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Research Article

# Clinical Practice: Estimating the Breakpoints for EUCAST Fast Antimicrobial Susceptibility Testing Using Flagged BacT/Alert Blood Culture Bottles

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## Abstract

*Introduction*: The escalating prevalence of multidrug resistance is a global threat to human health particularly in critically ill patients with bloodstream infections (BSIs). Delay in the administration of the appropriate antimicrobial treatment is associated with higher mortality rates and adverse consequences. This study attempted to estimate the rapid antimicrobial susceptibility testing (RAST) breakpoints directly from flagged BacT/Alert blood culture bottles in clinical practice. *Material & Methods*: A descriptive, cross-sectional study conducted at a tertiary care hospital in Delhi over a period of two months. The RAST was performed directly from the clinical samples for blood cultures received in our laboratory in parallel with the routine antimicrobial testing as per standard CLSI guidelines. Blood cultures were routinely incubated in BacT/Alert 3D. The inhibition zones were read at 4, 6, 8 and 16-20 hour of incubation as per European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The identification of the isolates was confirmed by Vitek-2 compact system. *Results*: In our study, the area of technical uncertainty (ATU) percentage was initially high at 4 hours but decreased significantly in later incubation periods. At 4 hours, none of the *S. aureus* isolates showed >90% categorical agreement (CA) for any antimicrobial tested. However, clindamycin achieved the highest CA (100%) at 6 hours and 90% thereafter, with no very major errors (VME) or major error (ME). Cefoxitin required 8 hours to reach >90% CA, with no VME observed at any time point, but up to 75% ME at 8 hours. At 4 hours, most antimicrobials had high (>1.5%) rates of VME among *Enterobacteriales*. By 6 hours, only Meropenem and Gentamicin had >90% CA, with no VME observed for other antibiotics. *Conclusion*: The RAST method is relatively easy to implement in clinical microbiology labs, offering cost-effectiveness, simplicity, and rapid results, especially in resource-limited settings. However, reporting RAST results can be complex due to potential challenges with CA, VME, and ME, particularly in the initial hours of incubation and within the ATU.

## Keywords

RAST, EUCAST, Breakpoints, Vitek-2 Compact System, Categorical Agreement

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# 1. Introduction

Sepsis is a life-threatening organ disfunction due to dysregulated host response to infection [1]. As per World Health Organization (WHO), in 2017, 48.9 million cases and 11 million sepsis-related deaths were reported worldwide, which accounted for almost 20% of total global mortality [2]. The burden of the disease is evidently varied across the geographical regions, approximately 85% of these sepsis cases and sepsis-related deaths occurred in low- and middle-income countries [2]. The striking increase in sepsis cases is largely attributed to health care-associated infections. Escalating prevalence of multi drug resistance bacteria serves to exacerbate the situation, much like adding fuel to the fire making it extremely challenging to manage such life threatening situations [3]. Prompt intervention become vital in saving the life of the patient, the Surviving Sepsis Campaign guidelines also recommends early initiation of antimicrobial therapy within an hour of the detection of septic shock [4]. Delay in the administration of the appropriate antimicrobial treatment is associated with higher mortality rates and adverse consequences. Reducing the time to identification and susceptibility testing is an essential prerequisite to speed up targeted antimicrobial therapy particularly in critically ill patients with bloodstream infections (BSIs) [5]. Rapid and reliable techniques for isolation, detection and antimicrobial susceptibility testing (AST) of the causative pathogen is need of the hour to adapt clinical intervention as fast as possible.

European Committee on Antimicrobial Susceptibility Testing (EUCAST) has defined a methodology for rapid antimicrobial susceptibility testing (RAST) directly from positive blood culture bottles by disk diffusion method with breakpoints for short incubations of 4hr, 6 hr and 8 hr. This method holds advantage of quicker turn-around time over conventional AST methods which usually requires a 16-20 hour of incubation for pure growth followed by 16-20 hour for AST [6, 7]. Clinical and Laboratory Standards Institute (CLSI) also included direct antimicrobial susceptibility testing (DAST) by disk diffusion method AST using short incubation times from the positive blood culture broth for gram negative bacteria (GNB) (*Enterobacterales* and *Pseudomonas aeruginosa*) [8]. The implementation of these novel approaches to reduces turn-around time (TAT) for AST can provide a reliable tool to improve clinical management of sepsis patients. These guidelines (EUCAST & CLSI) provide zone diameters for a limited number of bacteria while for others the zone diameters are yet to be established [6, 8]. Performed according to guidelines, these methods are both affordable, reliable and can be rapidly adapted to new antimicrobials making them particularly valuable in settings with limited resources where advanced AST systems are not accessible [9-11].

