

RESEARCH ARTICLE



Usage of 150 mm Mueller – Hinton Agar for the EUCAST rapid antimicrobial susceptibility test (RAST) directly from positive blood culture bottles

Serap Süzük Yıldız^{1*} , Sevgi Şahin¹ , Esra Tavukcu¹ ,
İpek Mumcuoğlu¹ , Can Hüseyin Hekimoğlu² ,
Ayşe Semra Güreşer¹ and Tuba Dal¹

¹ Department of Clinical Microbiology, Dr. Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital, University of Health Sciences, Ankara, Turkey

² Department of Communicable Diseases, Ministry of Health, General Directorate of Public Health, Ankara, Turkey

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ABSTRACT

In this study, we evaluated the performance of modified rapid antimicrobial susceptibility test (mRAST) with 150 mm Mueller Hinton Agar (MHA) plates which was earlier standardized for 90 mm MHA by EUCAST. Blood culture bottles spiked with ATCC quality control strains were prepared. For quality control *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 strains were used. By calculating and proportioning the surface areas of the plates comparing with 90 mm plates, 350 ± 50 µL undiluted blood culture samples were inoculated in 150 mm MHA, and 12 disks were placed. This process was repeated independently for three days and three times on each day for reproducibility. The mRAST test was performed on 50 samples with positive signals and gram-negative bacilli on Gram-stained samples (20 *Klebsiella pneumoniae*, 15 *E. coli*, 10 *Acinetobacter baumannii*, and five *P. aeruginosa*).

Comparison of 90 mm MHA and 150 mm MHA showed that the categorical agreement of ATCC strains and 50 gram negative isolates was 100% and >95%, respectively, for all antibiotics. For *K. pneumoniae*, only 0.4 major error (ME) was detected at 4 h. For *E. coli*, 3.2, 1.6, and 1.5 ME were detected at 4, 8, and 20 h, respectively, whereas 1.6 very major error (VME) was detected at 4 h and 1.0 VME was detected at both 8, and 20 h, respectively. No errors were detected for *P. aeruginosa* or *A. baumannii*.

These results indicated that 350 ± 50 µL of undiluted blood culture in 150 mm MHA was suitable for the mRAST test *in vitro*.

KEYWORDS

blood culture, rapid antimicrobial susceptibility test, antimicrobial susceptibility testing, disk diffusion

INTRODUCTION

Mueller Hinton agar (MHA) is a medium that was developed to determine antibiotic susceptibility in rapidly growing bacteria via the standard disk diffusion method [1]. It is also recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) for conducting the disk diffusion method to assess rapidly growing bacteria [2, 3]. The disk diffusion method is the most preferred method among antibiotic susceptibility tests in routine microbiology laboratories as it has excellent application features and low costs. Besides its application in

*Corresponding author.

E-mail: serapsuzuk@gmail.com

routine antibiotic susceptibility tests, it is used as a part of screening tests for resistance mechanisms and surveillance systems [4].

The ingredients and requirements of MHA are specified by the International Organization for Standardization (ISO) [5], the World Health Organization (WHO) [6], and the Food and Drug Administration (FDA) [7]. Powdered media prepared according to this procedure are offered to users in packaged form by companies. Many laboratories prepare powdered media for disk diffusion following the recommendations of the manufacturers.

To obtain accurate and reproducible test results in antibiotic susceptibility testing of fast-growing bacteria with MHA agar, standard media preparation methodologies should be followed. Therefore, the thickness, pH, and bacterial growth performance of the media of each production batch need to be determined and monitored [8].

The mortality and morbidity rates caused by bloodstream infections are high. For infections caused by multidrug-resistant bacteria, identifying bacteria at the species level and obtaining antibiotic susceptibility test results in an early period are extremely important for treating patients [9]. EUCAST has developed a standard for antibiotic susceptibility testing by direct disk diffusion from a positive-signaling bottle [10]. Although this method provides rapid and accurate results and is standardized, some difficulties are encountered in practice [9, 11]. Bloodstream infections are very common causes of sepsis. Treatment is closely related to the early initiation of effective antibiotics. Owing to the problem of antibiotic resistance, high resistance rates in bacteria may generally cause failure of empirical treatment. Therefore, the need for rapid antibiotic susceptibility tests has increased, and the use of these tests has become widespread. For this purpose, the rapid antimicrobial susceptibility test (RAST) study developed by EUCAST provides a very good turnover rate [12].

