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## Original Research Article

## Agreement between AST from minute colony (8-10h growth) and mature colony (16-18h/overnight incubation)

Sonali Padhy<sup>1</sup>, Ketan Priyadarshi<sup>2</sup>, Sarumathi Dhandapani<sup>3</sup>, Stessy Ann Punnen<sup>1</sup>, Apurba S Sastry<sup>1\*</sup><sup>1</sup>Dept. of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India<sup>2</sup>Dept. of Microbiology, All India Institute of Medical Sciences, Patna, Bihar, India<sup>3</sup>Dept. of Microbiology, Sri Devaraj Urs Medical College, Kolar, Karnataka, India

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## ABSTRACT

**Background:** Sepsis is a medical emergency where a successful patient outcome depends on early and appropriate antibiotic treatment. This study was conducted to evaluate agreement between antimicrobial susceptibility testing (AST) from minute colonies that can reduce the AST-TAT by as much as 8-10h as compared to the CLSI recommended protocol of performing AST from an overnight (16-18h) growth of mature colony.

**Materials and Methods:** In the present study, mDD results from minute colony (8-10h growth) were compared to the rDD results mature colony (16-18h/overnight incubation) CA and various types of errors were evaluated.

**Results:** 237 pathogens and 1597 organism-antibiotic combinations were evaluated, there was a CA of 93.30% which was extremely satisfactory and categorical disagreement was found only in 4.56% of organism-antibiotic combinations, which were mainly mE (4.56%) with nil VME (0%) and ME (0%).

**Conclusion:** We have found that minute colony (8-10h) AST is in agreement with reference mature colony (16-18h) AST, shortening TAT by (8-10h) earlier than the conventional reference method which is very helpful in treatment of sepsis patients.

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## 1. Introduction

Sepsis is a medical emergency currently defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection.”<sup>1</sup> It is a seven global health concern, with an estimated 11 million fatalities linked to it annually out of 48.9 million cases contributing to 20% of all deaths globally. This is more than 20 deaths every minute.<sup>2</sup> Although sepsis is a global illness, its prevalence is highest in low- and middle-income nations due to a lack of treatment resources. Hospitalized individuals with sepsis have a higher death rate, which is thought to be between

20 and 30 percent.<sup>3</sup> Treatment for sepsis can be lifesaving. High-quality clinical care is required for this, particularly in primary care clinics and hospitals with operation, critical, and emergency rooms. Furthermore, a successful patient outcome depends on early and appropriate antibiotic treatment.

A clinical microbiologist’s primary goal should be to provide patients with the best treatment possible by rapid turnaround time of blood culture reports.<sup>4</sup> Employing automated blood culture systems instead of conventional culture, sending at least two sets of culture before giving the first dose of antibiotics, collecting the recommended volume of blood aseptically for blood culture, and promptly

\* Corresponding author.

E-mail address: [drapurbasastry@gmail.com](mailto:drapurbasastry@gmail.com) (A. S. Sastry).

reporting the Gram stain report of positive blood culture bottles are a few strategies to shorten the turnaround time for blood culture reports.<sup>5</sup> Performing antimicrobial susceptibility testing (AST) from minute colonies can reduce the AST-TAT by as much as 8-10h as compared to the CLSI recommended protocol of performing AST from an overnight (16-18h) growth of mature colony. However, agreement of minute-colony AST with mature colony-AST has never been evaluated so far.

Therefore, we conducted this study to determine the agreement of minute-colony AST with mature colony-AST has never been evaluated so far.

## 2. Materials and Methods

Minute colony Antimicrobial susceptibility test (mDD) was performed according to CLSI guideline by Kirby -Bauer's disk diffusion test.<sup>6</sup> After 8-10 hours of colony growth about 3-5 fresh colonies from a non-selective medium like blood agar are touched to make a direct suspension in sterile normal saline. Turbidity is adjusted to 0.5 McFarland standard which is then lawn cultured onto Mueller Hinton Agar (MHA) by rotating the plate at an angle of 60° for 3 times within 15 minutes. Then the agar plates were dried for 2-5 minutes, and the antibiotic disks of appropriate panel were as per the organism identification were applied on to the MHA surface, using sterile forceps. The same procedure was followed for reference mature colony disk diffusion test (rDD) after 16-18 hours/overnight incubation of colony growth. The zone diameters were measured using a Vernier Caliper and interpreted as per CLSI breakpoints, after 16-18 hours of incubation according Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>7</sup> Along with this a control plate of ATCC Escherichia coli 25922 and ATCC *Staphylococcus aureus* 25923 were also subjected to AST to ensure the quality of the antimicrobial disks used.

