

Original Article

Effect of Incubation Time on Zone Size of Antimicrobials by Disk Diffusion Method for *Pseudomonas Aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a prominent pathogen in healthcare settings, notorious for its rapid development of resistance to multiple antimicrobial agents. Effective susceptibility testing is critical for managing infections caused by this bacterium. This study investigates the impact of varying incubation times on the efficacy of the disk diffusion method.

Objective: To evaluate the influence of incubation time on the zone sizes of antimicrobials against *Pseudomonas aeruginosa* using the disk diffusion method in a tertiary care setting.

Methods: Conducted at the Microbiology Department of Combined Military Hospital, Lahore, from October 2022 to March 2023, this descriptive study included 71 clinical isolates of *Pseudomonas aeruginosa*. Samples were inoculated on Mueller Hinton Agar and incubated at 35°C ±2°C for 6, 10, and 24 hours. Antimicrobial susceptibility was tested using a panel of clinically relevant antibiotics in triplicate, with zone sizes interpreted according to Clinical and Laboratory Standards Institute guidelines.

Results: Among the isolates, 59.15% were from males and 40.85% from females. The most affected age group was 15-60 years, comprising 59.52% of cases. Antimicrobial response varied with incubation times: Ceftazidime, Piperacillin-Tazobactam, and Cefepime showed increased zone sizes over time, while Tobramycin's zones steadily enlarged. Colistin consistently produced the largest zones at all time points.

Conclusion: The study highlights the significance of incubation time in disk diffusion testing for *Pseudomonas aeruginosa*. Adjusting incubation times can expedite results without incurring additional costs, enhancing the efficiency of antimicrobial susceptibility testing in clinical settings.

Keywords: Antimicrobial susceptibility, Disk diffusion testing, Incubation time, *Pseudomonas aeruginosa*, Tertiary care hospital.

INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative bacterium, has been recognized as a significant opportunistic pathogen, particularly within hospital environments. This pathogen is responsible for various infections, including those acquired in hospitals, communities, and through contaminated food (2). Notably, it has demonstrated an alarming capacity to develop resistance to multiple drugs, posing substantial challenges for treatment (1). Over the past two decades, multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) has emerged as a major health concern worldwide, becoming a leading cause of nosocomial infections, especially among patients with postoperative surgical injuries, those in intensive care units, burn and trauma units, and individuals suffering from chronic pulmonary conditions such as cystic fibrosis (1, 2).

Pseudomonas aeruginosa is particularly adept at surviving in diverse environments, withstanding a wide pH range (4.5 to 9.0) and high salinity, which contributes to its persistence in adverse conditions (4). Its resilience is further augmented by a unique outer membrane that acts as a formidable barrier to biocides and includes efflux pumps that expel antimicrobial agents, thereby increasing its survival and virulence (5). These attributes underscore the pathogen's capability to cause severe infections that are often associated with prolonged hospital stays and a spectrum of diseases including external otitis, endocarditis, ophthalmia, meningitis, septicemia, and pneumonia (2).

The survival and proliferation of *Pseudomonas aeruginosa*, despite the presence of commonly used solutions of disinfectants and antiseptics, can be attributed to its nutritional versatility and intrinsic and acquired mechanisms of antibiotic resistance. These factors complicate the treatment of infections caused by this pathogen, emphasizing the need for precise and effective susceptibility testing methods (5).

This study was conducted to evaluate the impact of incubation time on the size of antimicrobial inhibition zones determined by the disk diffusion method for *P. aeruginosa*. The rationale behind investigating the effect of incubation time on antimicrobial zone sizes stems from the necessity to optimize susceptibility testing protocols. Considering the increasing prevalence of multidrug-resistant strains of *P. aeruginosa*, understanding how incubation time influences antimicrobial efficacy is crucial for enhancing treatment strategies. Conducted in a tertiary care hospital setting, this study aims to offer valuable insights into the optimal incubation periods necessary for accurate susceptibility testing. Through meticulous analysis of zone sizes in response to varying incubation times, the research seeks to contribute to the refinement of clinical laboratory practices and ultimately improve patient outcomes.

