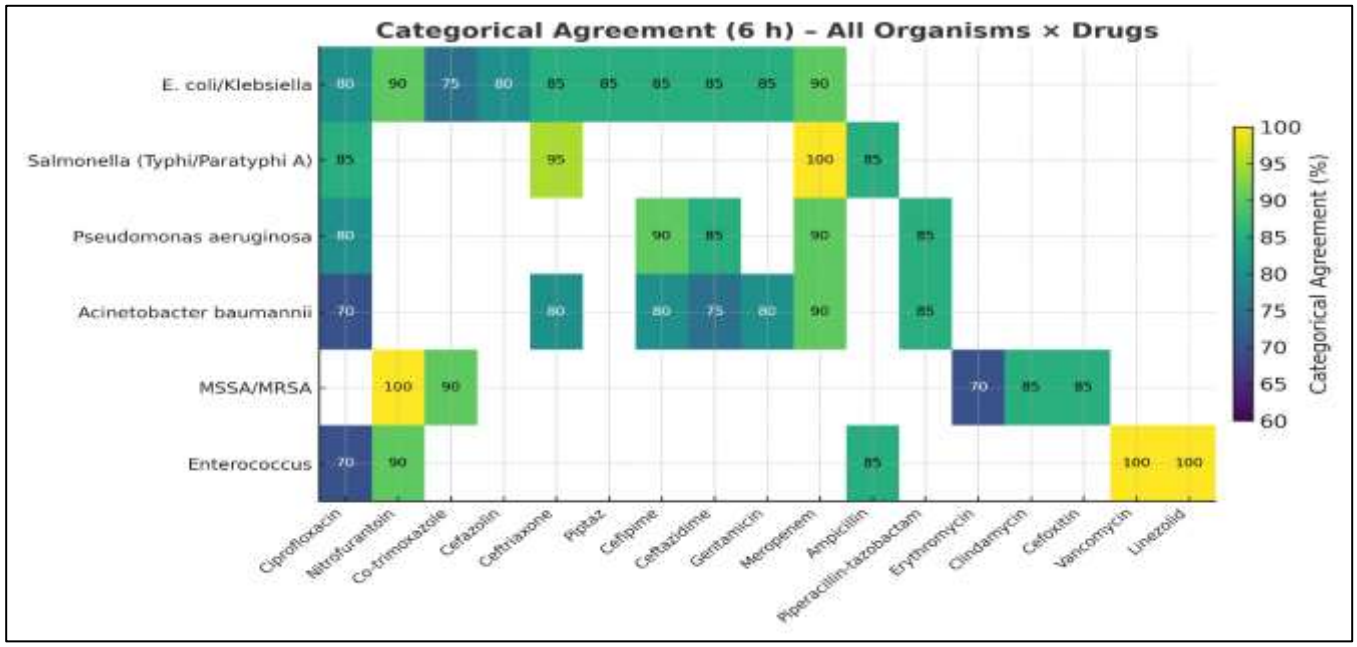

Figure I: Results of disk diffusion by rapid antibiotic susceptibility testing (rAST).

The figure shows categorical agreement for every organism-drug combination tested, at 6 hours. The intensity of the color depicts CA% with exact values written inside the boxes.

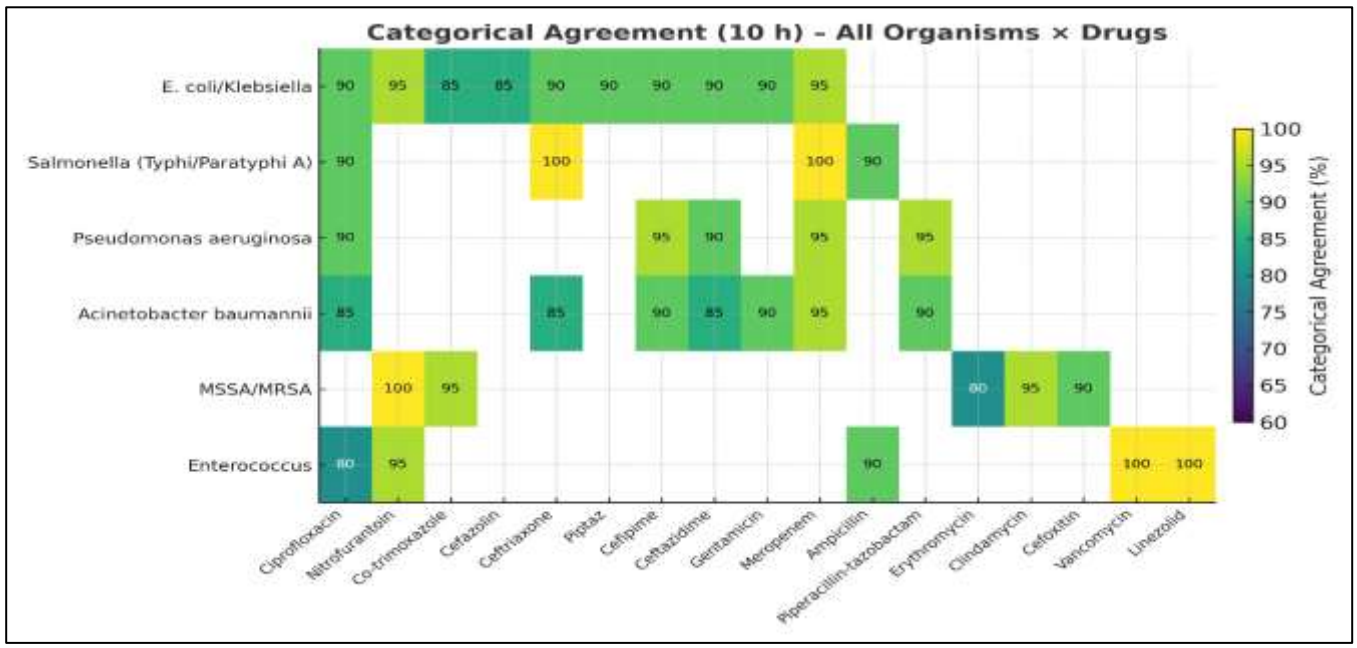

Figure-II: Results of disk diffusion by rapid antibiotic susceptibility testing (rAST).

