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ORIGINAL ARTICLE

# Tick-tock, beat the clock: comparative analysis of disc diffusion testing with 6-, 10-, and 24-h growth for accelerated antimicrobial susceptibility testing and antimicrobial stewardship

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## Abstract

Disc diffusion testing by Kirby-Bauer technique is the most used method for determining antimicrobial susceptibility in microbiological laboratories. The current guidelines by The Clinical and Laboratory Standards Institute (CLSI) 2022 specify using an 18- to 24-h growth for testing by disc diffusion. We aim to determine if using an early growth (6 h and 10 h) would produce comparable results, thus ultimately leading to reduced turnaround time. Six-hour, 10-h, and 24-h growths of 20 quality control strains and 6-h and 24-h growths of 48 clinical samples were used to perform disc diffusion testing using a panel of appropriate antimicrobial agents. Disc diffusion zone sizes were interpreted for all and comparative analyses were performed to determine categorical agreement, minor errors (mE), major errors (ME), and very major errors (VME) according to CLSI guidelines. On comparing with the standard 24 h of incubation, disc diffusion from 6-h and 10-h growths of quality control strains showed 94.38% categorical agreement, 5.10% mE, 0.69% MEs, and no VMEs. Disc diffusion testing for the additional 40 clinical samples yielded a similarly high level of categorical agreement (98.15%) and mE, ME, and VME of 1.29%, 1.22%, and 0% respectively. Disc diffusion testing using early growth is a simple and accurate method for susceptibility testing that can reduce turnaround time and may prove to be critical for timely patient management.

**Keywords** Early disc diffusion testing · Kirby-Bauer · Antimicrobial susceptibility testing · Rapid susceptibility

## Introduction

Antimicrobial susceptibility testing (AST) for micro-organisms can be done by multiple methods; one of those methods is disc diffusion which was first standardized by Bauer et al. in 1966 [1]. Disc diffusion method is simple and easy to adopt but relatively slow. The cost of supplies and materials is low and readily available, making it a reliable, reproducible, and low-cost AST method. Kirby-Bauer disc diffusion gives the flexibility to check different "panels" of antibiotics. It helps make antibiograms, hospital formulary, and local resistance trends. Alongside, when newer antimicrobials are approved for clinical use in the market, disc diffusion is frequently the first type of antibiotic susceptibility method that is available and cleared for clinical laboratories to use [2–5].

Inoculum preparation for disc diffusion method of AST, as specified by the Clinical and Laboratory Standards Institute (CLSI), indicates that 0.5 McFarland standard should be prepared from 18- to 24-h growth on a nonselective agar plate. Additional 16- to 24-h incubation of AST plate is required before reading and interpretation depending on the organism-antimicrobial combination [6].

The use of 18- to 24-h growth to prepare the initial testing inoculum is largely predicated on the norms of the human workday because most clinical microbiology laboratories are fully operational only during the "day shift" [7].

As the 24-h microbiology laboratory settings are now available, it is time to reexamine the need for 18 h of culture incubation before setting up AST. Reducing this incubation interval would be an inexpensive way to fasten AST results. This method of early disc diffusion (EDD) changes only the length of time of subculture growth from 18–24 h to 6–10 h,

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prior to disc diffusion setup and uses established guidelines like CLSI and EUCAST [7].

Thus, EDD can be easily introduced into the already existing workflows and can reduce the time to result by as much as 18 h without adding extra cost to the testing method. Faster antibiotic susceptibility testing can ultimately lead to better antibiotic stewardship and improved clinical outcome [8].

## Materials and methods

### Quality control strains

Twenty quality control strains of bacteria that are representative of bacteria commonly encountered in our clinical microbiology laboratory were chosen. The strains chosen included *Staphylococcus epidermidis*, *Staphylococcus hemolyticus*, methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus plazen*, *Enterococcus faecium*, *Enterococcus faecalis*, Group A Beta hemolytic *Streptococcus*, Group B Beta hemolytic *Streptococcus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, and *Salmonella* Typhimurium. These strains were inoculated on blood agar and incubated at 37 °C in an ambient air incubator. From the 6-h, 10-h, and 24-h growths of the same, suspensions were prepared at a concentration equal to 0.5 McFarland standard as measured by Densichek Plus (BioMérieux) turbidimeter. The bacterial suspensions were evenly spread in the form of a uniform lawn manually onto a 150-mm Mueller–Hinton agar plate using a sterile cotton swab. The excess moisture in the plates was allowed to evaporate for 3 to 5 min and appropriate antibiotic discs were applied onto the agar surface depending on the organism (Table 1). Care was taken not to place the discs closer than 24 mm center to center on the Mueller–Hinton agar plate. Once all discs were in place, the plates were inverted and incubated at 37 °C in an incubator for 18 h. When testing *Staphylococcus* spp. against vancomycin or *Enterococcus* spp. against vancomycin, we incubated for a full 24 h before reading. After incubation, the zone of inhibition around each antibiotic was measured manually and the zone sizes were interpreted for all using CLSI 2022 guidelines.