This study attempted to estimate/evaluate the performance of RAST breakpoints directly from flagged BacT/Alert blood culture bottles in clinical samples of suspected sepsis patients. We also evaluated for the presence of phenotypic drug resistance at the 4hr, 6hr and 8 hours of incubation.

# 2. Material & Methods

A descriptive cross-sectional study conducted at the bacteriology laboratory of University College of Medical Sciences, a tertiary care hospital in Delhi, India, over a period of two months (November – December, 2023). The non-duplicate clinical samples of blood received in our laboratory for routine culture were included in the study.

## 2.1. Procedure

The RAST was performed directly from the clinical samples for blood cultures in parallel with the routine testing. Blood cultures were routinely incubated in BacT/Alert 3D system. The gram stains from the flagged blood culture bottles were prepared to identify the mono-bacterial growth and eliminate poly-bacterial and mixed species growth. Within the 0-18 hours of the flag time, the RAST was performed as per EUCAST guidelines on flagged blood cultures showing mono-bacterial growth. About 125 to 150µL of undiluted blood culture broth was lawned onto Mueller- Hinton agar and antibiotic disks were placed evenly spaced across the MHA and plates were incubated at 35–37 ℃ under ambient condition. The antimicrobial disks used for RAST were Ceftazidime (10 µg), Cefotaxime (5µg), Piperacillin-Tazobactam (30/6 µg), Imipenem (10µg), Meropenem (10 µg), Ciprofloxacin (5 µg), Levofloxacin (5µg), Gentamicin (10 µg), Amikacin (30 µg), tobramycin (10 µg), and Amoxicillin-clavulanic acid (20/10 µg), for Gram-negative bacilli (GNB). For gram-positive cocci (GPC), Cefoxitin (30 µg), Norfloxacin (10 µg), Amikacin (30µg), Gentamicin (10µg and 30 µg), Tobramycin (10 µg), Clindamycin (2 µg), disks were used.

The isolates identified as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium Staphylococcus aureus*, and *Streptococcus pneumoniae* were included in the study. The identification of the isolates was confirmed by Vitek-2 compact system. Isolates other than as mentioned or displaying mixed growth were excluded from the study. In parallel with the RAST, routine testing was performed and AST was done as per CLSI guidelines.

The inhibition zones were read at 4hr, 6hr, 8hr and 16-20 hour of incubation for *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus.* Zones were read at 4hr, 6hr and 8hour of incubation for *Acinetobacter baumannii*, *Enterococcus faecalis* and *Enterococcus faecium* and at 6hr and 8 hours16-20 hours for *Pseudomonas aeruginosa* isolates. The area of technical uncertainty (ATU) is the zone-range where zone edges were not clearly visible or there is an overlap of breakpoints of susceptible and resistant isolates and

hence it cannot be determined whether the isolates are susceptible or not.

### 2.2. Phenotypic Drug Resistance

In addition to RAST, phenotypic drug resistance mechanism for MRSA (Methicillin-Resistant *Staphylococcus aureus*), ICR (Inducible Clindamycin Resistance), ESBL (Extended-Spectrum Beta-Lactamase) and CRE (Carbapenem Resistance *Enterobacterales*) were observed. For ESBL, double-disk synergy test by using Ceftazidime (30 µg) and Ceftazidime plus clavulanic acid (30/10 µg) disks were used. For CRE, carbaNP (RAPIDEC® CARBA NP test) test was used. In *Staphylococcus aureus* isolates, MRSA was detected by using Cefoxitin (30 µg) disks while performing RAST and for ICR, D-test (zone of inhibition around Clindamycin appears as a letter "D") was observed by placing Clindamycin (2 µg) and Erythromycin (15 µg) disks adjacent to each other.

