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Original article

Evaluating EUCAST rapid antimicrobial susceptibility test from positive neonatal blood culture directly against the golden standard disc diffusion method in a tertiary hospital

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ABSTRACT

Background: Neonatal sepsis is one of the leading causes of neonatal morbidity and mortality. Fast and accurate antibiotic susceptibility testing is very important for early diagnosis and treatment with appropriate antimicrobial agents. The European Committee of Antimicrobial Susceptibility Testing provides rapid antimicrobial susceptibility testing (RAST), based on the disk diffusion method, after 4, 6, and 8 hours of incubation. This susceptibility testing directly from positive blood culture bottles was prospectively evaluated against the golden standard CLSI disc-diffusion method. **Methods**: A cross-sectional diagnostic study was conducted at the NICU in Ain Shams University Hospitals from October 2022 until September 2023. Overall, 115 positive blood cultures for Gram positive and negative bacteria were isolated. Antibiotic discs used were Imipenem, Ceftazidime, Trimethoprim-sulphamethoxazole, Piperacillin/tazobactam, Cefotaxime, Tobramycin, Gentamycin, Cefoxitin, and Clindamycin. The results were assessed using the RAST breakpoints after 4,6 and 8 hours against disc diffusion method of CLSI. Categorical agreement of RAST with disc diffusion method for these antibiotics was evaluated. **Results**: Matching the results of the susceptibility of all organisms to different antibiotics between the conventional golden standard method and EUCAST RAST method, Categorical agreement was 76.8%, 92%, and 97.2% at 4, 6, and 8 hours respectively. **Conclusion**: RAST is a promising method for rapid antimicrobial susceptibility testing with a high rate of categorical agreement with the conventional disc diffusion method, particularly at 8-hour incubation. Thus, it promotes the appropriate use of antimicrobials, mitigates the emergence of antimicrobial resistance, and improves patient quality of care and outcome.

Introduction

Neonatal sepsis is a systemic condition that arises from bacterial, viral, or fungal origin. Neonatal sepsis is associated with hemodynamic changes and clinical findings causing severe morbidity and mortality [1]**.** Bloodstream infection (BSI) is a serious problem in newborns in neonatal intensive care units (NICUs). Out of 2.5 million babies who die within the first 4 weeks of life every year, 23% of them die because of infectious causes, including sepsis and pneumonia [2]. Therefore, rapid diagnosis of sepsis is crucial for the survival of hospitalized patients [3]**.** Each hour delay in the administration of the correct antibiotic is associated

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with an increase in the mortality and morbidity of septic patients. Thus, rapid identification of positive blood cultures and processing of antimicrobial susceptibility testing are essential for clinicians to properly treat patients with bloodstream infection [4]. The golden standard method of AST results needs minimum 48hours. Rapid, accurate results of antimicrobial susceptibility testing (AST) are critically needed to prevent escalating antibiotic resistance. Every hour delay in identifying multidrug-resistant organisms can cause neonatal mortality or morbidity [5]. European Committee on Antimicrobial Susceptibility Testing (EUCAST) released a methodology for performing rapid antimicrobial susceptibility testing (RAST) using disk diffusion performed directly from positive blood culture bottles, with shortened incubation time to 4, 6, and 8 h [6],[7]**.** This method has the potential to shorten the time of susceptibility testing results. In addition, the methodology of using disk diffusion is much less costly compared to implementing new technologies because the main equipment required are standard incubators. Thus, no need for new equipment in laboratories [8],[9]. The main advantage of RAST is the speed of the results compared to the conventional disc diffusion method of AST leading to great improvement in patient quality of care together with decreasing antimicrobial resistance in hospitals. However, the accuracy of RAST method is evaluated against the golden standard disc diffusion method in this study.

MATERIALS AND METHODS

This prospective comparative study was conducted in neonatal intensive care units (NICU) at Ain Shams University Hospitals from October 2022 until September 2023. Blood samples were collected from neonates who presented with septic signs as: apnea, lethargy, high fever, respiratory distress, poor feeding, and seizures. This study was done on 115 positive blood culture bottles. The ethical committee of Ain Shams University approved the study with Federal Wide Assurance number: 000017585, MS 514/2022 Measures were taken to ensure confidentiality and privacy of data.

