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ORIGINAL RESEARCH

Evaluation of EUCAST Rapid Antimicrobial Susceptibility Testing for Gram-Negative Bacteria Directly from Positive Blood Cultures

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Purpose: This study aims to evaluate the accuracy of EUCAST rapid antimicrobial susceptibility testing (RAST) for Gram-negative bacteria directly from positive blood cultures, comparing it with short-term incubation (5–7 hours) and conventional broth microdilution methods.

Methods: A total of 139 Gram-negative isolates were tested. RAST results were assessed at 4 h, 6 h against minimal inhibitory concentration results using the short-term incubation (5–7 h) method, while at 16–20 h, the RAST results were compared to conventional method. For those with interpretable results, CLSI M52 was used to define cutoffs for equivalence in antimicrobial susceptibility testing.

Results: Among all isolates, 80.6% (112/139) were successfully interpreted based on EUCAST RAST breakpoints, including *Escherichia coli* (81), *Klebsiella pneumoniae* complex (17), *Pseudomonas aeruginosa* (10) and *Acinetobacter baumannii* (4). The overall category agreements for all tested antibiotics were 98.9%, 99.5%, and 99.7% at 4, 6, and 16–20 hours, respectively, for *E. coli*, and 100% for *K. pneumoniae, P. aeruginosa*, and *A. baumannii*. The area of technical uncertainty rate significantly decreased over time, from 9.1% at 4 hours to 3.1% at 16–20 hours ($p < 0.05$). The method effectively identified extended-spectrum beta-lactamase (ESBL)-producing and carbapenem-resistant bacteria, demonstrating performance comparable to the BD system. Additionally, results for other *Enterobacterales* could be interpreted using the RAST breakpoints for *E. coli*. The integration of RAST into routine workflows provides rapid and accurate results without incurring additional costs or labor.

Conclusion: RAST is a reliable and cost-effective method for testing Gram-negative bacteria directly from blood cultures, significantly reducing turnaround time. Utilizing RAST at various reading times (6 hours and 16–20 hours) optimizes clinical workflows, enhances antimicrobial stewardship, and improves patient outcomes.

Keywords: blood culture, rapid antimicrobial susceptibility testing, EUCAST, gram-negative bacteria, bloodstream infection, short-term incubation

Introduction

Bloodstream infections (BSIs) are life-threatening conditions with high global morbidity and mortality rates.[1,2] Without timely and effective antimicrobial therapy, BSIs can progress rapidly to septic shock, resulting in a mortality rate exceeding 50%.[3] Blood culture (BC) remains the gold standard for diagnosing BSIs, but traditional methods for antimicrobial susceptibility testing (AST) require 24–48 hours to deliver results after a positive BC signal, delaying critical clinical decisions. Although molecular diagnostic techniques, such as multiplex polymerase chain reaction (mPCR), can shorten this time,[4] their high costs and demand for specialized equipment limit their widespread application, especially in resource-limited areas.

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Rapid identification (ID) and AST of the BSI pathogens are essential for timely selection of appropriate antimicrobial therapy, which may lead to better outcomes for patients.[5,6] Rapid antimicrobial susceptibility testing (RAST) provides a promising alternative by enabling direct AST from positive blood cultures within hours.[7,8] The European Committee on Antimicrobial Susceptibility Testing (EUCAST) RAST guidelines offer a standardized approach with flexible reading times (4 h, 6 h, 8 h), making it adaptable to various laboratory workflows.[8] Recent studies show that, for EUCAST RAST, prolonging the recommended incubation to 16–20 h is possible and can be used as a complement when the intended shorter incubation is not possible to achieve.[9,10] Unlike traditional methods, RAST requires minimal additional resources or specialized training, significantly reducing both turnaround time and operational costs.[8] These attributes make RAST especially advantageous in routine clinical microbiology laboratories, where rapid and accurate AST results are critical for optimizing patient management and improving antimicrobial stewardship.