**Table 1** Bacteria and corresponding antibiotics evaluated

| Organism encountered | Count (QC, clinical isolates) | Antibiotics tested (disc mass) |
|---|---|---|
| *Staphylococcus aureus* | 2, 2 | Gentamicin (10 µg), Penicillin (10 IU), Cefoxitin (30 µg), Vancomycin (30 µg), Teicoplanin (30 µg), Ciprofloxacin (5 µg), Linezolid (30 µg), Rifampicin (5 µg), Cotrimoxazole (1.25/23.75 µg), Clindamycin (2 µg), Chloramphenicol (30 µg), Erythromycin (15 µg) |
| *Staphylococcus epidermidis* | 1, 11 | |
| *Staphylococcus hominis* | 0, 9 | |
| *Staphylococcus hemolyticus* | 1, 5 | |
| *Staphylococcus plazen* | 1, 0 | |
| *Escherichia coli* | 1, 6 | Amikacin (30 µg), Netilmicin (30 µg), Amoxicillin-Clavulanate (20/10 µg), Piperacillin-Tazobactum (100/10 µg), Cefoperazone-Sulbactum (75/10 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Imipenem (10 µg), Meropenem (10 µg), Ciprofloxacin(5 µg), Polymyxin B(300 µg) |
| *Klebsiella pneumoniae* | 1, 6 | |
| *Proteus mirabilis* | 1, 0 | |
| *Proteus vulgaris* | 1, 0 | |
| *Serratia marcescens* | 1, 0 | |
| *Enterobacter hormaechei* | 0, 1 | |
| *Pseudomonas aeruginosa* | 1, 3 | Amikacin (30 µg), Netilmicin (30 µg), Piperacillin-Tazobactum (100/10 µg), Cefoperazone-Sulbactum (75/10 µg), Ceftazidime (30 µg), Imipenem (10 µg), Meropenem (10 µg), Ciprofloxacin(5 µg), Polymyxin B(300 µg) |
| *Salmonella spp.* | 4, 0 | Ciprofloxacin (5 µg), Cotrimoxazole (1.25/23.75 µg), Chloramphenicol (30 µg), Cefixime (5 µg), Ceftriaxone (30 µg), Ampicillin (10 µg), Azithromycin (15 µg), Cefotaxime (30 µg) |
| *Acinetobacter baumannii* | 1, 2 | Amikacin (30 µg), Augmentin (20/10 µg), Piperacillin-Tazobactum (100/10 µg), Cefoperazone-Sulbactum (75/10 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Imipenem (10 µg), Meropenem (10 µg), Ciprofloxacin(5 µg), Polymyxin B(300 µg) |
| *Streptococcus spp.* | 2, 0 | Penicillin (10 IU), Vancomycin (30 µg), Teicoplanin (30 µg), Linezolid (30 µg), Cotrimoxazole (1.25/23.75 µg), Clindamycin (2 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Levofloxacin (5 µg), Azithromycin (5 µg), Cefotaxime (30 µg) |
| *Enterococcus spp.* | 2, 3 | Gentamicin (120 µg), Tetracycline (30 µg), Penicillin (10 IU), Vancomycin (30 µg), Teicoplanin (30 µg), Ciprofloxacin (5 µg), Linezolid (30 µg), Erythromycin (15 µg) |

**Table 2** Comparison of disc diffusion testing from 6-h early growth method versus standard 24-h growth method of 20 quality control strains

| QC organism | No. of isolates | Susceptibility pattern of corresponding antibiotics evaluated | | | | | | Total | Categorical agreement (%) | Discrepancies (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6 h | | | 24 h | | | | | | | |
| | | S | I | R | S | I | R | | | Very major | Major | Minor |
| *Staphylococcus epidermidis* | 1 | 7 | 0 | 5 | 7 | 0 | 5 | 12 | 12/12 (100) | 0/5 (0) | 0/7 (0) | 0/12 (0) |
| *Staphylococcus hemolyticus* | 1 | 4 | 1 | 7 | 5 | 1 | 6 | 12 | 10/12 (83.33) | 0/6 (0) | 0/5 (0) | 2/12 (16.66) |
| *Staphylococcus aureus* | 2 | 19 | 1 | 4 | 19 | 1 | 4 | 24 | 24/24 (100) | 0/4 (0) | 0/19 (0) | 0/24 (0) |
| *Staphylococcus plazen* | 1 | 12 | 0 | 0 | 12 | 0 | 0 | 12 | 12/12 (100) | 0/0 (0) | 0/12 (0) | 0/12 (0) |
| *Enterococcus spp.* | 2 | 8 | 1 | 7 | 8 | 1 | 7 | 16 | 16/16 (100) | 0/7 (0) | 0/8 (0) | 0/16 (0) |
| *Streptococcus spp.* | 2 | 22 | 0 | 0 | 22 | 0 | 0 | 22 | 22/22 (100) | 0/0 (0) | 0/22 (0) | 0/22 (0) |
| *Escherichia coli* | 1 | 11 | 0 | 0 | 11 | 0 | 0 | 11 | 11/11 (100) | 0/0 (0) | 0/11 (0) | 0/11 (0) |
| *Klebsiella pneumoniae* | 1 | 5 | 5 | 1 | 8 | 2 | 1 | 11 | 8/11 (72.72) | 0/1 (0) | 0/8 (0) | 3/11 (27.27) |
| *Serratia marcescens* | 1 | 4 | 1 | 6 | 7 | 1 | 3 | 11 | 8/11 (72.72) | 0/11 (0) | 1/7 (14.28) | 2/11 (18.18) |
| *Acinetobacter baumannii* | 1 | 0 | 1 | 8 | 0 | 1 | 8 | 9 | 9/9 (100) | 0/8 (0) | 0/0 (0) | 0/9 (0) |
| *Pseudomonas aeruginosa* | 1 | 8 | 0 | 2 | 8 | 0 | 2 | 10 | 10/10 (100) | 0/2 (0) | 0/8 (0) | 0/10 (0) |
| *Proteus spp.* | 2 | 12 | 3 | 7 | 14 | 2 | 6 | 22 | 19/22 (86.36) | 0/6 (0) | 0/14 (0) | 3/22 (13.63) |
| *Salmonella spp.* | 4 | 22 | 1 | 1 | 22 | 1 | 1 | 24 | 24/24 (100) | 0/1 (0) | 0/22 (0) | 0/24 (0) |
| Total | 20 | 134 | 14 | 48 | 143 | 10 | 43 | 196 | 185/196 (94.38) | 0/43 (0) | 1/143 (0.69) | 10/196 (5.10) |

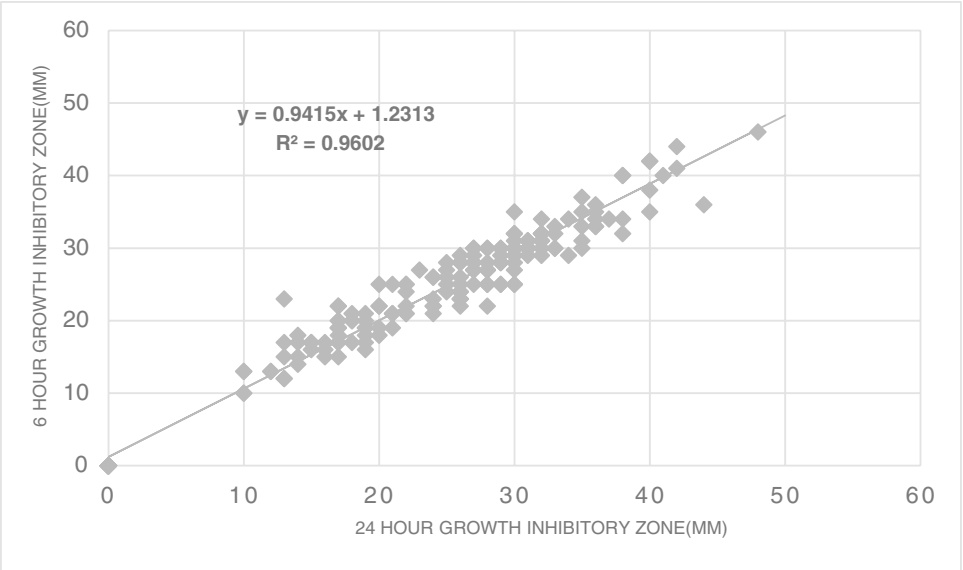



**Fig. 1** Linear regression analysis of inhibitory zones from EDD6 and St24 of QC strains. $R^2$ denotes goodness of fit