Samples were collected from neonates and processed as follows:

One isolate per patient was included in the study. Blood samples showing ≥ 2 different micro-organisms on Gram's staining or culture were excluded.

Preparation of blood culture bottles:

The main bacteriological laboratory at Ain Shams University Hospitals is functional round the clock and is equipped with BacTAlert® 3D (bioMerieux, Marcy l'Etoile, France) continuous blood culture monitoring system. Three ml of blood sample was inoculated in the Bact/Alert blood culture bottle, this bottle contained culture medium for microbial growth and sensors for detecting that growth. If microbial growth occurred, carbon dioxide gas was produced, and the color of gas-permeable sensor installed in the bottom of each culture bottle changed to yellow. Alarm produced in the form of audio and door screen light flash.

Positively flagged BC bottles were subjected to the following:

EUCAST RAST methodology:

Once blood culture (BC) was positive, Gram stain film was done. Bottles showing ≥ 2 different micro-organisms on Gram's staining were excluded. 125±25 µl of undiluted blood culture broth was taken from the positive blood culture to each Muller Hinton agar plate. The broth was spread gently over the Muller Hinton agar (MHA, Himedia®, Mumbai, India) plates surface by using cotton swab in three directions for RAST. Antibiotics were used according to Gram stain. In case of Gram-positive bacteria: gentamicin 10 µg, clindamycin 2 µg and cefoxitin 30 µg were used. If Gram-negative bacilli or coccobacilli were identified: imipenem 10µg, ceftazidime 10 µg, trimethoprim-sulphamethoxazole 1.25-23.75 µg, piperacillin/ tazobactam 30/6 µg, gentamicin 10 µg, cefotaxime 5 µg and tobramycin 10µg were used. Four to six antibiotic discs were used per plate. Plates were incubated for 4,_6 and 8 hours. Inhibition zones read at ±5 minutes of the stated reading time.

RAST disk diffusion reading results were interpreted according to organisms and incubation time-specific breakpoints into susceptible, resistant, or area of technical uncertainty (ATU) as per ***EUCAST RAST, 2023*** [10]**.** ATU is a range of inhibition zone diameters. There are ATUs for all organism-antimicrobial agent combinations with the EUCAST RAST method. The ATU represents an area where the separation between susceptibility categories is poor. Interpretative errors increase dramatically in this area and interpretation is not possible. Results above or below the ATU can reliably be reported.

Simultaneously, conventional identification and golden standard antimicrobial susceptibility testing by Clinical and Laboratory Standards Institute (CLSI-M100**, 2023) were done:**

Parallelly, inoculum from BC bottles was inoculated on blood agar and MacConkey agar (Himedia®, Mumbai, India) plates for standard identification method [11]**.** Plates were examined daily for evidence of growth for 48 hours. Gram-stained films of the revealed isolates were examined. Gram-negative bacteria were identified using the following biochemical reactions: Oxidase test, triple sugar iron test, citrate test, urease test and indole test, where Gram-positive bacteria were identified using Catalase test and coagulase test. The isolated coagulase-negative Staphylococci were excluded as no cut points were found in RAST method according to European Committee.

Golden standard antimicrobial susceptibility testing by Kirby-Bauer disk-diffusion method was done. Results of antimicrobial susceptibility were interpreted as per **CLSI**-M100**,** 2023**)** Results were reported by measuring the zone of inhibition size and interpreted according to CLSI guidelines table [12]**.**

Statistical analysis

Evaluating Rapid antimicrobial susceptibility testing (RAST) method is performed using a comparison with the reference golden standard method, as per the ISO 20776-2 standard: a categorical agreement (CA) is obtained when the strain is in the same clinical category (R, I, S). A very major error (VME) corresponds to a false susceptibility result and is calculated using the resistant strains tested, and a major error (ME), in the case of false resistance, is calculated on the number of susceptible strains. Finally, a minor error (MiE) occurs when a strain is classified as Intermediate (I) instead of S or R, or S or R instead of I. A reliable method will obtain the following scores: CA≥ 90%, EA≥ 90%, VME ≤ 3%, and ME ≤ 3% ***(ISO, 2018)***. Using Statistical package for Social Science (SPSS 26), data were presented, and Fisher's exact test was used for categorical variables, where appropriate. $p \leq 0.05$ was considered as "statistically significant".