For quality control, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25921 were used with each batch as per the EUCAST guidelines. A purity plate was put to observe contamination during the procedure.

### 2.3. Statistical Analysis

For statistical analysis, Microsoft SPSS version 2.0 was used. The results from the RAST method were compared using CLSI M100 guidelines as the reference method. The categorical agreement (CA), very major errors (VME), and major errors (ME) were determined. The CA was defined as agreement in the interpretation of the RAST and the reference method. VME (false susceptibility) determines the percentage of false susceptible results by RAST method as compared to the reference method whereas ME (false resistance) determines the percentage of false resistant results by RAST methods as compared to the reference method. As per CLSI recommendations, a new system can be acceptable when it meets the standards as follows: CA ≥ 90%, VME ≤ 1.5%, and ME ≤ 3% (CLSI, 2021). The ATU were not included in the calculation of CA, VME and ME [10, 11].

## 3. Results

### 3.1. Distribution of Isolates

Over a period of two months (November – December, 2023), among flagged BacT/Alert blood culture bottles, 42 showed mono-bacterial isolates that qualified for further testing by RAST method. Among these 42 clinical isolates, 22 (52.4%) were gram positive cocci (GPC) including *Staphylococcus aureus* 20 (47.6%) and *Enterococcus faecalis* 2 (4.8%) and 20 (47.6%) were GNB including *Klebsiellapneumoniae*10 (23.8%), *Escherichia coli* 7 (16.7%) and *Acinetobacter baumannii* 3 (7.1%).

***Table 1.*** *Readings of RAST method of Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli and isolates at 4,6,8 and 16-20 hour as per EUCAST guidelines and Acinetobacter baumannii at 4,6 and 8 hour as per EUCAST guidelines and at 16-20 hour as per CLSI guidelines.*

| | S | ATU | R | S | ATU | R | S | ATU | R | S | ATU | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S. aureus (n= 20) | 4 Hour | | | 6 Hour | | | 8 Hour | | | 16-20 Hour | | |
| Cefoxitin | 5 | 8 | 7 | 4 | 1 | 15 | 4 | 0 | 16 | 3 | 0 | 17 |
| Norfloxacin | 4 | 6 | 10 | 4 | 0 | 16 | 4 | 0 | 16 | 4 | 0 | 16 |
| Amikacin | 14 | 4 | 2 | 18 | 0 | 2 | 18 | 0 | 2 | 18 | 1 | 1 |
| Gentamicin | 9 | 10 | 1 | 11 | 4 | 5 | 13 | 2 | 5 | 13 | 2 | 5 |
| Tobramycin | 8 | 8 | 4 | 10 | 4 | 6 | 12 | 3 | 5 | 12 | 2 | 6 |
| Clindamycin | 10 | 7 | 3 | 15 | 1 | 4 | 15 | 0 | 5 | 15 | 0 | 5 |
| K. pneumoniae (n=10) | 4 hour | | | 6 hour | | | 8 hour | | | 16-20 hour | | |
| Amoxicillin-clavulanic acid | 0 | 6 | 4 | 1 | 3 | 6 | 1 | 2 | 7 | 0 | 0 | 10 |
| Cefotaxime | 1 | 1 | 8 | 2 | 0 | 8 | 2 | 0 | 8 | 2 | 0 | 8 |
| Ceftazidime | 1 | 3 | 6 | 0 | 2 | 8 | 1 | 1 | 8 | 0 | 2 | 8 |
| Imipenem | 6 | 2 | 2 | 5 | 3 | 2 | 4 | 3 | 3 | 7 | 1 | 2 |
| Meropenem | 2 | 2 | 6 | 1 | 1 | 8 | 1 | 1 | 8 | 1 | 1 | 8 |
| Ciprofloxacin | 1 | 2 | 7 | 2 | 0 | 8 | 2 | 0 | 8 | 2 | 1 | 7 |
| Levofloxacin | 0 | 2 | 8 | 1 | 1 | 8 | 1 | 1 | 8 | 1 | 1 | 8 |