## Clinical isolates

Forty-eight clinical samples for blood culture were collected from patients attending a tertiary care hospital, in Delhi. The blood culture bottles were incubated in BacT/Alert (BioMérieux, Marcy-l'Étoile, France) automated system. Bottles flagged as positive by the BacT/Alert system were subcultured on blood agar. Six-hour and 24-h growths of the same were used to perform disc diffusion testing using a panel of appropriate antimicrobial agents as done with the quality control strains. Similarly, the disc diffusion zone sizes were interpreted for all using CLSI 2022 guidelines. From the 24-h growths, species identification by MALDI-TOF (VITEK-MS system, BioMérieux, Marcy-l'Étoile, France) was carried out.

## Analysis of disc diffusion breakpoints

Categorical agreement, minor errors (mEs), major errors (MEs), and very major errors (VMEs) were calculated per the approved guidelines by CLSI for the development of in vitro susceptibility testing criteria and quality control parameters [9]. Categorical agreement (CA) means similar interpretive criteria (susceptible/intermediate/resistant) was agreed upon between the two methods. Minor error (mE) means a susceptible or resistant result was shown as intermediate and vice versa. Major error (ME) denotes a susceptible isolate shown as resistant and calculated only for susceptible isolates. Very major error (VME) suggests a resistant isolate was shown susceptible and calculated only for resistant isolates.

Furthermore, quantitative agreement between methods was evaluated by performing a linear regression analysis of inhibitory zones from 6-h and standard 24-h disc diffusion and the values of the slope and $r^2$ were determined which denote the goodness of fit. Similar analyses were carried out for the 10-h inhibitory zones. The difference in inhibitory zone diameters for the clinical isolates was calculated and compared among the different organisms encountered as well as among the different drugs used.

# Results

## Quality control strains

Overall, 98 Gram-positive isolate-drug combinations were evaluated. Out of them, 22.45% ($n=22$) were found to be resistant by using standard methods of 24-h growth AST (St24). While using 6-h early disc diffusion testing (EDD6), 23.47% ($n=23$) were found to be resistant. Likewise, out of the 98 Gram-negative isolate-drug combinations evaluated, 21.42% ($n=21$) were resistant using standard 24-h growth AST, whereas 25.5% ($n=25$) were resistant using 6-h early disc diffusion testing.

On comparing the EDD6 zone sizes for the quality control strains to the standard 24-h of incubation, 6-h growth showed 5.10% mE and 0.69% MEs and no VMEs. Categorical agreement with standard incubation was 94.38% (Table 2).

A linear regression analysis of inhibitory zones from EDD6 and St24 revealed an $r^2$ value of 0.96 and a slope

**Table 3** Comparison of disc diffusion testing from the 10-h early growth method versus standard 24-h growth method of 20 quality control strains

| QC organism | No. of isolates | Susceptibility pattern of corresponding antibiotics evaluated | | | | | | Total | Categorical agreement (%) | Discrepancies (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 h | | | 24 h | | | | | | | |
| | | S | I | R | S | I | R | | | Very major | Major | Minor |
| *Staphylococcus epidermidis* | 1 | 7 | 0 | 5 | 7 | 0 | 5 | 12 | 12/12 (100) | 0/5 (0) | 0/7 (0) | 0/12 (0) |
| *Staphylococcus hemolyticus* | 1 | 4 | 1 | 7 | 5 | 1 | 6 | 12 | 10/12 (83.33) | 0/6 (0) | 0/5 (0) | 2/12(16.66) |
| *Staphylococcus aureus* | 2 | 19 | 1 | 4 | 19 | 1 | 4 | 24 | 24/24 (100) | 0/4 (0) | 0/19 (0) | 0/24 (0) |
| *Staphylococcus plazen* | 1 | 12 | 0 | 0 | 12 | 0 | 0 | 12 | 12/12 (100) | 0/0 (0) | 0/12 (0) | 0/12 (0) |
| *Enterococcus spp.* | 2 | 8 | 1 | 7 | 8 | 1 | 7 | 16 | 16/16 (100) | 0/7 (0) | 0/8 (0) | 0/16 (0) |
| *Streptococcus spp.* | 2 | 22 | 0 | 0 | 22 | 0 | 0 | 22 | 22/22 (100) | 0/0 (0) | 0/22 (0) | 0/22 (0) |
| *Escherichia coli* | 1 | 11 | 0 | 0 | 11 | 0 | 0 | 11 | 11/11 (100) | 0/0 (0) | 0/11 (0) | 0/11 (0) |
| *Klebsiella pneumoniae* | 1 | 9 | 1 | 1 | 8 | 2 | 1 | 11 | 10/11 (90.90) | 0/1 (0) | 0/8 (0) | 1/11 (9.09) |
| *Serratia marcescens* | 1 | 4 | 1 | 6 | 7 | 1 | 3 | 11 | 8/11 (72.72) | 0/11 (0) | 1/7 (14.28) | 2/11 (18.18) |
| *Acinetobacter baumannii* | 1 | 1 | 0 | 8 | 0 | 1 | 8 | 9 | 8/9 (88.89) | 0/8 (0) | 0/0 (0) | 1/9 (11.11) |
| *Pseudomonas aeruginosa* | 1 | 8 | 0 | 2 | 8 | 0 | 2 | 10 | 10/10 (100) | 0/2 (0) | 0/8 (0) | 0/10 (0) |
| *Proteus spp.* | 2 | 12 | 3 | 7 | 14 | 2 | 6 | 22 | 19/22 (86.36) | 0/6 (0) | 0/14 (0) | 3/22 (13.63) |
| *Salmonella spp.* | 4 | 23 | 0 | 1 | 22 | 1 | 1 | 24 | 23/24 (95.83) | 0/1 (0) | 0/22 (0) | 1/24 (4.16) |
| Total | 20 | 140 | 8 | 48 | 143 | 10 | 43 | 196 | 185/196 (94.38) | 0/43 (0) | 1/143 (0.69) | 10/196 (5.10) |


**Fig. 2** Linear regression analysis of inhibitory zones from EDD10 and St24 of QC strains

value of 0.94 which suggests a high level of correlation between the two (Fig. 1).