### 2.1. Study design and analysis

mDD results were compared to the rDD results from the subculture plates. Categorical agreement (CA) was evaluated, using breakpoints mentioned in CLSI M100 ED33-2023, following the exclusion of any antibiotics to which the pathogen is known to have intrinsic resistance.

A panel of eight antibiotics was tested on Gram-negative bacteria such as Amikacin 30µg (AK), Ciprofloxacin 5µg (CIP), Ceftriaxone 30µg (CTR), Ceftazidime 30 µg (CAZ), Cefoperazone Sulbactam 75/30µg (CFS), Piperacillin-tazobactam 100/10µg (PIT), and Meropenem 10 µg(MERO), Tigecycline 15µg (TIGE), Minocycline 30µg (MINO). If the pathogen was identified to belong to the *Acinetobacter* species or *Enterobacteriaceae* family, then all of these were included for study with the exception of CAZ for members of *Enterobacteriaceae* family. For *Pseudomonas* species CTR was excluded and Aztreonam

(AZTR) was included in the analysis. For *Acinetobacter* species CTR and CIP were excluded from analysis. For *Providencia stuartii* TIGE was excluded from analysis. All other non-fermenters were not included in the study's analysis since the antibiotic panel employed in it was different. The antibiotic panel used for *Stenotrophomonas maltophilia* included Levofloxacin, Co-trimoxazole 1.25/23.75µg and Minocycline and for *Salmonella enterica* subsp. enterica Chloramphenicol, Ceftriaxone 30µg (CTR), Ampicillin 10µg (AMP), Co-trimoxazole 1.25/23.75µg (COT) and Ciprofloxacin 5µg (CIP).

The antibiotic panel used for *Staphylococcus aureus* comprised of 7 disks such as cefoxitin 30 µg (OX), erythromycin 15µg (ER), co-trimoxazole 1.25/23.75µg (COT), clindamycin 2µg (CN), tetracycline 30µg (TE), levofloxacin 5µg (LE) and linezolid 30µg (LZ) and for Enterococcus species ampicillin 10µg (AMP), high level gentamicin 120µg (HLG), tetracycline 30µg (TE), and linezolid 30µg (LZ), vancomycin 30µg (VAN), minocycline 30µg (MINO).

The minute colony disk Diffusion Test results were compared with the reports of reference mature colony disk diffusion test and the closeness of agreement was analysed, thereby establishing the efficiency of minute colony Disk Diffusion Test from positively flagged blood culture. Comparisons between mDD and rDD's performance were made using categorical disagreement and CA. The categorical disagreement was further characterized into minor error (mE), major error (ME), and very ME (VME) as depicted in (Table 1). Microsoft Excel document contained all of the collected data. IBM SPSS Statistics for Windows, Version 19.0, was used to analyse the data.

**Table 1:** Terminologies used for comparison of performance of minute colony disk diffusion test with reference disk diffusion tests

CA	Categorical disagreement						
	mE		ME		VME		
r DD	S	I	R	R or S	I	S	R
m DD	S	I	R	I	R or S	R	S

R=Resistant, S=Sensitive, I=Intermediate, mE=Minor error, ME=Major error, VME=Very ME, rDD=Reference disk diffusion, mDD=Minute Colony disk diffusion, CA=Categorical agreement

## 3. Results

During the study period, 237 freshly flagged positive blood cultures from patients suspected of having bloodstream infections (BSIs) were identified by the routine (reference) laboratory method. Antimicrobial susceptibility test made from the minute colony (minute colony-AST or mDD) results were compared to the results of reference mature colony disk diffusion test (rDD) performed from

the subculture plates. Categorical agreement (CA) was evaluated, using breakpoints mentioned in CLSI M100 ED33-2023, following the exclusion of any antimicrobials to which the pathogen is known to possess intrinsic resistance.