Results

The Main Microbiology laboratory, at Ain Shams University tertiary hospital received 610 blood culture (BC) bottles, from NICU, during the study period between October 2022 until September 2023, of which 115 (18.8%) flagged positive.

The mean age of the studied group was 12.85 days, age ranged from 1 to 28 days. 40.9% of neonates were males and 59.1% were females.

Fifty-five of 115 (47.8%) positive bottles showed Gram-negatives on Gram's staining on which RAST was performed.

Fifty-seven of 115 (49.6%) positive bottles showed Gram-positives on Gram's staining. *Coagulase-negative Staphylococci* (44) isolates were excluded according to European Committee on Rapid Antimicrobial Susceptibility Testing. The isolated coagulase-positive *Staphylococci* (13) on which RAST was performed.

Three of 115 (2.6%) positive bottles showed Gram-negative coccobacilli on which RAST was performed as shown in table 1.

Out of 115 isolates, 71 (61.7%) had monomicrobial growth of *E.coli* (n = 24, 20.8%), *K. pneumoniae* (n = 31, 27%), *Staph.aureus* (n = 13, 11.3%) *and A. baumannii* (n = 3, 2.6%) were eligible for inclusion as shown in table 1.

The overall antimicrobial susceptibility pattern of these isolates against tested antimicrobials, as per conventional methodology in CLSI is given in Figure 1.

The proportion of each category of results as per RAST at different timepoints and conventional methods are shown in Table 2. Results of both methods were compared and shown in Table 3.

There was an agreement more than 90% between both methods regarding the susceptibility to all antibiotics with major error less than 3 in the eighth hour except piperacillin- tazobactam as shown in table 3. The average results of AST of all organisms to different antibiotics compared to the conventional golden standard method, was 76.8% at four hours, 92% at six hours and 97.2% at eight hours respectively.

Table 1. The number and the percentage of the studied organisms:

		N	%
Gram	**Negative bacilli**	55	47.8%
	Positive cocci	57	49.6%
	negative Coccobacilli	3	2.6%
Organism	**Klebsiella pneumoniae**	31	27%
	Coagulase negative staphylococci	44	38.3%
	E coli	24	20.8%
	Staph aureus	13	11.3%
	Acinetobacter baumannii	3	2.6%

Table 2. Summary of Results of RAST methodology at different timepoints and the conventional method.

RAST method								**Conventional disc diffusion method**	
4h			**6h**			**8h**		**R**	**S**
R	**ATU**	**S**	**R**	**ATU**	**S**	**R**	**S**	**304 (69.7%)**	**132 (30.3%)**
354 (81.2%)	**42 (9.6%)**	**40 (9.2%)**	**327 (75 %)**	**9 (2.1%)**	**100 (22.9%)**	**314 (72.1%)**	**122 (27.9%)**		