Likewise, 10-h growth (EDD10) comparisons yielded a similar pattern of 5.10% mE and 0.69% MEs and no VMEs. Categorical agreement with standard incubation was 94.38% (Table 3). A linear regression analysis of inhibitory zones from EDD10 and St24 revealed an $r^2$ value of 0.96 and a slope value of 0.95 indicating a high level of correlation between the two (Fig. 2).

## Clinical isolates

Overall, 348 Gram-positive isolate-drug combinations were evaluated. Out of them, 39.36% ($n = 137$) were found to be resistant by St24, while in EDD6, 41.21% ($n = 143$) were found to be resistant. Out of the 192 Gram-negative isolate-drug combinations evaluated, it was observed that 68.75% ($n = 132$) were resistant using both standard 24-h growth AST and 6-h early disc diffusion testing.

Disc diffusion testing for the 48 clinical samples yielded a high level of categorical agreement {530 of 540 measurements (98.15%)} and mE, ME, and VME of 1.29%, 1.22%, and 0% respectively (Table 4).

Comparison of various organism/antibiotic class combinations revealed that 90.74% (49/54) of the combinations showed a 100% categorical agreement. The categorical agreement for the remaining combinations (5/54) ranged from 60 to 90.91% (Table 5).

A linear regression analysis of inhibitory zones from EDD6 and St24 revealed an $r^2$ value of 0.96 and a slope value of 1.04 which suggests a high level of correlation between the two (Fig. 3).

Similar analyses were done for drugs and organisms between EDD6 and St24 (Figs. 4 and 5). The coefficient of determination $r^2$ was found to range from 0.84 in *Staphylococcus aureus* to 0.99 in *Pseudomonas aeruginosa.* The mean slope was 1.01 with a standard error of 0.02 (95% confidence interval 0.98 to 1.05). Likewise, analyses for individual drugs showed an $r^2$ range from 0.94 in Penicillins to 0.98 in Aminoglycosides. The mean slope was 1.05 with a standard error of 0.01 (95% confidence interval 1.01 to 1.09). Nevertheless, all tested organisms and drugs showed a high level of correlation between 6-h and 24-h testing.

The mean difference in inhibitory zone diameters was observed to be 1.16 mm with the zone at 6-h being smaller than the 24-h controls in most of the cases. The majority of the isolate-drug combinations (84.45%) showed a less than 3 mm difference between 6-h and 24-h inhibitory zones. The maximum variation in zone diameters was seen with the drugs Meropenem (2 mm) and Teicoplanin (1.89 mm); nevertheless, this variation was found not to alter the sensitivity pattern, as all but one of them were observed in sensitive strains only (Fig. 6).

## Discussion

The pressing priority for microbiologists around the world has been to reduce the turnaround time for antimicrobial susceptibility testing. This seems of paramount importance, especially in patients suffering from bloodstream infections

**Table 4** Comparison of disc diffusion testing from 6-h early growth method versus standard 24-h growth method of 48 Clinical isolates

| Organism isolated from clinical samples | No. of isolates | Susceptibility pattern of corresponding antibiotics evaluated | | | | | | Total | Categorical agreement (%) | Discrepancies (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6 h | | | 24 h | | | | | Very major | Major | Minor |
| | | S | I | R | S | I | R | | | | | |
| *Staphylococcus hominis* | 9 | 56 | 8 | 44 | 56 | 11 | 41 | 108 | 105/108 (97.22) | 0/41 (0) | 0/56 (0) | 3/108 (2.7) |
| *Staphylococcus epidermidis* | 11 | 72 | 10 | 50 | 75 | 7 | 50 | 132 | 129/132 (97.72) | 0/50 (0) | 0/75 (0) | 3/132 (2.27) |
| *Staphylococcus hemolyticus* | 5 | 25 | 2 | 33 | 28 | 2 | 30 | 60 | 57/60 (95) | 0/30 (0) | 3/28 (10.71) | 0/60 (0) |
| *Staphylococcus aureus* | 2 | 20 | 3 | 1 | 20 | 3 | 1 | 24 | 24/24 (100) | 0/1 (0) | 0/20 (0) | 0/24 (0) |
| *Enterococcus faecium* | 3 | 9 | 0 | 15 | 9 | 0 | 15 | 24 | 24/24 (100) | 0/15 (0) | 0/9 (0) | 0/24 (0) |
| *Escherichia coli* | 6 | 30 | 1 | 35 | 31 | 0 | 35 | 66 | 65/66 (98.48) | 0/35 (0) | 0/31 (0) | 1/66 (1.51) |
| *Klebsiella pneumoniae* | 6 | 11 | 2 | 53 | 11 | 2 | 53 | 66 | 66/66 (100) | 0/53 (0) | 0/11 (0) | 0/66 (0) |
| *Enterobacter hormaechei* | 1 | 1 | 0 | 10 | 1 | 0 | 10 | 11 | 11/11 (100) | 0/10 (0) | 0/1 (0) | 0/11 (0) |
| *Acinetobacter baumannii* | 2 | 2 | 0 | 20 | 2 | 0 | 20 | 22 | 22/22 (100) | 0/20 (0) | 0/2 (0) | 0/22 (0) |
| *Pseudomonas aeruginosa* | 3 | 11 | 2 | 14 | 11 | 2 | 14 | 27 | 27/27 (100) | 0/14 (0) | 0/11 (0) | 0/27 (0) |
| Total | 48 | 237 | 28 | 275 | 244 | 27 | 269 | 540 | 530/540 (98.15) | 0/269 (0) | 3/244 (1.22) | 7/540 (1.29) |

where timely administration of antibiotics is essential to improve outcomes of the patients [10].

Several developments in the recent past have addressed this, including staffing the microbiology laboratory around the clock, and rapid phenotypic and genotypic susceptibility testing, as well as total laboratory automation [11]. A summary of a few of the various phenotypic methods that can be implemented in microbiology laboratories to reduce the time to result in reporting antimicrobial susceptibility testing (AST) from blood cultures is represented in Fig. 7 [12–21]. While the various approaches have produced accurate and rapid susceptibility results, their utility in resource-limited settings has been put into question and the dire need of the hour is a simple, cost-effective method of AST that can deliver results faster than traditional methods and can be deployed in laboratories with limited resources.

Direct-from-blood-culture disc diffusion testing, also known as the rapid antimicrobial susceptibility test (RAST), may be one of the methods to address this issue. In this, disc diffusion testing is performed directly from positive blood culture bottles and read after 4, 6, 8, and 16–20 h. A CLSI report from 2018 showed that this method had a categorical agreement with standard disc diffusion that ranged from 86.3 to 90.4% when performed on 20 Gram-negative isolates, spiked into three commonly used blood culture systems (BacT/Alert, Bactec, and VersaTREK) [22] which prompted the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to develop method-specific breakpoints for RAST [23, 24]. This was followed by the introduction of direct susceptibility breakpoints by CLSI that can be read at 8–10 and 16–18 h [2].