A maximum of six disks can be placed in a 90 mm medium according to EUCAST standards. This is one of the challenges encountered in RAST. Ours is an oncology hospital, where most patients receiving treatment are immune-specific. To start the correct antibiotic treatment, RAST is routinely performed in patients with bloodstream infections. We aimed to perform a validation study using 150 mm media, which we frequently use in routine practice instead of 90 mm media to conduct our processes; moreover, in our method, 12 disks can be evaluated in a single plate.

MATERIALS AND METHODS

Spiked blood culture bottle

In this study, spiked blood culture bottles were prepared with *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 standard strains and 50 samples (20 *Klebsiella pneumoniae*, 15 *E. coli*, 10 *Acinetobacter baumannii* and five *P. aeruginosa* isolates). The standard

strains and samples passaged in 5% sheep blood agar were incubated overnight. Then, serial dilutions of them were prepared, and 1 mL of the bacterial suspension of 10^5 CFU/mL was inoculated into a BacT/Alert Standard Aerobic (Biomérieux, France) bottle, after which 5 mL of sterile blood was added [13]. The blood culture bottles were incubated in BacT/Alert 3D (bioMérieux, St. Laurent, Quebec). Positive signals were obtained between 4 and 18 h on average. Antibiotic susceptibility tests were performed following the EUCAST RAST methodology for bottles with positive signals between 0 and 14 h.

Preparation of 90 and 150 mm media and disks for RAST

As per the EUCAST medium preparation standard, 25 mL of prepared MHA (Oxoid, UK) was added to Petri dishes 90 mm in diameter, and 71 mL of MH was added to a Petri plate 150 mm in diameter. Sterility, pH, thickness, and performance tests were conducted for each production [14]. Ampicillin (10 µg), amoxicillin-clavulanic acid (20-10 µg), piperacillin tazobactam (30-6 µg), cefotaxime (5 µg), ceftazidime (10 µg), ceftazidime-avibactam (10-4 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg) and trimethoprim-sulfamethoxazole (1.25–23.75 µg) (Oxoid, UK) were used for RAST and mRAST.

Quality control study

Routine quality control studies of the media and disks were performed using *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212.

Study of antibiotic susceptibility tests

Each bottle with a positive signal was inoculated on routine 5% sheep blood agar and EMB medium. After overnight incubation, the EUCAST standard disk diffusion antibiotic susceptibility test was performed for each bacterial strain.

RAST

From each positive-signaling bottle, 125 ± 25 µL of blood culture sample was inoculated in MHA medium on 90 mm plates according to the guidelines of EUCAST. The disks were placed following the EUCAST RAST procedure, with a maximum of six disks in one plate [9]. The surface areas of the plates were calculated and proportioned to 350 ± 50 µL 150 mm plates. Accordingly, 350 ± 50 µL of undiluted blood was inoculated in 150 mm agar. Both 90 and 150 mm media were spread with an automatic plate rotator, after which, the disks were placed. Each sample was prepared in triplicate during the day, and the experiments were repeated on three different days.

After conducting this validation study, the mRAST study was performed using 50 blood cultures with positive signals with gram-negative bacteria in Gram stain. These positive blood culture bottles were analyzed using the FilmArray BCID panel (The BIOFIRE BCID2 Panel, France) and

bacteria were identified by VITEK Mass Spectrometry System (Vitek MS; bioMerieux, France) from the 4 h. Additionally, an EUCAST disk diffusion study was performed on bacteria grown in subculture, and the results were compared to the RAST results. This study included 20 *K. pneumoniae* strains, 15 *E. coli* strains, 10 *A. baumannii* strains, and five *P. aeruginosa* strains.

Analyses of data

The Area of Technical Uncertainty (ATU) (uninterpretable) results were not considered in the categorical error rates. The results were compared with the standard disk diffusion test (sDD) results determined as the reference method, and the very major errors (VMEs; mRAST = S and sDD = R), major errors (MEs; mRAST = S and sDD = R) and minor errors [mEs; mRAST = S or R and sDD = susceptible, increased exposure (I)] were determined [10].