Gram-negative bacteremia, frequently associated with multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens, necessitates prompt and precise AST to inform appropriate treatment.[11,12] New β-lactams/β-lactamase inhibitors (eg, ceftazidime/avibactam (CZA)) are favored as a first-line anti-infective agent for treating carbapenemase-producing *K. pneumoniae* (CRKP) Infections.[13] Previous studies have showed that EUCAST RAST was accurate to determine ceftazidime/avibactam susceptibility of carbapenemase-producing *K. pneumoniae* and *E. coli* directly from BC bottles.[10,14] Delays in AST can lead to inappropriate antibiotic use, exacerbating resistance and patient outcomes. A rapid method by short-term incubation of positive blood culture samples on solid culture medium for 5–7 h followed by ID and AST was established.[15,16] Furthermore, this rapid method is routinely employed in our laboratory. By integrating short-term incubation and RAST into clinical workflows, laboratories can achieve faster ID and AST reporting without compromising accuracy, allowing clinicians to initiate targeted therapies earlier. This study aims to evaluate the accuracy and clinical practicality of EUCAST-based RAST for Gram-negative bacteria, comparing its performance to short-term incubation and conventional methods, with an emphasis on its potential to enhance clinical workflows and reduce diagnostic delays.

Materials and Methods

Clinical Sample Collection and Laboratory Processing

This study was conducted at the First Affiliated Hospital of Yangtze University in Hubei Province, China, from September 2023 to September 2024. A total of 452 positive blood culture specimens from patients with suspected bloodstream infections were included. All specimens were processed in the clinical microbiology laboratory following standardized protocols. Cultures were monitored using the Bactec FX system (Becton Dickinson, USA) until the instrument signaled positive or reached a maximum culture period of 5 days. Upon positivity, Gram staining was immediately performed. Of these, 139 specimens (139/452, 30.7%) containing single Gram-negative bacterial isolates were enrolled, while the remaining 313 were excluded due to Gram-positive bacteria, fungi, polymicrobial growth, or unmet RAST criteria. For eligible specimens, RAST was initiated within 3 hours post-alert, with selective interpretation at 4 h, 6 h, and 16–20 h based on instrument alert timing and laboratory staffing availability (not all isolates were analyzed at all timepoints). Simultaneously, positive samples were subcultured onto vancomycin-supplemented blood agar plates (BAP) and chocolate agar plates (CAP) and incubated at 35°C in 5% CO^2 for routine species identification and downstream analyses. The experimental workflow is illustrated in Figure 1.

Rapid Antimicrobial Susceptibility Testing (RAST)

Rapid disk diffusion (DD) testing was performed based on EUCAST guidelines for RAST (Version 6.1). A 100 μL sample of undiluted blood culture broth from the positive bottle was placed onto each 90-mm Mueller–Hinton (MH) agar plate, which was then incubated at 35°C under aerobic conditions. For bottles flagged positive between 5:00 PM and 10:00 AM, RAST was conducted by 10:00–11:00 AM, with results interpreted at 4 and 6 hours, according to EUCAST RAST breakpoints. ID of isolates was done using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) under the short-term incubation method. For bottles flagged after 10:00 AM, RAST was

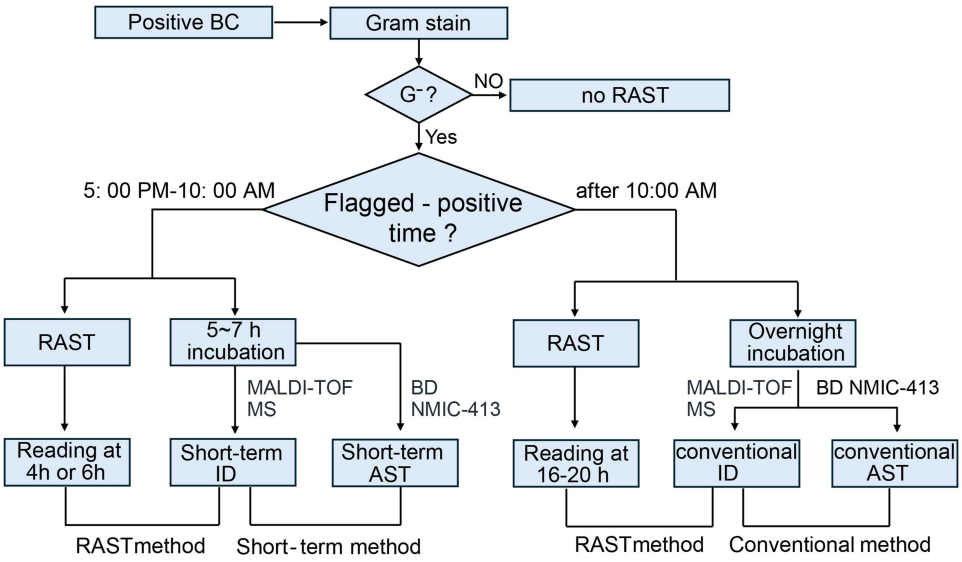

Figure 1 Methodology flowchart of RAST for detecting Gram-negative bacteria in positive blood cultures and its comparison with short-term and conventional methods.

processed within 3 hours with results interpreted at 4 hours on the same day and/or the 16–20-hour readings the following morning. Pure colonies were identified using conventional incubation methods.