This approach has proven to be an accurate method of susceptibility testing in various multilaboratory studies [25] and also provides a complete panel of AST, contrary to the MS based and calorimetric methods. Conversely, there are a few disadvantages to this method; the initial incorporation into the existing laboratory workflow might require additional efforts due to the reliance on alternative breakpoints. Furthermore, this method needs round-the-clock available trained staff; while EUCAST breakpoints are available for a wider range of organisms, CLSI provides breakpoints for only Enterobacterales and *Pseudomonas aeruginosa*. Nevertheless, RAST has emerged as a widely accepted method of rapid and accurate AST owing to its major advantage of not needing a subculture from the positive blood culture bottle; this effectively saves 24 h in providing the susceptibility report (Fig. 7).

In the pursuit of other rapid phenotypic AST methods, Fitzgerald, C. et al. introduced a novel idea of utilizing early growths at 4–6 h for antimicrobial susceptibility testing as

**Table 5** Comparison of disc diffusion testing of various organism/antibiotic class combinations from 6-h early growth method versus standard 24-h growth method of 48 clinical isolates

| Organism isolated from clinical samples | Categorical agreement % | | | | | | |
|---|---|---|---|---|---|---|---|
| | Aminoglycosides | Penicillins | Cephalosporins | Glycopeptide | Macrolides | Fluroquinolones | Carbapenems |
| *Staphylococcus hominis* | 100 | 100 | 100 | 100 | 77.77 | 77.77 | NA * |
| *Staphylococcus epidermidis* | 100 | 100 | 100 | 100 | 90.91 | 100 | NA * |
| *Staphylococcus hemolyticus* | 60 | 100 | 100 | 100 | 100 | 100 | NA * |
| *Staphylococcus aureus* | 100 | 100 | 100 | 100 | 100 | 100 | NA * |
| *Enterococcus faecium* | 100 (high level Aminoglycoside) | 100 | NA * | 100 | 100 | 100 | NA * |
| *Escherichia coli* | 100 | 100 | 100 | NA * | NA * | 83.33 | 100 |
| *Klebsiella pneumoniae* | 100 | 100 | 100 | NA * | NA * | 100 | 100 |
| *Enterobacter hormaechei* | 100 | 100 | 100 | NA * | NA * | 100 | 100 |
| *Acinetobacter baumannii* | 100 | 100 | 100 | NA * | NA * | 100 | 100 |
| *Pseudomonas aeruginosa* | 100 | 100 | 100 | NA * | NA * | 100 | 100 |

*The group of antibiotics are not recommended for testing in the respective organisms

opposed to the standard 24-h growth as recommended by CLSI [26]. Comparative analysis of the rapid and standard AST results showed an overall interpretive category error rate of 7.7% (6.7% minor errors, 0.6% major errors, and 0.4% very major errors). Webber, DM. et al. further expanded on the idea and compared disc diffusion testing done on 6- and 10-h growth with 24-h growth [7]. They observed that the disc diffusion performed on 6- and 10-h growth (EDD6 and EDD10 respectively) has a good categorical and quantitative agreement with standard disc testing (St24) when applied to 21 clinical and QC isolates as well as 100 clinical isolates.

In our study evaluating this EDD testing, we observed a similarly high level of correlation between EDD6 and St24 as well as between EDD10 and St24. For the 20 quality control strains, we noticed that the categorical agreement of EDD6 with standard incubation was 94.38% with 5.10% mE and 0.69% MEs and no VMEs. Likewise, 10-h growth comparisons yielded a similar pattern of 5.10% mE and 0.69% MEs and no VMEs with 94.38% categorical agreement with standard incubation. Inhibitory zone size from 6-h ($r^2 = 0.96$) and 10-h ($r^2 = 0.96$) growth correlated well with results from standard conditions.