RESULTS

Quality control studies of media and disks

The quality control studies of media and disks were evaluated according to the EUCAST quality document. The performance of the media and disks was adequate and appropriate for the study.

Spiked blood culture RAST results

The precision and accuracy of the spiked blood culture prepared by QC strains were assessed according to the range provided in the EUCAST RAST QC tables. The results were evaluated within and between days. Accordingly, 95.2% agreement was determined for *E. coli* and *P. aeruginosa*, and 100% agreement was determined for *S. aureus* and *E. faecalis*. In the accuracy study, the categorical agreement was 100% for all three bacterial species.

RAST results from blood culture bottles with positive signals

The mRAST and RAST tests were performed with positive signals with gram-negative bacilli on Gram stain. The results were compared to those of the standard disk diffusion method. According to the results obtained with mRAST, no readable zone was detected for *P. aeruginosa* at the fourth hour; thus, the first evaluation was performed at the sixth hour for *P. aeruginosa*. For all other bacterial species, a readable zone was detected for all antibiotics at the fourth hour. No minor error was observed. The ME detected in *K. pneumoniae* was associated with amikacin. The VME detected in *E. coli* was associated with ceftazidime and the ME was associated with ciprofloxacin and levofloxacin. No errors were detected for *P. aeruginosa* or *A. baumannii*. Among all bacteria, the area of technical uncertainty (ATU) was most frequently detected with piperacillin-tazobactam. The results of the mRAST test for bacteria according to standard disk diffusion are provided in Table 1. The results

Table 1. Comparison of the zone diameters detected by mRAST at 4, 8, and 20 h and the standard disk diffusion clinical breakpoints and VME and ME error rates in mRAST

Incubation Time Species (number of isolates)	4 h*		8 h		20 h	
	n	%	n	%	n	%
<i>K. pneumoniae</i> (20)	Number of completed test: 260					
Not interpreted to S or R (ATU) (%)	8	3.1	4	1.5		0.0
Interpreted to S (%)	58	22.3	61	23.5	65	25.0
Interpreted to R (%)	194	74.6	195	75.0	195	75.0
mE (%)	0	0.0	0	0.0	0	0.0
ME (%)	1	0.4	0	0.0	0	0.0
VME (%)	0	0.0	0	0.0	0	0.0
Total Errors (%)	1	0.4	0	0.0	0	0.0
<i>E. coli</i> (15)	Number of completed test: 195					
Not interpreted to S or R (ATU) (%)	15	7.7	5	2.6	4	2.1
Interpreted to S (%)	108	55.4	119	61.0	120	61.5
Interpreted to R (%)	72	36.9	71	36.4	71	36.4
mE (%)	0	0.0	0	0.0	0	0.0
ME (%)	6	3.2	3	1.6	3	1.5
VME (%)	3	1.6	2	1.0	2	1.0
Total Errors (%)	9	4.8	5	2.6	5	2.5
<i>A. baumannii</i>** (10)	Number of completed test: 70					
Not interpreted to S or R (ATU) (%)	9	12.9	3	4.3	2	2.9
Interpreted to S (%)*	14	20.0	12	17.1	3	4.3
Interpreted to R (%)	47	67.1	55	78.6	65	92.9
mE (%)	0	0.0	0	0.0	0	0.0
ME (%)	0	0.0	0	0.0	0	0.0
VME (%)	0	0.0	0	0.0	0	0.0
Total Errors (%)	0	0.0	0	0.0	0	0.0
<i>P. aeruginosa</i>** (5)	Number of completed test: 35					
Not interpreted to S or R (ATU) (%)	20	44.4	5	11.1	5	11.1
Interpreted to S (%)	20	44.4	35	77.8	35	77.8
Interpreted to R (%)	5	11.1	5	11.1	5	11.1
mE (%)	0	0.0	0	0.0	0	0.0
ME (%)	0	0.0	0	0.0	0	0.0
VME (%)	0	0.0	0	0.0	0	0.0
Total Errors (%)	0	0	0	0.0	0	0.0

*6 h for *P. aeruginosa*.
**Including species-agent combinations for which WT isolates are categorized as I.
S, susceptibility; R, resistance; mE: minor error; ME, major error; VME, very major error.

of mRAST and ssDD were shown in [Supplementary Table 1](#) and the rate of susceptibility of isolates were in the [Table 2](#).