[Table 2] shows the distribution of bacteria isolated from the positive blood cultures for which both mDD and rDD tests were performed. GNB accounts for 53.16% (126) of total isolates; and Gram-positive cocci 23.63% (56). Among Gram-negative bacilli, *Escherichia coli* was the most common isolate (24.47%), followed by *Klebsiella pneumoniae* (19.41%), *Acinetobacter baumannii* (12.24%), *Pseudomonas aeruginosa* (9.28%), *Enterobacter cloacae* (4.64%), *Salmonella enterica* subsp *enterica* (4.64%) and *Stenotrophomonas maltophilia* (1.69%). *Staphylococcus aureus* (15.61%) was the most prevalent isolate among Gram-positive cocci followed by *Enterococcus faecium* (7.17%) and *Enterococcus faecalis* (0.84%). They were subjected to further analysis of CA between mDD and rDD.

As shown in Table 3, overall, mDD performed excellent with a CA of 93.30% with rDD; mE of 4.56% and ME, VME both 0% fulfilling the performance criteria, is considered acceptable (ME  $\leq$ 3%; VME  $\leq$ 3%). mE were highest in *Klebsiella pneumoniae* (8.42%).

Upon analyzing the discrepancy in the quantity of antibiotics used in each isolate, it was found that the majority of the organisms exhibited disagreements at <2 antibiotics per isolate (20.20%); significant disagreement at  $\geq$ 2 antibiotics was observed with *Klebsiella pneumoniae* (13.04%).

Among *Escherichia coli* (Table 4), CA was >90% for all the antibiotics in the panel AND 100% for TIGE. VME and ME both were 0%. However higher mE was observed for CF (8.62%) with kappa value of 0.798, CFS (6.90%) with kappa value of 0.830. There was a CA of > 95% in *Klebsiella pneumoniae* for all the antibiotics tested except for CF (93.48%). mE was high for MINO (23.91%) with kappa value of 0.537. VME and ME both were 0%. For *Enterobacter cloacae*, the CA was 100% for all the antibiotics in the panel except for AK and PIT both (90.91%). And mE was 9.09% for both AK and PIT. However due to lesser number of isolates of *Enterobacter cloacae* statistical significance could not be determined and so kappa value is denoted as NA (not available) in the (Table 4).

Among *Pseudomonas aeruginosa* (Table 5), CA was 100% for all the antibiotics in the panel except CAZ and PIT (95.45%) and CFS (90.09%). VME and ME both were 0%. However higher mE was observed for CFS (9.09%).

For *Acinetobacter baumannii* (Table 6), the CA was 100% for CFS, MINO with >95% for all other antibiotics in the panel except for TIGE (89.66%). mE was high for TIGE (10.34%) with kappa value of 0.284.

Among other GNB like *Salmonella enterica* subsp. *enterica* and *Stenotrophomonas maltophilia* CA was 100% for all antibiotics tested although statistical significance could not be determined due to lesser number of isolates.

Among Gram-positive cocci, *Enterococcus faecalis* had CA of 100%. *Staphylococcus aureus* (Table 7) had CA of 100% for all antibiotics tested except TE (97.29%) and both ER, LEVO (89.19%). Higher mE of 10.81% was observed for both ER and LEVO. *Enterococcus faecium* (Table 8) had CA of 100% for all the antibiotics tested except HLG and MINO (94.12%).

Statistical significance was determined with p value < 0.001 for *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus* for all the antibiotics tested and not for other organisms as the number of isolates were insufficient for statistical analysis. The data was analysed using SPSS software version 19.0.

#### 4. Discussion

Developing novel diagnostic methods for antimicrobial susceptibility is one of the goals of the Global Action Plan on Antimicrobial Resistance of the World Health Organization, which aims to decrease bacterial resistance.<sup>8</sup> According to a research by Baltas et.al. in 2020, patients who received efficient antibiotic therapy early had a better chance of surviving than those who did not, with the latter group ultimately facing a larger risk of mortality.<sup>9</sup> Additionally, this study demonstrated that antimicrobial resistance was frequently the cause of treatment failure, highlighting the necessity of faster ASTs. Published research to date has demonstrated the need of treating patients with BSI with antimicrobials as soon as possible to lower death rates and hospital expenses associated with these infections.<sup>10</sup> Therefore, it is crucial to provide rapid diagnostic techniques to identify the microorganisms causing BSI as well as early implementation of targeted antimicrobial therapy which is considered as one of the crucial stewardship intervention. Our study was done to determine the agreement of minute-colony AST with mature colony-AST which are the first of its kind research work, which has never been studied to the best of our knowledge. This would reduce the AST-TAT by 8-10h.