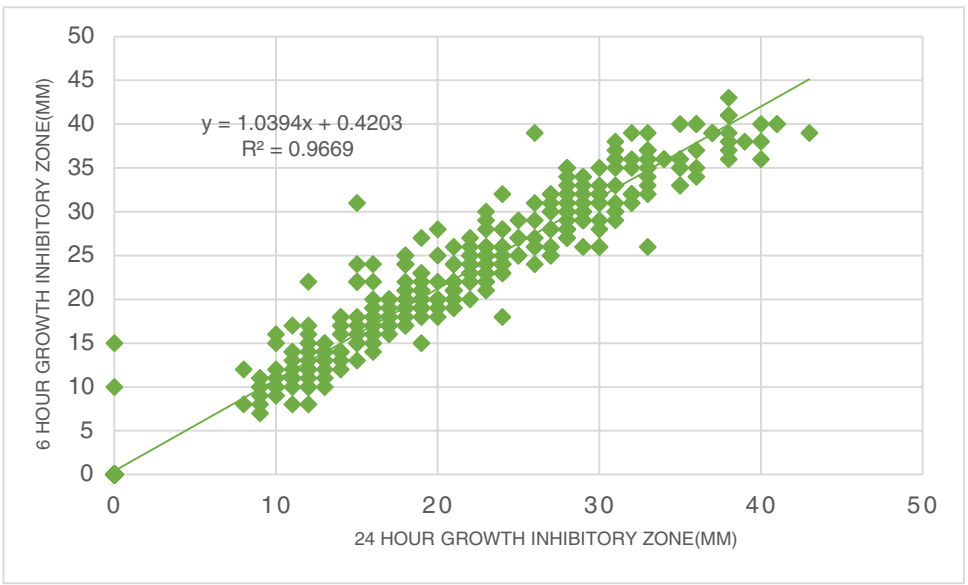



**Fig. 3** Linear regression analysis of inhibitory zones from EDD6 and St24 of all clinical isolates

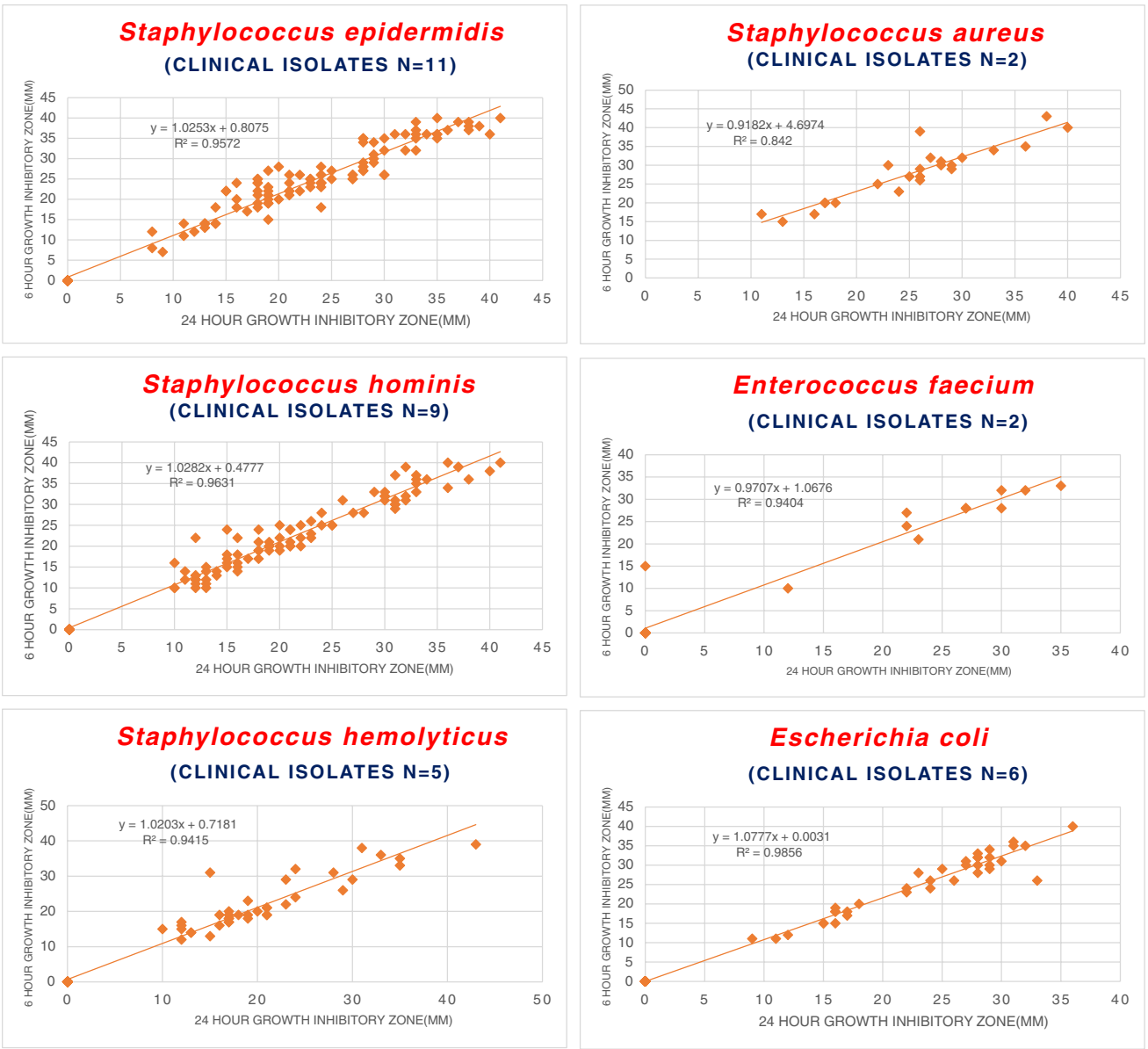


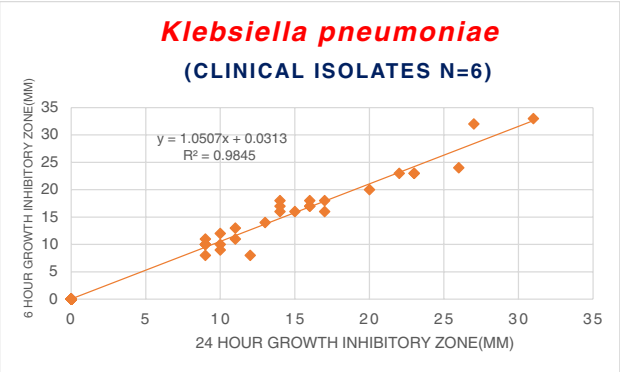

**Fig. 4** Linear regression analysis of inhibitory zones from EDD6 and St24 of individual organisms

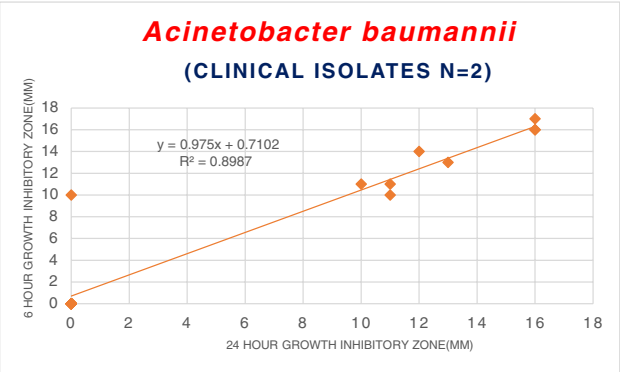
Early disc diffusion testing (EDD6) for the additional 48 clinical samples yielded a good categorical agreement (98.15%) with St24 and mE, ME, and VME of 1.29%, 1.22%, and 0% respectively with an $r^2$ value of 0.96 from the inhibitory zone sizes which suggests a high level of correlation between the two.

These values of categorical agreement were well above the threshold (90% or more) provided by the FDA Class II Special Controls Guidance for AST systems. Likewise, AST results from EDD6 and EDD10 met the FDA-recommended threshold of ME of 3% or less and VME upper and lower 95% CIs less than or equal to 7.5 and 1.5%, respectively. We also noticed that early disc diffusion testing performed well across a range of micro-organisms, antibiotic classes, and resistance patterns.

Our results were highly concordant with the previous study from 100 clinical isolates where they noticed a 96.5% categorical agreement between EDD6 and St24 and no VME, no ME, and 3.5% mE [7].

Thus, these results demonstrate that early disc diffusion testing is an accurate method for antimicrobial susceptibility testing with reduced turnaround time. It also has the added



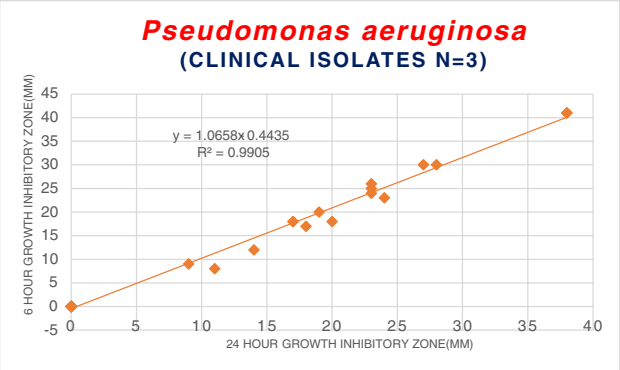




**Fig. 4** (continued)

benefit of only changing the length of time for subculture growth (18- to 24-h incubation time prior to disc diffusion setup to 6- to 10- h incubation time prior to disc diffusion setup) and uses already established antimicrobial breakpoints, which are regularly updated and are broadly available through package inserts, CLSI guidelines, and EUCAST publications. As a result, it could be incorporated into existing laboratory workflows with utmost ease.