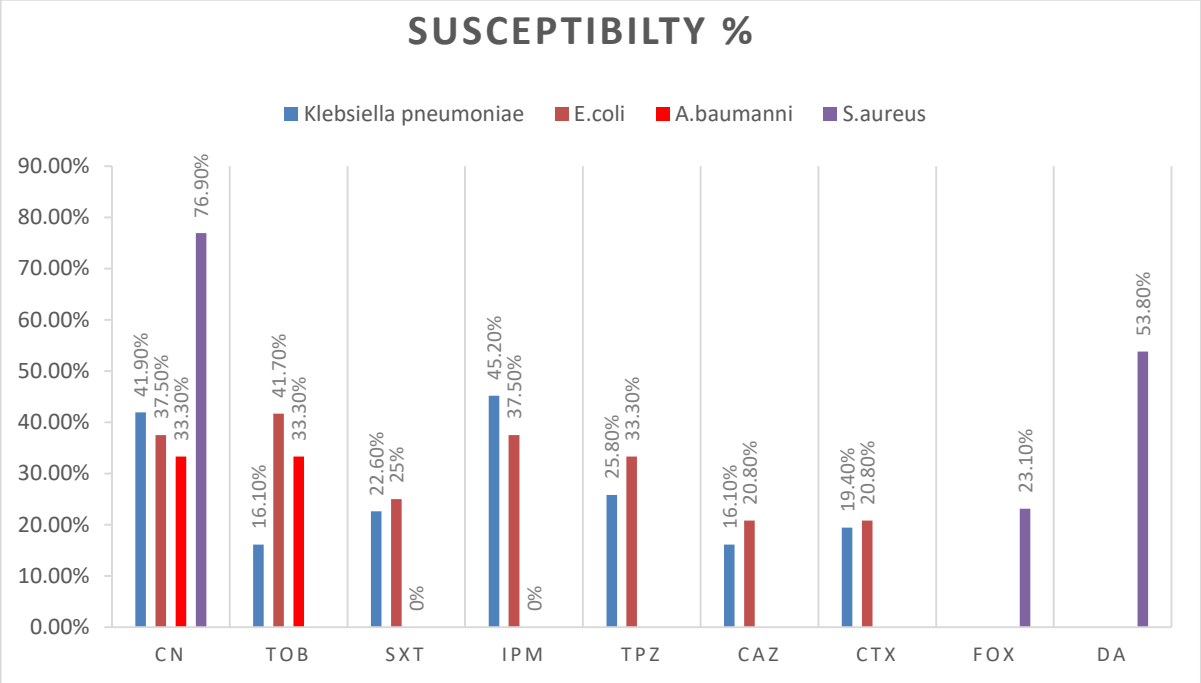
RAST: Rapid Antimicrobial Susceptibility Testing; CLSI: Clinical Laboratory Standards Institute; S: Susceptible; ATU: Area of Technical Uncertainty; R: Resistant; I: Intermediate

Table 3. Comparison of RAST Methodology (EUCAST, version6.1) with disc diffusion Method (CLSI) in terms of Categorical Agreement, Major Errors, Very Major Errors and Minor Errors at 4, 6- and 8-hour of incubation regarding individual antibiotics used on the different isolated organisms (*Klebsiella pneumoniae, E.coli, Staphylococcus aureus and Acinetobacter baumannii).*

	CA		**ME**		**VME**	
	N	**%**	**N**	**%**	**N**	**%**
Gentamycin 4h	48	67.6%	13	18.3%	0	0.0%
Gentamycin 6h	64	90.1%	5	7.0%	0	0.0%
Gentamycin 8h	69	97.2%	2	2.8%	0	0.0%
Imipenem 4h	39	67.2%	10	17.2%	0	0.0%
Imipenem 6h	49	84.5%	6	10.3%	0	0.0%
Imipenem 8h	57	98.3%	1	1.7%	0	0.0%
Piperacillin-Tazobactam 4h	40	72.7%	4	7.3%	0	0.0%
Piperacillin-Tazobactam 6h	49	89.1%	3	5.5%	0	0.0%
Piperacillin-Tazobactam 8h	52	94.5%	3	5.5%	0	0.0%
Tobramycin 4h	45	77.6%	9	15.5%	0	0.0%
Tobramycin 6h	52	89.7%	5	8.6%	0	0.0%
Tobramycin 8h	57	98.3%	1	1.7%	0	0.0%
Trimethoprim/Sulfamethoxazole 4h	48	82.8%	8	13.8%	0	0.0%
Trimethoprim/Sulfamethoxazole 6h	55	94.8%	3	5.2%	0	0.0%
Trimethoprim/Sulfamethoxazole 8h	57	98.3%	1	1.7%	0	0.0%