| | S | ATU | R | S | ATU | R | S | ATU | R | S | ATU | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amikacin | 3 | 2 | 5 | 4 | 0 | 6 | 4 | 0 | 6 | 4 | 0 | 6 |
| Gentamicin | 6 | 1 | 3 | 7 | 0 | 3 | 7 | 0 | 3 | 7 | 0 | 3 |
| Tobramycin | 6 | 2 | 2 | 7 | 0 | 3 | 7 | 0 | 3 | 7 | 0 | 3 |
| E. coli (n= 7) | 4 HOUR | | | 6 HOUR | | | 8 HOUR | | | 16-20 HOUR | | |
| Amoxicillin-clavulanic acid | 4 | 1 | 2 | 5 | 0 | 2 | 3 | 0 | 4 | 3 | 1 | 3 |
| Cefotaxime | 4 | 1 | 2 | 4 | 1 | 2 | 4 | 1 | 2 | 5 | 0 | 2 |
| Ceftazidime | 1 | 4 | 2 | 4 | 0 | 3 | 3 | 1 | 3 | 2 | 2 | 3 |
| Imipenem | 5 | 1 | 1 | 6 | 1 | 0 | 6 | 1 | 0 | 6 | 1 | 0 |
| Meropenem | 5 | 2 | 0 | 5 | 1 | 1 | 5 | 0 | 2 | 5 | 0 | 2 |
| Ciprofloxacin | 0 | 2 | 5 | 2 | 0 | 5 | 2 | 0 | 5 | 2 | 0 | 5 |
| Levofloxacin | 0 | 3 | 4 | 1 | 2 | 4 | 2 | 1 | 4 | 2 | 1 | 4 |
| Amikacin | 3 | 3 | 1 | 5 | 2 | 0 | 4 | 1 | 2 | 7 | 0 | 0 |
| Gentamicin | 5 | 1 | 1 | 6 | 0 | 1 | 6 | 0 | 1 | 6 | 0 | 1 |
| Tobramycin | 6 | 1 | 0 | 7 | 0 | 0 | 7 | 0 | 0 | 7 | 0 | 0 |
| A.baumannii (n= 3) | 4 HOUR | | | 6 HOUR | | | 8 HOUR | | | CLSI (Reference Method) | | |
| Imipenem | 1 | 0 | 2 | 1 | 0 | 2 | 1 | 0 | 2 | 0 | 0 | 3 |
| Meropenem | 1 | 0 | 2 | 1 | 0 | 2 | 1 | 0 | 2 | 1 | 0 | 2 |
| Ciprofloxacin | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 0 | 3 |
| Levofloxacin | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 |
| Amikacin | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 0 | 3 | 0 | 0 | 3 |
| Gentamicin | 1 | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 |
| Tobramycin | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 |

Note ATU: Area of technical uncertainty; S: Susceptible; R: Resistant; CLSI: Clinical and Laboratory Standards Institute, RAST: Rapid antimicrobial susceptibility testing

***Table 2.*** *The comparison of results of RAST to the reference method (as per CLSI guidelines).*

| S. aureus (20) | CLSI (Reference method) | | 4 Hour | | | 6 Hour | | | 8 Hour | | | 16-18 Hour | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S | R | CA% | VME% | ME% | CA% | VME% | ME% | CA% | VME% | ME% | CA% | VME% | ME% |
| Cefoxitin | 16 | 4 | 61.5 | 0 | 18.8 | 89.4 | 0 | 68.8 | 90 | 0 | 75.0 | 95 | 0 | 81.3 |
| Norfloxacin | 5 | 15 | 70.5 | 20 | 25 | 95 | 0 | 25 | 95 | 0 | 25 | 95 | 0 | 25 |
| Gentamicin | 16 | 4 | 50 | 0 | 37.5 | 63 | 0 | 6.3 | 73.6 | 0 | 6.3 | 75 | 0 | 6.3 |
| Clindamycin | 15 | 5 | 63.1 | 0 | 13.3 | 100 | 0 | 0 | 90 | 0 | 0 | 90 | 0 | 0 |
| Enterobacteriales (17) | | | | | | | | | | | | | | |
| Amoxicillin-clavulanic acid | 3 | 14 | 90 | 7.1 | 0 | 78.57 | 21.4 | 0 | 93.3 | 7.1 | 0 | 100 | 0 | 0 |
| Cefotaxime | 0 | 17 | 66.6 | 29.4 | 0 | 66.60 | 35.3 | 0 | 62.5 | 35.3 | 0 | 62.5 | 41.2 | 0 |