METHODS

The study was conducted at the Microbiology Department of Combined Military Hospital (CMH), Lahore, from October 2022 to March 2023, following the approval from the institutional ethics review committee (letter no.433/2023). The research aimed to assess the effect of varying incubation times on the turnaround time for disk diffusion antimicrobial susceptibility testing. A total of 71 clinical isolates of *Pseudomonas* species, collected from both indoor and outdoor departments across various specialties, were included in this descriptive analysis.

Initially, isolates were cultivated on Mueller Hinton Agar and subjected to incubation at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for durations of 6, 10, and 24 hours. Following incubation, disk diffusion tests were performed, adhering to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2, 7). Colonies demonstrating rapid oxidase positivity were selected for further testing. A bacterial suspension equivalent to a 0.5 McFarland standard was prepared using three to five selected colonies or a small area of growth. This suspension was evenly spread onto a 150-mm Mueller-Hinton agar plate using a sterile cotton swab. After allowing the excess moisture to evaporate for 3 to 15 minutes, antimicrobial disks appropriate for the organism were applied. Plates were then inverted and incubated under the conditions previously specified.

The zones of inhibition surrounding each antibiotic disk were manually measured post-incubation. The interpretation of these test results was guided by the principles set forth by the CLSI. Data from the study were statistically analyzed using SPSS version 24. Quantitative variables, including the distribution of specimens from male and female patients across different age groups, were presented as frequencies and percentages, offering a comprehensive overview of the demographic involvement in the study. Through rigorous adherence to standardized testing protocols, the research aimed to elucidate the optimal incubation times necessary to maximize the efficacy and efficiency of the disk diffusion method in clinical settings.

RESULTS

The study on *Pseudomonas aeruginosa*, comprising 71 clinical isolates, revealed a gender disparity in infection rates, with males accounting for 59.15% and females for 40.85%. In terms of age distribution, the majority of infections were observed in the 15-60 years age group, which accounted for 59.52% of the total cases. In contrast, the youngest age group (0-14 years) and the oldest age group (>60 years) exhibited lower infection rates of 19.04% and 21.42% among males, and 13.79% and 48.27% among females, respectively.

From the disk diffusion tests performed after 6, 10, and 24 hours of incubation, varying responses were noted based on the duration of incubation and the type of clinical specimen. Blood specimens showed limited zone enlargement over time, maintaining minimal growth across all time points. Pus samples, however, demonstrated a gradual decrease in zone size as the incubation period increased, with initial sizes reducing significantly by the 24-hour mark. Wound swabs and sputum also followed a similar pattern, with notable reductions in zone size from the 6-hour to the 24-hour readings. Urine samples presented an interesting variation with zones diminishing entirely by the 24-hour interval.

The analysis of antimicrobial susceptibility showed differential responses across antibiotics. Ceftazidime and Piperacillin-Tazobactam (Tzp) exhibited steady increases in zone size, plateauing at 10 hours. Cefepime and Tobramycin displayed continuous growth in efficacy over time, with notable increments in zone size up to the 24-hour measurement. In contrast, Ciprofloxacin showed minimal change throughout the periods, suggesting a lower susceptibility to time-dependent zone size alteration. Both Meropenem and Imipenem recorded progressive increases in their zones, aligning with the trend of enhanced effectiveness with prolonged incubation. Colistin stood out for maintaining the highest zone readings across all durations, illustrating its robust activity against *Pseudomonas aeruginosa*, achieving near maximum efficacy by the end of the study period.

Table 1: Demographic Distribution of *Pseudomonas aeruginosa* infection (n=71)

Gender distribution n=71 (%)	Age 0-14 years n=12 (%)	Age 15-60 years n=36 (%)	Age >60 years n=23 (%)
Male, n=42 (59.15)	8 (19.04)	25 (59.52)	9 (21.42)
Female, n=29 (40.84)	4 (13.79)	11 (37.93)	14 (48.27)

Table 2: Antimicrobial zones of *Pseudomonas aeruginosa* after 6, 10, and 24 hours from different clinical specimens (n=71)

Sr No	Specimen n= 71 (%)	6 hour	10 hour	24 hour
1	Blood n=7 (9.85)	5	1	1
2	Pus n=33 (46.47)	14	9	5
3	Wound swab n=21 (29.57)	12	4	2
4	Sputum n=6 (8.45)	3	0	2
5	Urine n=4(5.63)	2	2	0