In the present study, we evaluated 237 pathogens and 1597 organism-antibiotic combinations. Overall, there was a CA of 93.30% which was incredibly satisfactory. [Table 3] The categorical disagreement was found only in 4.56% of organism-antibiotic combinations, which were mainly mE (4.56%) with nil VME (0%) and ME (0%). (Table 3) Percentages of errors (mE, ME, and VME) were overall much lower than the acceptable performance criteria of International Standard ISO 20776-2 (ME  $\leq$ 3%; VME  $\leq$ 3%). There are paucity of recent literature comparing mDD with rDD, as most of the studies focused on the comparison of mDD with AST from colonies by automated

**Table 2:** Distribution of bacteria isolated from positive blood cultures for which both mDD and rDD were performed

Organisms	Number of isolates tested, n (%)
GNB	126(53.16%)
<i>Escherichia coli</i> ( <i>E. coli</i> )	58(24.47%)
<i>Klebsiella pneumoniae</i> ( <i>K. pneumoniae</i> )	46(19.41%)
<i>Enterobacter cloacae</i> ( <i>Ent.cloacae</i> )	11(4.64%)
<i>Salmonella enterica</i> subsp. <i>enterica</i>	11(4.64%)
<i>Pseudomonas aeruginosa</i>	22(9.28%)
<i>Acinetobacter baumannii</i>	29(12.24%)
<i>Stenotrophomonas maltophilia</i>	4(1.69%)
GPC	56(23.63%)
<i>Staphylococcus aureus</i>	37(15.61%)
<i>Enterococcus faecium</i>	17(7.17%)
<i>Enterococcus faecalis</i>	2(0.84%)
Total	237

**Table 3:** Performance of mDD compared to rDD for various groups of organisms

Organisms and antibiotics tested (n×Ab=N)	CA, (n%)	Categorical disagreement, n (%)					
		Among isolate-antibiotic combinations tested				Among the isolates tested	
		Minor error	Major error	Very Major error	Total	Disagreement at <2 antibiotics	Disagreement at ≥2 antibiotics
<i>Escherichia coli</i> (58x7=406)	95.3% (387/406)	4.91% (19/406)	0%	0%	4.91% (19/406)	22.41% (13/58)	5.17% (3/58)
<i>Klebsiella pneumoniae</i> (46x7= 322)	92.24% (297/322)	8.42% (25/322)	0%	0%	8.42% (25/322)	28.26% (13/46)	13.04% (6/46)
<i>Enterobacter cloacae</i> (11x7=77)	97.40% (75/77)	2.67% (2/77)	0%	0%	2.67% (2/77)	18.18% (2/11)	0%
<i>Salmonella enterica</i> subsp. <i>enterica</i> (11 x 5=55)	100% (55/55)	0%	0%	0%	0%	0%	0%
<i>Pseudomonas aeruginosa</i> (22 x7=154 )	97.40% (150/154)	2.67% (4/154)	0%	0%	2.67% (4/154)	18.18% (4/22)	0%
<i>Acinetobacter baumannii</i> (29 x 7=203)	96.55% (196/203)	3.57% (7/203)	0%	0%	3.57% (7/203)	17.24% (5/29)	3.45% (1/29)
<i>Stenotrophomonas maltophilia</i> (4 x3=12)	100% (12/12)	0%	0%	0%	0%	0%	0%
<i>Staphylococcus aureus</i> (37 x 7=259)	96.52% (250/259)	3.6% (9/259)	0%	0%	3.6% (9/259)	24.32% (9/37)	0%
<i>Enterococcus faecium</i> (17 x5=85 )	80% (68/85)	2.94% (2/85)	0%	0%	2.94% (2/85)	11.76% (2/17)	0%
<i>Enterococcus faecalis</i> (2x5=10)	100% (10/10)	0%	0%	0%	0%	0%	0%
Overall (1597)	93.30% (1490/1597)	4.56% (68/1597)	0%	0%	4.56% (68/1597)	20.20% (48/237)	2.53% (6/237)