Ceftazidime 4h	48	87.3%	4	7.3%	0	0.0%
Ceftazidime 6h	52	94.5%	3	5.5%	0	0.0%
Ceftazidime 8h	54	98.2%	1	1.8%	0	0.0%
Cefotaxime 4h	47	85.5%	5	9.1%	0	0.0%
Cefotaxime 6h	53	96.4%	2	3.6%	0	0.0%
Cefotaxime 8h	54	98.2%	1	1.8%	0	0.0%
Cefoxtin 4h	10	76.9%	3	23.1%	0	0.0%
Cefoxtin 6h	13	100%	0	0.0%	0	0.0%
Cefoxtin 8h	13	100%	0	0.0%	0	0.0%
Clindamycin 4h	9	69.2%	4	30.8%	0	0.0%
Clindamycin 6h	13	100%	0	0.0%	0	0.0%
Clindamycin 8h	13	100%	0	0.0%	0	0.0%

Figure 1. The overall antimicrobial susceptibility profile of these isolates against tested antimicrobials, as per the conventional disc diffusion method of CLSI-M100-S29.



Discussion

Neonatal sepsis is one of the leading causes of neonatal morbidity and mortality in resource-limited settings [13]**.** Using conventional methods, species identification and antimicrobial susceptibility testing may take up to 72 hours after sample collection [14]. Thus, broad-spectrum antimicrobials are often used as empiric therapy which, in turn, has led to increasing rates of microbial resistance [15], [16]. Most of neonatal sepsis-related morbidity and mortality can be decreased by early diagnosis and treatment with appropriate antimicrobial agents [17].

EUCAST released a new rapid antimicrobial susceptibility testing (RAST) using disk diffusion directly from positive blood culture bottles. RAST results appear after 4 hours nevertheless, more reliable after 6 and 8 hours [6],[7]**.** There is flexibility in the choice of antibiotics for RAST compared with preformed commercially available AST panels. In addition, RAST is compatible with WHO ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid, and robust, equipment free, deliverable to users) [18]**.** Timely and reliable susceptibility results facilitate deescalating the empiric antibiotic treatment, with optimal antimicrobial choice, and

adjusting the antimicrobial stewardship program accordingly [19].

This study aimed to compare the rapid antimicrobial susceptibility test (RAST) against golden standard disc diffusion method by CLSI.

The current study was conducted on 115 neonates with neonatal sepsis according to the Bact/Alert blood culture results (bioMerieux, France). We included only 71 positive BC bottles yielding Gram-positive and negative isolates. Patients with *coagulase-negative staphylococci* bloodstream infections were not included because of the unavailable recommendations for them. The study was conducted at the Main Microbiology laboratory, Ain Shams University Tertiary Hospital from October 2022 to September 2023.

No study was found evaluating the RAST methodology in neonates. However, several studies confirmed that the results of the RAST are in good agreement with those of the conventional method of CLSI.

The present study revealed that, the results of RAST at 8 hours correspond to disk diffusion method, with a categorical agreement more than 90% with major error rates less than 3 except for piperacillin-tazobactam. The average results of AST of all organisms to different antibiotics compared to the golden standard method, was 77% at four hours, 91.5% at six hours and 96.8% at eight hours. Eight-hour RAST results came in agreement with *Najeeb et al.* who also compared RAST methodology by EUCAST for Positive BC bottles with disc diffusion method by CLSI [20].

Regarding *E. coli, Klebsiella, and Acinetobacter* species susceptibility to imipenem (IPM) at 4, 6, and 8 hours; categorical agreement (CA) was more than 90% at eight-hour with low major and very major error rates in contrast to four and six hours that showed low categorical agreement with high major and very major error rates. Similarly, *Najeeb et al.* found imipenem (IPM) susceptibility testing by RAST at four hour not significant (CA less than 90%) and also at six hour as VME rates were more than 3 while at eight hours CA was more than 90%[20].

As for piperacillin-tazobactam (TZP) results at 4, 6, and 8 hours, categorical agreement (CA) was consistently greater than 90% at four and six hours. However, major errors (ME) were more than 3% at 8 hours. This partly agreed with *Najeeb et al.* who showed poor CAs for piperacillin-tazobactam due to high number of MEs at all time points[20]. The same findings were observed in other studies such as *Jasuja et al.* and *Soo et al.* [21], [22].