| S. aureus (20) | CLSI (Reference method) | | 4 Hour | | | 6 Hour | | | 8 Hour | | | 16-18 Hour | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S | R | CA% | VME% | ME% | CA% | VME% | ME% | CA% | VME% | ME% | CA% | VME% | ME% |
| Ceftazidime | 3 | 14 | 58.3 | 0 | 0 | 75 | 7.1 | 0 | 88.23 | 7.1 | 0 | 92.3 | 0 | 0 |
| Imipenem | 7 | 10 | 42.1 | 40.0 | 0 | 69.2 | 0 | 0 | 64.28 | 30.0 | 0 | 61.53 | 60 | 0 |
| Meropenem | 6 | 11 | 66.6 | 9.1 | 0 | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| Ciprofloxacin | 4 | 13 | 92.3 | 23.1 | 0 | 88.8 | 0 | 0 | 88.2 | 0 | 0 | 87.5 | 0 | 0 |
| Levofloxacin | 7 | 10 | 73.3 | 0 | 28.6 | 86.6 | 0 | 28.6 | 82.3 | 0 | 28.6 | 93.3 | 0 | 28.6 |
| Amikacin | 5 | 12 | 61.5 | 8.3 | 0 | 57.1 | 33.3 | 0 | 68.75 | 25.0 | 0 | 52.94 | 50 | 0 |
| Gentamicin | 12 | 5 | 82.3 | 0 | 0 | 94.11 | 20 | 0 | 94.11 | 0 | 0 | 94.11 | 20 | 0 |
| Tobramycin | 5 | 12 | 31.2 | 58.3 | 0 | 42.1 | 0 | 0 | 47 | 75.0 | 0 | 47 | 75 | 0 |

CA: Categorical agreement, VME: Very major error, ME: Major error, AST: Antimicrobial susceptibility testing, RAST: Rapid antimicrobial susceptibility testing, CLSI: Clinical and Laboratory Standards Institute, and EUCAST: European Committee on Antimicrobial Susceptibility Testing.

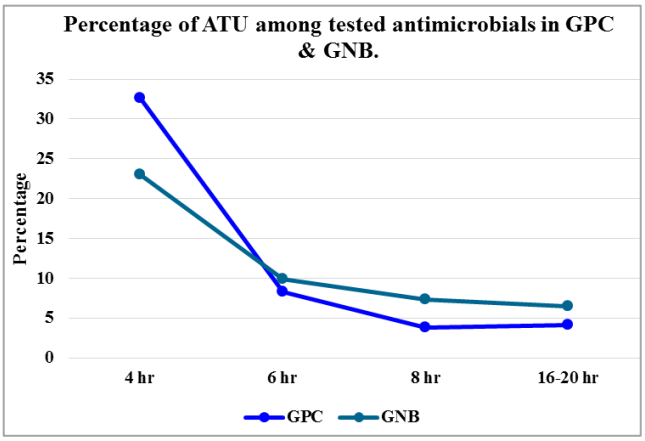



***Figure 1.*** *Percentage of ATU among tested antimicrobials in GPC and GNB at the time of reading.*

ATU: Area of Technical Uncertainty, GPC: Gram-positive cocci, GNB: Gram negative bacilli.

A total of 42 clinical isolates, and 6 to 10 antimicrobials tested per isolate resulted in overall 1259 inhibition zone diameters which were read at 4hr, 6hr, 8hr and 16-20 hour. The results of RAST are summarized in Table 1. At 4 hours of incubation, a thin but noticeable growth was observed. However, by 6 hour to 8 hours, the growth became clear and comparable to that of 16-20 hour of incubation. The ATU, which cannot be determined whether the isolates are susceptible or not were also noted. The percentage of results in the ATU was more observed in early readings, especially at 4 hour incubation. The percentage of ATU at 4 hour was more observed in GPC (32%) as compared to GNB (23%). However, their occurrence significantly reduced at 6-hour incubation (8% in GPC and 10% in GNB) and subsequent incubation periods. Figure 1 illustrates the percentage of ATU observed at 4hr, 6hr, 8hr and 16-20 hour of incubation in GPC and GNB.