Table 3: Positivity of zones of *Pseudomonas aeruginosa* isolates (n=71) against different Antimicrobial agents in accordance with time

Sr. no.	Antibiotics (S*= zone size)	6 hour Zone reading (%)	10 hour Zone reading	24 hour Zone reading
1	Ceftazidime ()	48 (67.60)	57 (80.28)	57 (80.28)
2	Tzp ()	57 (80.28)	61 (85.91)	64 (90.14)
3	Cefepime	53 (74.64)	58 (81.69)	63 (88.73)
4	Tobramycin	49 (69.01)	56 (78.87)	61 (85.91)
5	Ciprofloxacin	7 (9.85)	7 (9.85)	8(11.26)
6	Meropenem	47 (66.19)	55 (77.46)	62 (87.32)
7	Imipenem	37 (52.11)	49 (69.01)	59 (83.09)
8	Colistin	65 (91.54)	67 (94.36)	69 (97.18)

DISCUSSION

The study explored the stability and reliability of disk diffusion antibiotic susceptibility testing for *Pseudomonas aeruginosa* by assessing the inhibition zone sizes following incubation periods of 6, 10, and 24 hours. Particularly, this research sought to understand the potential benefits of early zone size reading for critical samples derived from neonatal and adult ICUs, surgical ICUs, and dialysis units. The findings indicated that early susceptibility testing can be crucial in clinical settings where prompt and accurate results are essential for effective patient management and infection control.

Table 1 revealed distinct gender and age distribution patterns among *P. aeruginosa* infections, with middle-aged males and elderly females showing the highest frequency of infections (11, 12). These demographic patterns suggest potential influences from varying lifestyle factors, including differential hand hygiene practices and physiological differences between genders. The distribution of *P. aeruginosa* across different clinical specimens, as presented in Table 2, showed a predominance in pus samples, aligning with previous research findings that indicate a similar trend in the occurrence of this pathogen (13, 14).

A critical evaluation of antimicrobial efficacy, as shown in Table 3, revealed variable responses among different antibiotics. Agents such as Ceftazidime, Piperacillin-Tazobactam, and Cefepime exhibited increased zone sizes with longer incubation times, reaching a plateau at 24 hours (15, 16, 17). This pattern suggests that the efficacy of these antibiotics against *P. aeruginosa* may be enhanced by extended incubation. Conversely, Ciprofloxacin displayed minimal changes across different incubation periods, indicating a limited impact of time on its activity against *P. aeruginosa*. In contrast, both Meropenem and Imipenem showed significant increases in zone sizes, with Imipenem exhibiting more pronounced effects (17, 18). The consistent efficacy of Colistin across all incubation durations underscores its robust activity against this pathogen (19, 20).

The strengths of this study include its comprehensive analysis of a significant number of clinical isolates from a diverse patient population and the meticulous adherence to standardized methodologies for disk diffusion testing. However, the study is not without limitations. The variability in clinical sample types and the intrinsic differences in bacterial load across samples could affect the

generalizability of the findings. Additionally, the study was conducted within a single institution, which may limit the applicability of the results to other settings with different antimicrobial resistance profiles.

Overall, these findings underscore the importance of considering incubation time in the disk diffusion method to optimize antimicrobial treatment strategies against *Pseudomonas aeruginosa*. The results contribute valuable insights to clinical microbiology practices, particularly in refining laboratory protocols to improve the accuracy and timeliness of antibiotic susceptibility testing.

CONCLUSION

In conclusion, our research underscores the critical role of incubation time in optimizing antimicrobial susceptibility testing for *Pseudomonas aeruginosa*. We observed variable antibiotic efficacy with different incubation durations, with some antibiotics like Colistin showing consistent effectiveness across all tested periods. This study demonstrates that early growth detection in disk diffusion tests can notably expedite the delivery of results—reducing turnaround times by as much as 18 hours compared to conventional methods. Such advancements hold significant potential for enhancing patient care by enabling quicker therapeutic decisions, all while maintaining cost-efficiency and not necessitating specialized equipment. These findings advocate for revising standard laboratory protocols to incorporate variable incubation times for better clinical outcomes.

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