DISCUSSION

This study implies that quick results of AST by disk diffusion testing at shorter incubation times, specifically at 10 hours, can be as reliable and accurate as standard incubation times. There has been a strong agreement with low error rates, indicating the possibility of reporting the findings earlier in clinical settings. The findings are consistent with those of previous studies that proved the utility and benefits of using shorter incubation periods.

Our study found highly reliable and accurate results when the readings were compared between early and standard incubation timings. This aligns with older studies from the 1970s and 1980s. In 1972, Boyle et al. demonstrated that incorporating dyes to increase the distinction between zones of growth and inhibition allowed accurate readings after 6.5 hours of incubation, comparable to those obtained from control plates incubated overnight.[12] Kluge et al. found that when 8-hour data were compared to 18-hour readings, the category accuracy rate for Enterobacterales in cultures with apparent growth was 89.0-95.1%. [9] Liberman *et al*. and Midtvedt *et al*. observed categorical agreement of 84.0% and 75%, respectively, when they compared earlier zone measurements with zones after 18-20 hours of incubation, and the majority of errors were minor.[10,13] Although these findings align with the current study's results, one should be cautious when interpreting data from older studies, as the antibiotics used are no longer tested, and interpretations differ significantly from those of today. Additionally, the patterns of antimicrobial resistance have evolved, increasing the likelihood of misleading susceptible categorization.

Le Page *et al*. determined the minimal incubation time required to ascertain the ESBL status and imipenem susceptibility of 25 Gram-negative bacteria using a high-resolution real-time video imager.[14] Although it took 6.5 hours for all strains to be accurately classified as susceptible, intermediate, or resistant, they discovered that the first imipenem-resistant strain had already been spotted after 3 hours. It took 4.5 hours to correctly identify strains that were ESBL-positive. Study results of Le Page *et al*. showed that, as compared with 24-hour readings, an early reading at 6-hour or 8-hour intervals in Enterobacterales exhibited significant concordance, with an error rate of 3% (0.4% ME and VME) after 6 hours and 1% (0.0% ME and 0.3% VME) after 8 hours. [15]

The results of an analysis of 88 difficult-to-treat organisms were reported in another investigation by van den Bijllaardt et al. After 10 hours of incubation, zone measurement provided precise susceptibility data where the essential error rate was 6.7% and mE, ME, and VME were 1.6%, 0.2%, and 0.7%, respectively.[5] Although this study was advanced in terms of using imaging techniques and getting data hourly, it was limited to Enterobacterales only.

Cao et al also checked the rAST methods on the positive blood culture bottles. The results were consistent with our study, signifying the importance of early zone readability. However, they mentioned that the proportion of readable zones increased with the extension of the incubation period.[16]

Although shortening the conventional disk diffusion method remains the most economical methodology for low- and middle-income countries, emerging rapid AST technologies are paving the way forward. These include the adenosine triphosphate (ATP) bioluminescence-based assay, which enables susceptibility results within approximately 3 hours[17], and the fully automated ASTar system (Q-linea, Uppsala, Sweden), capable of performing microdilution AST directly from positive blood cultures in about 6 hours.[18] These revolutionary methods promise to deliver reliable results in the shortest time so that clinicians are provided with accurate and rapid information without unnecessary delays.

Implementation of shortened incubation protocols will benefit antimicrobial stewardship through earlier de-escalation or optimization. It can potentially save the laboratory time and expedite contact with healthcare workers, particularly in higher-volume or resource-poor facilities. Based on our results, the initial reading after 10 hours appears to be appropriate for meropenem, nitrofurantoin, vancomycin, and linezolid for all the assessed species, with a minimal risk of misinterpretation.

The study has multiple limitations that need to be looked at in the future. The limited sample size per organism lowers the power of subgroup analysis. Only a subset of antibiotics has been investigated; therefore, the results may not reflect the full range of clinical decision-making. The findings are limited in generalizability because they were conducted in a single tertiary-care hospital. Future studies using automated technologies for early result interpretation may reduce observer

bias and improve diagnostic accuracy. A broader range of antibiotics and organisms would improve the understanding and refine guidelines for early AST reporting.

01CONCLUSION

For several drug-organism combinations, early measurement of disk diffusion breakpoints at 10 hours is sufficiently accurate to direct therapy. This can assist in containing AMR by reducing the use of broad-spectrum empirical antibiotics. Before routinely implementing shorter incubation methods, laboratories must validate them for particular organism-drug combinations.

CONFLICT OF INTEREST

None

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHOR CONTRIBUTION

Nida Safdar: Acquisition, analysis, and interpretation of data, drafting of work, critical revisions, final approval, and accountable for all aspects of publication

Aqib Sultan: Conceived the idea, designed the study and collected data, final approval, accountable for all aspects of publication

Nasrullah Malik: Critical revisions, final approval, accountable for all aspects of publication

Summiya Nizamuddin: Manuscript writing, critical revisions, final approval, accountable for all aspects of publication

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