## 3.2. Rapid Antimicrobial Susceptibility Testing in Gram-Positive Cocci

Among the *S. aureus* isolates, none of the tested antimicrobial presented with > 90% of Categorical Agreement (CA) for any tested antimicrobial at 4 hour of incubation. However the highest CA (100%) was presented by Clindamycin 6 hour incubation and 90% at subsequent incubation with no VME and ME. Cefoxitin, on the other hand, achieved the CA >90% only after 8 hours of incubation. There was no VME observed at 4,6,8 hour of incubation for any Cefoxitin, Gentamicin and Clindamycin. However, up to 75% ME was observed at 8 hour of incubation for Cefoxitin. For Norfloxacin, the VME of 20% was observed at 4 hour incubation however no VME were observed at 6, 8 hour. Due to the absence of zone diameter for Amikacin, and Tobramycin for *Staphylococcus aureus* as per CLSI M100 guidelines, calculations for categorical agreement, very major errors, and major errors were not possible.

Among *E. faecalis*, both the isolates exhibited zone diameter within susceptibility category for Linezolid and susceptible increased exposure (Si) category for Imipenem at 4hr, 6hr and 8 hour of incubation. The zone diameters for Vancomycin were in ATU category and were not resistance for both the isolates at 4hr, 6hr and 8 hour of incubation. Additionally, for high level amino glycoside resistance (HLAR) testing, one isolate was positive and one was negative for HLAR by using Gentamicin (30ug) disk at 4hr, 6hr and 8 hour of incubation. On comparing

the RAST results with the reference method, both the isolates were susceptible for Imipenem, Vancomycin, Linezolid as well as to high level Gentamicin (120ug) when AST was performed as per CLSI guidelines.

## 3.3. Rapid Antimicrobial Susceptibility Testing in Gram-Negative Bacilli

*K. pneumoniae* was the most common isolate among GNB, followed by *E.coli*. Majority of antimicrobials (except Amoxicillin-clavulanate& Ciprofloxacin) had CA of < 90% at 4 hour incubation for *Enterobacterales*. The VME observed at 4 hour incubation was high (>1.5%) for most of the antimicrobials (Except for Ceftazidime, Levofloxacin and Gentamicin). While the ME was high for Levofloxacin (28.6%), and rest of the antimicrobials showed no ME (0%). At 6 hour incubation only 2 antimicrobials (Meropenem& Gentamicin) had CA of > 90%. No VME was observed for 5 antimicrobials (Imipenem, Meropenem, Ciprofloxacin, Levofloxacin and Tobramycin) and high (>1.5%) VME was observed for rest of the antimicrobials. No ME was observed at 6 hour incubation for most of the antimicrobials (except Levofloxacin). No much variation in results of CA, VME and ME was observed at 8 hour incubation as compared to the results at 6 hour incubation.

Among gram negative isolates, 3 were *Acinetobacter baumannii*, on comparing RAST results with reference method, Levofloxacin, Meropenem and Tobramycin achieved same result at 4, 6 and 8 hour. However Gentamicin and Amikacin achieved the same result at 6 hour and 8 hour incubation respectively.

## 3.4. Antimicrobial Resistance

Antimicrobial resistance (MRSA, ICR, ESBL & CRE) was observed. At 6 hour incubation, 32% of AMR was recorded and remaining 68% at 8 hour of incubation. The percentage of antimicrobial resistance detected is shown in figure 2.