Comparing Gram-negative microorganisms' sensitivity test results to tobramycin (TOB) in both methods, Categorical agreement (CA) was consistently more than 90% at 8 hours. This indicates that TOB susceptibility testing by RAST was reliable at the mentioned time. This was in concordance with *Martin et al.* that showed the same results at 8 hours [23].

As for trimethoprim-sulfamethoxazole (SXT), results revealed high Categorical agreement (CA) with 98.3% at 8 hours, however, high major error rates were recorded at 4 and 6 hours. These results agreed with *Najeeb et al.* at 4 and 6 hours, but opposing it at 8 hours. This may be attributed to the later study had large sample size [20].

Furthermore, *E. coli and Klebsiella pneumoniae* susceptibility to ceftazidime (CAZ) revealed 98.2% categorical agreement at 8 hours with major error rates 1.8 which indicates the reliability of RAST at 8 hours. A study was conducted in 2020 by *Soo et al.* strongly agreed with this study at 8 hours. Lastly, *E. coli and Klebsiella pneumoniae* susceptibility to cefotaxime (CTX) using both methods demonstrated findings of categorical agreement (CA) higher than 90% at 8 hours. These results indicate that cefotaxime testing by RAST is reliable at 8 hours [22]. Similarly, a study in Japan by *Uechi et al.* showed CAs 95.6% after 8 hours incubation [24].

Comparing *Staph aureus* susceptibility to cefoxitin and clindamycin, categorical agreement was 100% at 6 and 8 hours with no VME or ME errors. This came in agreement with *Park et al.* who reported the same results at the mentioned times[25].

In 2020, *Jonasson et al.* compared the RAST results with broth microdilution method (BMD) results. They evaluated the parameters such as the use of blood culture bottles of four different commercial companies, processing time of positive bottles, and reported that these variables caused no systematic difference, and had minimal effect on the interpretation of the results [6].

Similar to our findings, *Jonasson et al.* concluded that the zone diameters were increased with the prolongation of the incubation time, zone diameters could be more easily readable at eighth-

hour and the error rates for all bacteria were reduced when reading was performed at 8 hours[6].

Moreover, in 2022, *Martin et al.* evaluated the impact of EUCAST RAST on the management of Gram-negative bloodstream infections; implicated high degree of categorical agreement of RAST and CLSI. It also suggested that RAST led to quicker prescription of effective antibiotic therapy, showcasing its potential life-saving benefits in management of antibiotic therapy in patients with Gram-negative sepsis. It reduces the time factor for escalating or de-escalating the empiric antimicrobial treatment[23].

However, the manual processing of the EUCAST RAST and the need to read the inhibition zone diameters at strictly defined time points is a labor-intensive method [26]. This can be overcomed by fully automated RAST as recommended by *Jonasson et al. and Cherkaoui et al.* In fully automated RAST, inoculated media are transferred to the incubator immediately after deposition of the antibiotic disks. The digital images are taken at the defined time points. This decreases the man-error in reading the inhibition zones at the exact different time points in larger number of isolates[6], [27].

Conclusion:

This study showed high categorical agreement >90% with the golden disc diffusion method especially at eight-hour. RAST is a promising accurate method for antimicrobial susceptibility testing compared to the golden standard method. RAST reduces the turnaround time in reaching the results of antimicrobial susceptibility tests from 72 hours up to 8 hours. Earlier accurate results can improve patient's outcome particularly in such vulnerable group of patients (Newborns). Thus, urgently needed in critical cases. RAST is cost effective method. It is suitable for resource-limited settings where newer technological methods cannot be implemented.

Recommendation and limitation:

Adaptation of RAST in routine lab work since it is an accurate and reliable method. However, this method requires accurate manual reading at 4, 6, 8 hours intervals that can be overcome by using fully automated RAST. This study is the first of its type in Egypt to evaluate the EUCAST RAST from positive blood culture. Further wider-scale multicentric studies are needed for other vulnerable groups of patients, for example septic patients from pediatric ICU, or geriatric units,in resource-limited settings.

Conflict of interest:

The authors declare that there is no conflict of interest.

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