**Table 4:** Performance of mDD compared to rDD test for Enterobacterales

Antibiotics	Organism	CA, n(%)	Categorical disagreement, n (%)				Kappavalue (95% CI)
			Minor	Major	Very Major	Total	
Amikacin (AK)	<i>E. coli</i> (n=58)	55 (94.83)	3(5.17)	0	0	3(5.17)	0.812
	<i>K. pneumoniae</i> (n=46)	45(97.82)	1(2.17)	0	0	1(2.17)	0.957
	Ent. Cloacae (n=11)	10(90.91)	1(9.09)	0	0	1(9.09)	NA
Ciprofloxacin (CF)	<i>E. coli</i> (n=58)	53(91.38)	5(8.62)	0	0	5(8.62)	0.798
	<i>K. pneumoniae</i> (n=46)	43(93.48)	3(6.52)	0	0	3(6.52)	0.886
	Ent. Cloacae (n=11)	11(100)	0	0	0	0	NA
Cefoperazone-Sulbactam (CFS)	<i>E. coli</i> (n=58)	54(93.10)	4(6.90)	0	0	4(6.90)	0.830
	<i>K. pneumoniae</i> (n=46)	46(100)	0	0	0	0	1
	Ent. Cloacae (n=11)	11(100)	0	0	0	0	NA
Piperacillin-tazobactam (PIT)	<i>E. coli</i> (n=58)	55(94.83)	3(5.17)	0	0	3(5.17)	0.812
	<i>K. pneumoniae</i> (n=46)	44(95.65)	2(4.35)	0	0	2(4.35)	0.865
	Ent. Cloacae (n=11)	10(90.91)	1(9.09)	0	0	1(9.09)	NA
Meropenem (MERO)	<i>E. coli</i> (n=58)	57(98.28)	1(1.72)	0	0	1(1.72)	0.956
	<i>K. pneumoniae</i> (n=46)	46(100)	0	0	0	0	1
	Ent. Cloacae (n=11)	11(100)	0	0	0	0	NA
Tigecycline (TIGE)	<i>E. coli</i> (n=58)	58(100)	0	0	0	0	1
	<i>K. pneumoniae</i> (n=46)	38(82.60)	8(17.39)	0	0	8(17.39)	0.537
	Ent. Cloacae (n=11)	11(100)	0	0	0	0	NA
Minocycline (MINO)	<i>E. coli</i> (n=58)	55(94.83)	3(5.17)	0	0	3(5.17)	0.820
	<i>K. pneumoniae</i> (n=46)	35(76.10)	11(23.91)	0	0	11(23.91)	0.571
	Ent. Cloacae (n=11)	11(100)	0	0	0	0	NA

**Table 5:** Performance of mDD compared to rDD test for *Pseudomonas aeruginosa*

<i>Pseudomonas aeruginosa</i> (n=22)	CA,n (%)	Categorical disagreement, n (%)				Total
		Minor	Major	Very Major		
Ceftazidime (CAZ)	21(95.45)	1(4.55)	0	0	1(4.55)	
Ciprofloxacin(CF)	22(100)	0	0	0	0	
Cefoperazone-Sulbactam (CFS)	20(90.09)	2(9.09)	0	0	2(9.09)	
Piperacillin-tazobactam (PIT)	21(95.45)	1(4.55)	0	0	1(4.55)	
Amikacin (AK)	22(100)	0	0	0	0	
Meropenem (MERO)	22(100)	0	0	0	0	
Aztreonam (AZTR)	22(100)	0	0	0	0	

**Table 6:** Performance of mDD compared to rDD test for *Acinetobacter baumannii*

<i>Acinetobacter baumannii</i> (n=29)	CA, n(%)	Categorical disagreement, n (%)				Kappa value (95% CI)
		Minor	Major	Very Major	Total	
Ceftazidime (CAZ)	28(96.55)	1(3.45)	0	0	1(3.45)	0.858
Piperacillin-tazobactam (PIT)	28(96.55)	1(3.45)	0	0	1(3.45)	0.772
Cefoperazone-Sulbactam (CFS)	29(100)	0	0	0	0	1
Amikacin(AK)	28(96.55)	1(3.45)	0	0	1(3.45)	0.871
Meropenem(MERO)	28(96.55)	1(3.45)	0	0	1(3.45)	0.884
Tigecycline (TIGE)	26(89.66)	3(10.34)	0	0	3(10.34)	0.284
Minocycline(MINO)	29(100)	0	0	0	0	1