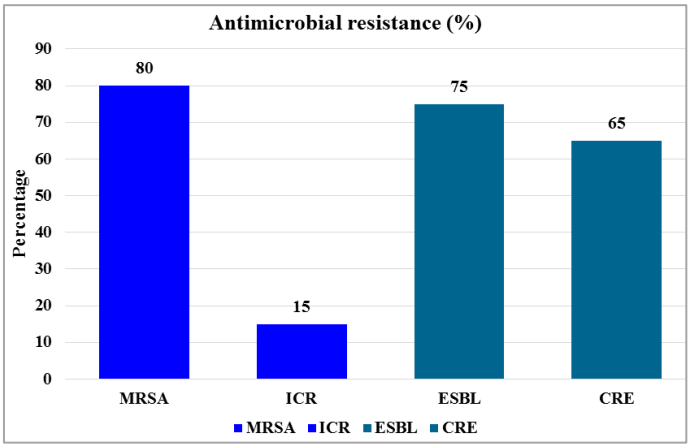
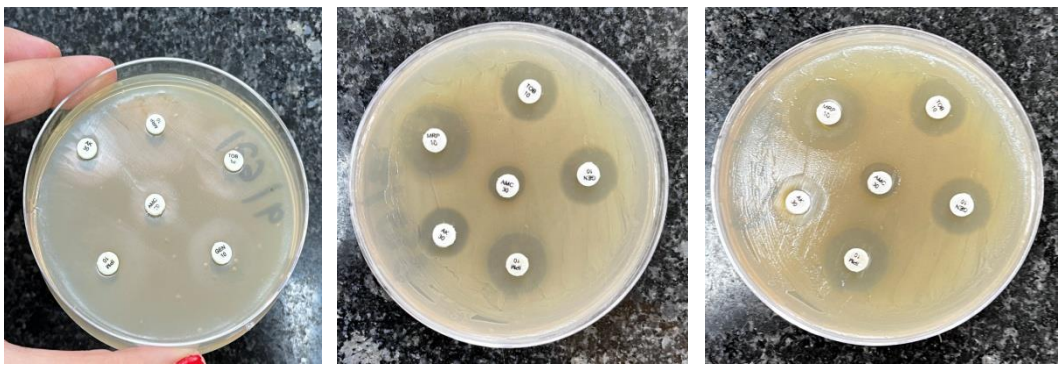



***Figure 2.*** *Distribution of antimicrobial resistance among tested isolates in the study group.*

MRSA: Methicillin Resistant *Staphylococcus aureus*; ICR: Inducible Clindamycin resistance; ESBL: Extended Spectrum Beta-Lactamase; CRE: carbapenem-resistant *Enterobacterales.*



a) b) c)

RAST: Rapid antimicrobial susceptibility testing

***Figure 3.*** *RAST of gram negative bacteria at 4 hour incubation (a); at 6 hour incubation (b); at 8 hour incubation (c).*

## 4. Discussion

The foundation of sepsis management relies on promptly administering suitable and efficient antimicrobial therapy. However, the prevalence of MDR pathogens complicates the empirical antibiotic treatment choices, increases the risk of inappropriate treatments. The conventional methods usually take 2-3 days for identification and susceptibility testing of the causative pathogens from positive blood cultures. The utilization of RAST method reduces the duration of susceptibility testing, thereby expediting the implementation of targeted antimicrobial therapy. In this study we observed and compared the results of RAST by EUCAST with reference method (CLSI) along with phenotypic antimicrobial resistance.

The EUCAST guidelines defines ATU as zone range where the edges of zones are not distinctly visible or where breakpoints of susceptible and resistant isolates overlap, making it challenging to determine the susceptibility of the isolates. In our study, the ATU percentage was high at 4 hour incubation which reduces drastically at subsequent incubation period. The similar findings were observed in another study by Cherkaoui*et al* (2023) [12]. They observed a high rate of ATU for *E. coli*, *Klebsiella* spp., *A. baumannii* and *S. aureus* isolates was observed at 4 h, but this rate declined over time. In contrast to Cherkaoui*et al*, we observed a higher occurrence of ATU in GPC as compared to GNB after 4 hour incubation.

In our study, for *S.aureus*, the CA of < 90 % and no VME was observed at 4 hour of incubation with high ME for most of the tested antimicrobial. The CA remained same for the GNBs however the high VME, and no ME was observed in *Enterobacteriales* for most of the antimicrobials tested. In another study by Soo*et al* (2019), They compared the RAST results of 194 gram negative isolates against the routine method, Vitek-2. They observed high ME rates for Piperacillin-Tazobactam and Ceftazidime in gram negative isolates (*E.coli* and *K. pneumoniae*) similar to our study. However the VME rates were low at 4 hour incubation and CA of > 90% was achieved in their which is not achieved in our study [13, 14].