DISCUSSION

As this study was conducted in an oncology hospital, most patients receiving treatment were immunosuppressed. Such patients are prone to infections during cancer treatment, and the mortality and morbidity rates of bloodstream infections are especially high [15]. Antibiotic resistance rates in Turkey

Table 2. The antimicrobial resistance rates of bacteria in mRAST and standard disk diffusion

Bacteria (n)	Antibiotics	4h* (S + I)	8 h (S + I)	20 h (S + I)	sDD (S + I)
<i>K. pneumoniae</i> (20)	Amikacin	57.9	60.0	60.0	60.0
	Amoxicillin-clavulanic acid	0.0	0.0	0.0	0.0
	Cefotaxime	5.0	5.0	5.0	5.0
	Ceftazidime	5.0	5.0	5.0	5.0
	Ceftazidime-avibactam	40.0	40.0	40.0	40.0
	Ciprofloxacin	15.0	15.0	15.0	15.0
	Gentamicin	66.7	70.0	70.0	70.0
	Imipenem	50.0	50.0	55.0	55.0
	Levofloxacin	15.0	15.0	15.0	15.0
	Meropenem	38.9	38.9	45.0	45.0
	Piperacillin-tazobactam	15.8	15.0	15.0	15.0
	Trimethoprim-sulfamethoxazole	0.0	0.0	0.0	0.0
<i>E. coli</i> (15)	Amikacin	100.0	100.0	100.0	100.0
	Amoxicillin-clavulanic acid	0.0	0.0	0.0	0.0
	Ampicillin	0.0	0.0	0.0	0.0
	Cefotaxime	46.7	46.7	46.7	46.7
	Ceftazidime	38.5	60.0	60.0	60.0
	Ceftazidime-avibactam	100.0	100.0	100.0	100.0
	Ciprofloxacin	42.9	42.9	42.9	42.9
	Gentamicin	86.7	86.7	86.7	86.7
	Imipenem	100.0	93.3	93.3	93.3
	Levofloxacin	46.2	50.0	50.0	50.0
	Meropenem	100.0	93.3	93.3	93.3
	Piperacillin-tazobactam	71.4	92.9	93.3	93.3
	Trimethoprim-sulfamethoxazole	46.7	46.7	46.7	46.7
<i>A. baumannii</i> (10)	Amikacin	28.6	20.0	20.0	20.0
	Ciprofloxacin	20.0	20.0	20.0	20.0
	Gentamicin	20.0	20.0	20.0	20.0
	Imipenem	20.0	20.0	20.0	20.0
	Levofloxacin	20.0	20.0	20.0	20.0
	Meropenem	20.0	20.0	20.0	20.0
	Trimethoprim-sulfamethoxazole	50.0	28.6	50.0	50.0
<i>P. aeruginosa</i> (5)	Amikacin	100.0	100.0	100.0	100.0
	Ceftazidime	0.0	0.0	0.0	0.0
	Ciprofloxacin	–	100.0	100.0	100.0
	Imipenem	–	100.0	100.0	100.0
	Levofloxacin	–	100.0	100.0	100.0
	Meropenem	100.0	100.0	100.0	100.0
	Piperacillin-tazobactam	–	–	–	40.0

sDD: standard disk diffusion.

S + I: Susceptible, standard dosing regimen + Susceptible, increased exposure.

are quite high, especially for gram-negative bacilli [16]. Along with the advancements in technology in microbiology laboratories, speed of turn over the tests has become the most important factor. Most of these tests are syndromic tests that can be studied molecularly [17]. However, the costs of these tests are also high, which increases the financial burden for the patients [18]. The RAST test standardized by EUCAST is a highly effective method that can be used in routine microbiology laboratories [9]. We use the EUCAST RAST procedure in our routine laboratory examinations. As the blood culture contamination rates were high in our routine practice (unpublished data), syndromic panels and RAST studies were performed from bottles in which gram-negative bacilli were detected via Gram staining. The EUCAST RAST study was standardized on 90 mm plates. In our routine practice, at least three plates were