However, there are some major limitations to our current study. Firstly, the present study involved only 20 quality control strains and 48 clinical isolates. While the results from their testing were concordant with the previous study with 121 isolates, the small sample size of both studies restricts the knowledge of the possible limitations that might be encountered in everyday practice if early disc diffusion testing is to be implemented as a routine system in laboratories. Secondly, this EDD testing can be used only for sterile samples as it might not be possible to discern a mixed culture from a 6-h growth. Thirdly, a practical limitation that was encountered during the study was the lack of adequate growth at 6 h from samples other than blood. Thus, further studies with greater sample sizes are required to estimate the utility of EDD testing in other sterile as well as non-sterile samples.

In conclusion, disc diffusion testing by the Kirby-Bauer technique has been the most used method for determining antimicrobial susceptibility (AST) in microbiological laboratories owing to its simplicity, cost-effectiveness, and reliability [27]. EDD testing is a simple and accurate method that can reduce turnaround time while at the same time, retaining the beneficial attributes of this method. This approach shows enough promise for it to be considered by laboratories and may prove critical for timely patient management.

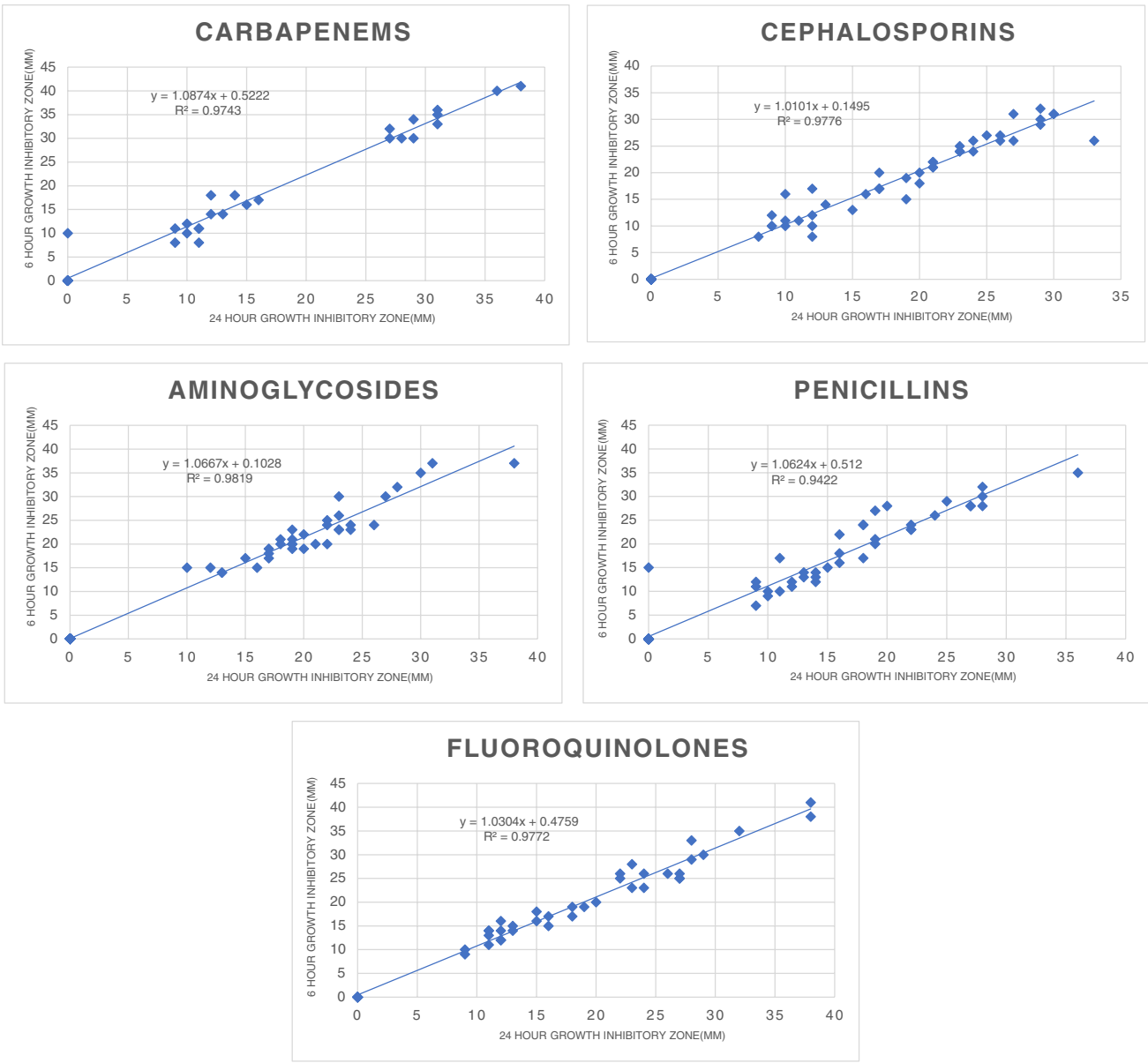



**Fig. 5** Linear regression analysis of inhibitory zones from EDD6 and St24 of individual drugs

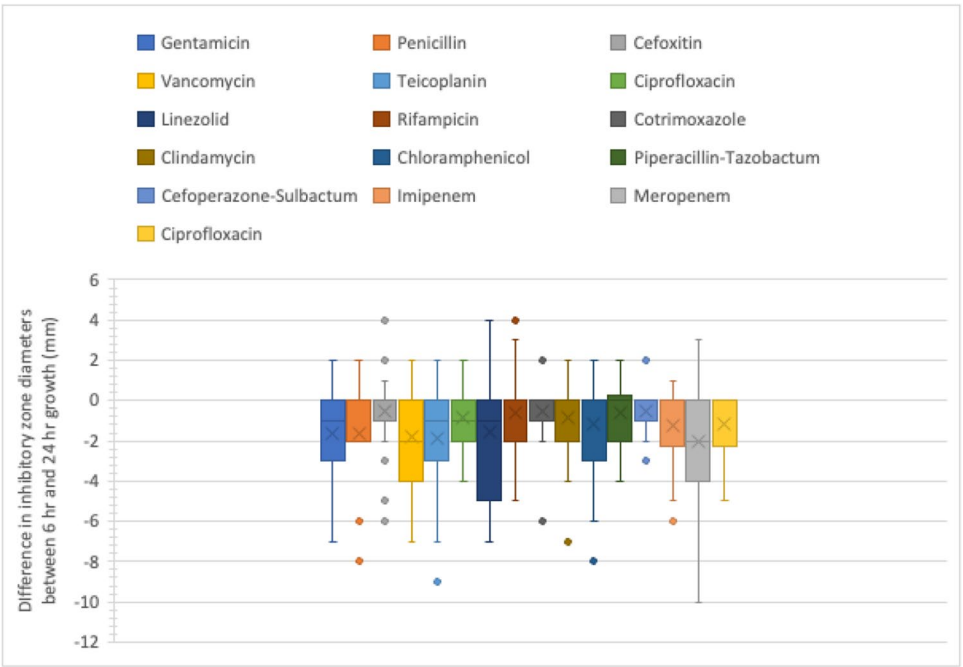



**Fig. 6** Difference in inhibitory zone diameters between the EDD6 and St24. Central lines denote median differences, crosses denote mean differences, boxes surround the interquartile range (IQR), whiskers extend to the farthest nonoutliers, and data points represent outliers that are 1.5 times the IQR

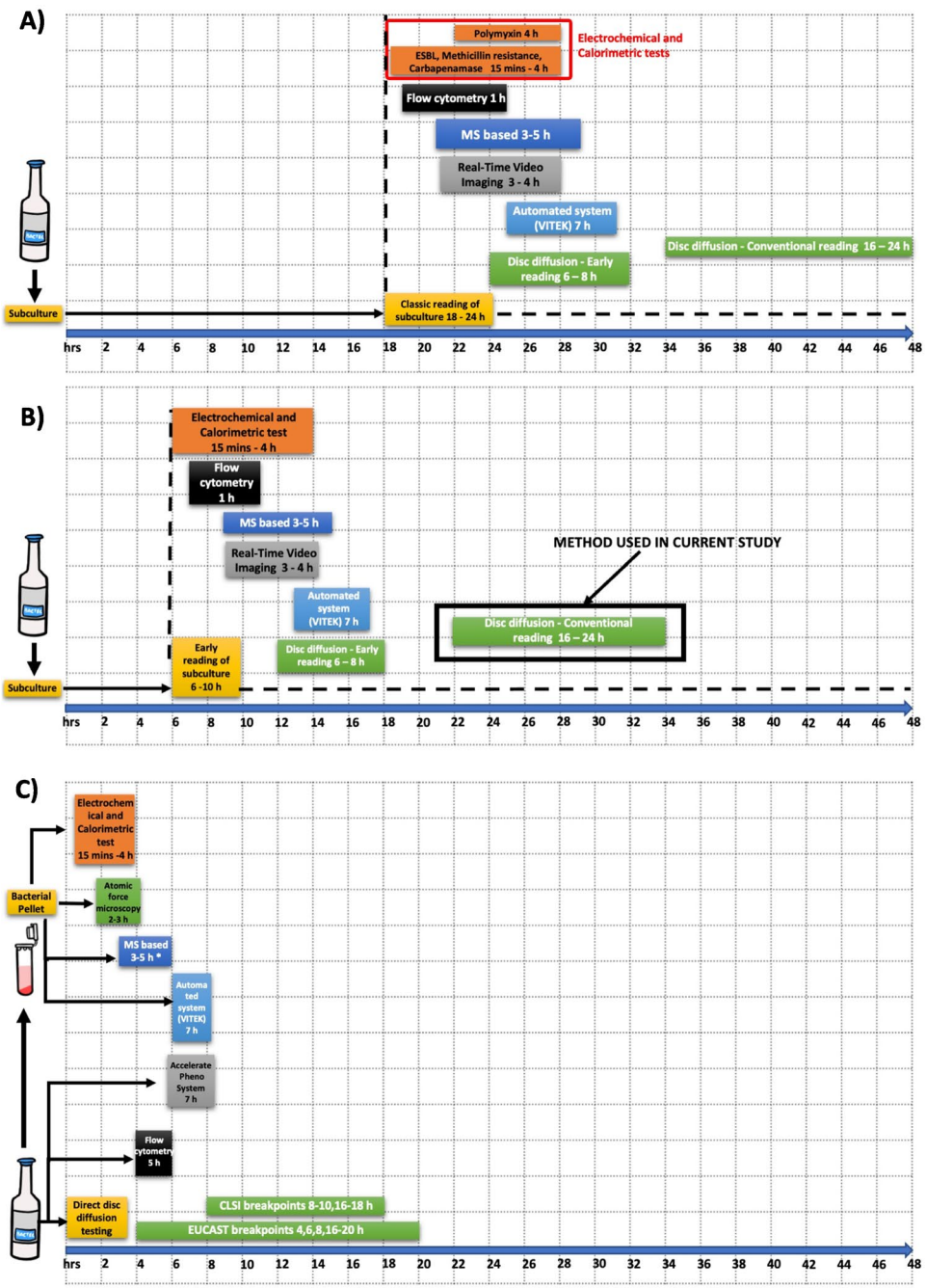