**Table 7:** Performance of mDD compared to rDD test for *Staphylococcus aureus*

<i>Staphylococcus aureus</i> (n=37)	CA,n (%)	Categorical disagreement, n (%)				Kappa value (95% CI)
		Minor	Major	Very Major	Total	
Cefoxitin (OX)	37(100)	0	0	0	0	1
Erythromycin(ER)	33(89.19)	4(10.81)	0	0	4(10.81)	0.806
Clindamycin(CN)	37(100)	0	0	0	0	1
Co-trimoxazole (COT)	37(100)	0	0	0	0	1
Tetracycline (TE)	36(97.29)	1(2.70)	0	0	1(2.70)	0.905
Levofloxacin(LEVO)	33(89.19)	4(10.81)	0	0	4(10.81)	0.806
Linezolid(LZ)	37(100)	0	0	0	0	1

**Table 8:** Performance of mDD compared to rDD test for *Enterococcus faecium*

<i>Enterococcus faecium</i> (n=17)	CA,n (%)	Categorical disagreement, n (%)			
		Minor	Major	Very Major	Total
Ampicillin (AMP)	17(100)	0	0	0	0
High level gentamicin (HLG)	16(94.12)	1(5.88)	0	0	1(5.88)
Tetracycline (TE)	17(100)	0	0	0	0
Linezolid (LZ)	17(100)	0	0	0	0
Vancomycin (VAN)	17(100)	0	0	0	0
Minocycline (MINO)	16(94.12)	1(5.88)	0	0	1(5.88)

systems (Vitek2, Phoenix, or Micro scan).<sup>11–15</sup>

We conducted a unique analysis of the categorical disagreement at  $\leq 2$  and  $\geq 2$  antibiotics. We observed that the categorical disagreement at  $< 2$  and  $\geq 2$  antibiotics were 20.20% and 2.53%, respectively (Table 3), by which we can derive that even if there is categorical disagreement, majority will have discrepancies with  $< 2$  antibiotics.

Among *Escherichia coli* (Table 4), CA was  $> 90\%$  for all the antibiotics in the panel AND 100% for TIGE. There was a CA of  $> 95\%$  in *Klebsiella pneumoniae* for all the antibiotics tested except for CF (93.48%). (Table 4) For *Enterobacter cloacae* (Table 4), the CA was 100% for all the antibiotics in the panel except for AK and PIT both (90.91%). Among *Pseudomonas aeruginosa* (Table 5), CA was 100% for all the antibiotics in the panel except CAZ and PIT (95.45%) and CFS (90.09%). For *Acinetobacter baumannii* (Table 6), the CA was 100% for CFS, MINO with  $> 95\%$  for all other antibiotics in the panel except for TIGE (89.66%). *Staphylococcus aureus* (Table 7) had CA of 100% for all antibiotics tested except TE (97.29%) and both ER, LEVO (89.19%). *Enterococcus faecium* (Table 8) had CA of 100% for all the antibiotics tested except HLG and MINO (94.12%). For all the organisms tested mE was less than 10% except for *Acinetobacter baumannii* for TIGE (10.34%), *Staphylococcus aureus* for both ER and LEVO (10.81%) and VME and ME both were 0% which is the most important conclusion of our study. Another significant finding in our study was among other GNB like *Salmonella enterica* subsp. *enterica* and *Stenotrophomonas maltophilia* and GPC like *Enterococcus faecalis* CA was 100% for all antibiotics tested. To the best of our knowledge, there is no additional literature available to compare the outcomes.

## 5. Conclusion

In critically ill patients, BSI presents a high risk of morbidity and death. Early intervention with the right antimicrobial drugs combined with supportive care leads to better patient outcomes.<sup>8</sup> Nevertheless, conventional culture methods are laborious, and results are frequently not available for 48 hours following the patient’s disease presentation. Early detection and tailored therapeutic intervention for sepsis patients may be possible with prompt identification of bacteria grown in blood cultures along with AST.

## 6. Ethical Approval

This study was done after taking Ethics committee approval from the Institutional Ethics Committee (Human Studies), JIPMER, Puducherry. REG. NO. EC/NEW/INST/2020/331.

## 7. Source of Funding

None.

## 8. Conflicts of Interest


None.

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### Author biography

**Sonali Padhy**, Junior Resident  <https://orcid.org/0009-0009-2499-9932>

**Ketan Priyadarshi**, Assistant Professor

**Sarumathi Dhandapani**, Assistant Professor

**Stessy Ann Punnen**, Project Officer

**Apurba S Sastry**, Additional Professor

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