prepared for each patient. We aimed to validate 150 mm MHA medium, which we use more frequently in our routine examinations, for the RAST study. Using this technique, we aimed to use a single plate for each patient. We predicted that using a single plate would reduce the cost and workload. However, cost and workload were not evaluated in this study. Considering the method standardized for the 90 mm medium, the amount of undiluted sample used was calculated as $350 \pm 50 \mu\text{L}$ for the 150 mm medium, which is proportional to the area calculation. Studies were performed from spiked blood culture bottles prepared with standard strains and from blood culture bottles of patients with positive signals. The results obtained were statistically evaluated, and $350 \pm 50 \mu\text{L}$ of undiluted blood sample was determined to be an appropriate amount for 150 mm MHA.

Among the limitations of this study, workload assessment for differences 90 mm MHA and 150 mm MHA could not be made within the laboratory. Additionally, another important limitation of the study was that running samples with five-day repetitions would be more appropriate for the accuracy and precision study; however, owing to the cost of the bottles, single repetitions were limited to three days, but for this purpose, the tests were run in three repetitions each day. Another important limitation of our hospital was that the bacteria evaluated were generally resistant bacteria due to the high resistance rates in our hospital. Although our number of isolates was limited, we found that the results were useful for the infection control committee to evaluate patients; hence, we decided to publish the first results of the mRAST study.

In a study evaluating RAST, reliable and clinically significant results were obtained for the antibiotics tested at the end of 4 h of incubation, especially for the *E. coli* and *K. pneumoniae* isolates. In this study, in which ESBL-producing isolates were evaluated, the test had high sensitivity in determining cefotaxime resistance. A study reported that empirical treatment was also effective [19].

In our study, VME was detected for ciprofloxacin and levofloxacin in one isolate of *E. coli* at the fourth hour of incubation. In a multicenter study, ciprofloxacin VME was reported in *E. coli* and *K. pneumoniae* isolates, although VME rates were very low [10].

The RAST results contribute to the modification of ineffective empirical treatment [20]. The RAST is an effective rapid sensitivity test for reducing mortality, especially in BSI patients [21]. In this study, gram-negative bacteria with positive blood cultures detected by Gram staining in the morning were included in the RAST group, and those with gram-negative growth at other times of the day were included in the control group. The effects of the RAST results in the clinic were evaluated. In the study conducted with the RAST, categorical concordance based on the results of the antibiotic susceptibility test was reported to the clinic [22]. Antibiotic management and patient outcomes were compared between the two groups. RAST improved the management of antibiotic therapy in patients with gram-negative sepsis, but mortality and length of hospitalization did not differ significantly between the groups [23].

As the use of rapid identification systems can be limited, in this study, we evaluated the application of RAST without rapid identification and investigated the accuracy and applicability of the RAST method and the effects of the results obtained using this method on the treatment decision of clinicians. The effects of categorical results obtained with RAST at 4, 6, and 8 h of incubation on the treatment decision of clinicians were retrospectively evaluated. Even in a microbiology laboratory with limited facilities, reliable antimicrobial susceptibility test results can be obtained in a short time with the RAST method without using automated systems, and antimicrobial selection can be directed in a shorter time [24].

RAST is a reliable method to improve the clinical management of sepsis patients as it provides rapid

phenotypic susceptibility data. It contributes significantly to antimicrobial management by allowing early detection of resistant isolates and early infection control measures for isolates [25].

The microbiology laboratory plays a critical role in managing the treatment of immunosuppressed patients in our hospital. For this purpose, fast, reliable, and effective testing methods are used in our laboratory, and the results are shared with the infection control committee through constant communication. The microbiology laboratory aims to obtain fast, reliable, and accurate results to provide evidence-based data for treating immunosuppressed patients. We conducted a verification study for the use of 150 mm MHA, which we routinely use in the laboratory for the EUCAST RAST. In this modified method, we argue that inoculating and reading on a single MHA can decrease the microbiological workload. We found that performing mRAST with $350 \pm 50 \mu\text{L}$ of undiluted blood culture in 150 mm MHA is a rapid and reliable *in vitro* method in immunosuppressed patients with gram-negative bacilli in blood culture.

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SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at <https://doi.org/10.1556/030.2025.02538>.

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