**Fig. 7** Phenotypic methods that are approved and being evaluated for reporting antimicrobial susceptibility testing (AST) from blood cultures, plotted against a timeline in which their results can be made available: **A** Methods that require a subculture—reading of the subculture taken at 18–24 h. **B** Methods that require a subculture—reading of the subculture taken at 6–10 h. **C** Methods not needing a subculture. The individual boxes extend over the time in which the results may be available; the left margin of the box represents the earliest possible time at which the phenotypic method can provide a result; the right margin of the box corresponds to the maximum time needed by the respective tests. The time written within the boxes indicates the time needed for the individual tests or the time periods at which breakpoints are available. The method used in the current study is highlighted. *Also includes MALDI-TOF (mass spectrometry–based direct-on-target microdroplet growth assay)

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**Author contribution** All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Dr. Kavi Priya Appasami and Dr. Jaya Biswas. The first draft of the manuscript was written by Dr. Kavi Priya Appasami and Dr. Jaya Biswas and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data availability** All data generated or analyzed during this study are included in this published article.

**Code availability** Not applicable.

## Declarations

**Ethics approval** This study involved the use of quality control strains and established clinical isolates. The AIIMS Research Ethics Committee has confirmed that no ethical approval is required.

**Consent to participate** Not applicable as this study involved the use of quality control strains and established clinical isolates.

**Consent for publication** Not applicable as no confidential patient information has been included in the manuscript.

**Conflict of interest** The authors declare no competing interests.

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