In a study by Park *et al*. (2023), for *S. aureus*, they observed high CA, (100%) at 4, 6, and 8 hours. There were no ME or VME at 4, 6, and 8 hours. All categories were consistent between the two methods when compared with the broth microdilution (Sensitizer) results based on the CLSI criteria. In our study discrepancy among the CA and ME were observed in *S. aureus*, though the CA of > 90% was achieved by most of the antimicrobials at 8 and 16-18 hour [15].

At results of RAST at 6 hour and 8 hour incubation is almost similar with most of the antimicrobials. In a study by Mancini *et al*, (2020) the overall better separation of the susceptible and resistant strains after 8 h incubation resulted in generated a higher number of interpretable results as compared to results after 6 hour incubation unlike our study [16]. This may be attributed to the small sample size and the utilization of automated techniques for inoculum preparation (such as flow cytometry using the UF-4000 system) and streaking to minimize human errors, aspects that were not present in our study [16].

With the suggested breakpoints of RAST, up to 32% of AMR (MRSA, ICR, ESBL & CRE) can be detected phenotypically at 6 hour, and remaining 68% can be detected at 8 hour of incubation. Similar results were found in study by Jonson *et al.* (2020) [6].

RAST is a not a complicated method to introduce in standard clinical microbiology laboratories. It is cost-effective, simple to execute, and provides rapid results, particularly in settings with limited resources where automated methods like Vitek and Maldi-TOF are unavailable. Implementing this method demands adaptation of work- flow to reduce the human errors and enhance precision. The reporting of the RAST results can be challenging due to the increased likelihood of poor CA, VME and ME during the initial hours of incubation along with the ATU. Though the incorporation of the ATU in the guidelines itself prevents unavoidable variation from causing VME and ME to some extent. We noted an overall increase in CA and more favourable VME and ME for selected antimicrobials. Hence, RAST can be used to for selected antimicrobials not all, but caution is advised, particularly regarding early readings, especially the 4-hour reading. To enhance this approach moving forward, focusing on establishing specific breakpoints for a wider range of species and expanding the study to multiple centers will enhance the diversity of isolates. Additionally, automating the reading of inhibition zones can reduce interpretation errors and improve accuracy.

## 5. Conclusion

The RAST method offers rapid results, yet there are some limitations in evaluating the applicability of this method. Unlike CLSI, EUCAST-RAST method cannot be performed for various strains and antimicrobial agents. As of now, the RAST method has only undergone validation for eight species and is not applicable to species beyond this validated set. In this study we observed that the number of ATU was high after 4 hour incubation making it unreliable for reporting. The discrepancy among CA, VME and ME undermines the reliability of the test for certain antimicrobials at 6 and 8 hours of the test. Though in general, the numbers of VME and ME decreased over time and CA increases with subsequent incubations. The early phenotypic detection of antimicrobial resistance at 6-8 hours significantly aids in initiating antimicrobial treatment. This study indicates that RAST method can be used to for certain antimicrobials not all, also the early readings, especially the 4-h reading, using the RAST breakpoints proposed by EUCAST cannot be used clinical micro-

biology laboratories. The primary lacunae of this study is its limited sample size. Increased sample size is imperative to ensure more precise results.

## Abbreviations

| | |
|---|---|
| RAST | Rapid Antimicrobial Susceptibility Testing |
| CLSI | Clinical and Laboratory Standards Institute |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| AMR | Antimicrobial Resistance |
| MRSA | Methicillin Resistant *Staphylococcus aureus* |
| ICR | Inducible Clindamycin Resistance |
| ESBL | Extended Spectrum Beta-Lactamase |
| CRE | Carbapenem-Resistant *Enterobacterales* |
| ATU | Area of Technical Uncertainty |
| S | Susceptible |
| R | Resistant |
| GPC | Gram-Positive Cocci |
| GNB | Gram Negative Bacilli |
| CA | Categorical Agreement |
| VME | Very Major Error |
| ME | Major Error |
| AST | Antimicrobial Susceptibility Testing |

## Author Contributions

**Seema Gangar:** Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing

**Kirti Nirmal:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing

**Avinash Kant Lakra:** Funding acquisition Investigation, Project administration, Resources

**Kalyani Swain:** Data curation, Methodology

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## Conflicts of Interest

The authors declare no conflicts of interest.

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