We tested 11 antibiotic disks (OXOID®, United Kingdom; Biokont, China): 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), Gentamicin (10 μg), Tobramycin (10 μg), Trimethoprim-sulfamethoxazole (1.25/23.75 μg). The inhibition zones were measured independently by two operators, with divergences of only 1 mm considered not significant. *E. coli* ATCC 25922 was used as a quality control organism to validate the method.

Short-Term Incubation Method

The short-term incubation method was performed as described in our previous report.[15] When bottles flagged positive between 5:00 PM and 10:00 AM, they were subcultured immediately. Following 5 to 7 hours of incubation, microbial ID was performed directly from blood agar plates (BAP) or chocolate agar plates (CAP) using the Zybio EXS2000 mass spectrometer (MS). Bacterial colonies were transferred to the MS target plates using 1 μL of 70% formic acid. Once dried, an additional 1 μL of matrix solution was added for subsequent ID by MALDI-TOF MS. If the identified isolates were confirmed to be clinically significant rather than contaminants in the blood culture, colonies from the CAP were selected for AST. AST results were obtained using BD NMIC-413 plates after an incubation period of 5 to 7 hours and were interpreted according to the current CLSI standards.

Conventional Method

When bottles flagged positive after 10:00 AM, pure overnight bacterial colonies from BAP were utilized for conventional AST. A standardized inoculum was prepared and adjusted to a McFarland standard of 0.5. This suspension was then applied to BD NMIC-413 panels to determine minimum inhibitory concentrations (MICs). The MIC values were interpreted according to the CLSI M100 standards, providing categorical results as susceptible (S), intermediate (I), or resistant (R). The method for detecting ESBLs was BD system confirmatory testing, which is included in the NMIC-413 panels.

Data Analysis and Quality Control

The comparisons of RAST results with short-term and conventional incubation AST were categorized as follows: category agreement (CA), very major error (VME), major error (ME), and minor error (mE). CA was calculated as (Number of categorical result matches/Total tested) × 100. VME, indicating false susceptibility, was calculated as

(Number of resistant isolates misclassified as susceptible/Total number of resistant isolates) × 100. ME, representing false resistance, was calculated as (Number of susceptible isolates misclassified as resistant/Total number of susceptible isolates) × 100. mE, involving susceptible/resistant versus intermediate misclassification, was calculated as (Number of intermediate isolates misclassified as susceptible or resistant/Total number of isolates) × 100. Broth microdilution (BMD) with BD M50 system was used as a reference. Since the intermediate category does not apply to RAST, EUCAST introduced the concept of an area of technical uncertainty (ATU), within which interpretation as susceptible or resistant is not feasible. Unreadable inhibition zone (UIZ) was noted without further interpretation. Both ATU and UIZ results were excluded from the denominator when calculating CA, VME, ME, and mE. Cefotaxime and ceftriaxone were regarded as equivalent agents, as cross-resistance and cross-susceptibility were nearly complete when assessed by conventional methods in *Enterobacterales*. Standard strains, including *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853, were employed as internal quality controls for the NMIC-413 panels.

Comparisons involving dichotomous variables were tested using the $\chi 2$ test (SPSS 22.0). Statistical significance was set at a p-value < 0.05.

Results

Interpretation of RAST Results for Gram-Negative Bacteria

A total of 139 Gram-negative organisms underwent RAST directly from positive blood cultures, as shown in Figure 2. Among these, 80.6% (112/139) were successfully interpreted based on EUCAST RAST breakpoints. The interpretable isolates included 81 *Escherichia coli* (including 30 ESBL-producing), 17 *Klebsiella pneumoniae* complex (including 1 carbapenem-resistant *K. pneumoniae*, and 2 ESBL-producing *K. pneumoniae*), 10 *Pseudomonas aeruginosa* (including 1 carbapenem-resistant *P. aeruginosa*), and 4 *Acinetobacter baumannii* (including 1 carbapenem-resistant *A. baumannii*). The remaining 27 isolates, which comprised 16 other *Enterobacterales* (excluding *E. coli* and *K. pneumoniae* complex), 4 other species of *Acinetobacter*, 2 non-*Enterobacterales*, and 5 polymicrobial samples, could not be interpreted as they fell outside the scope of current EUCAST RAST breakpoint criteria.

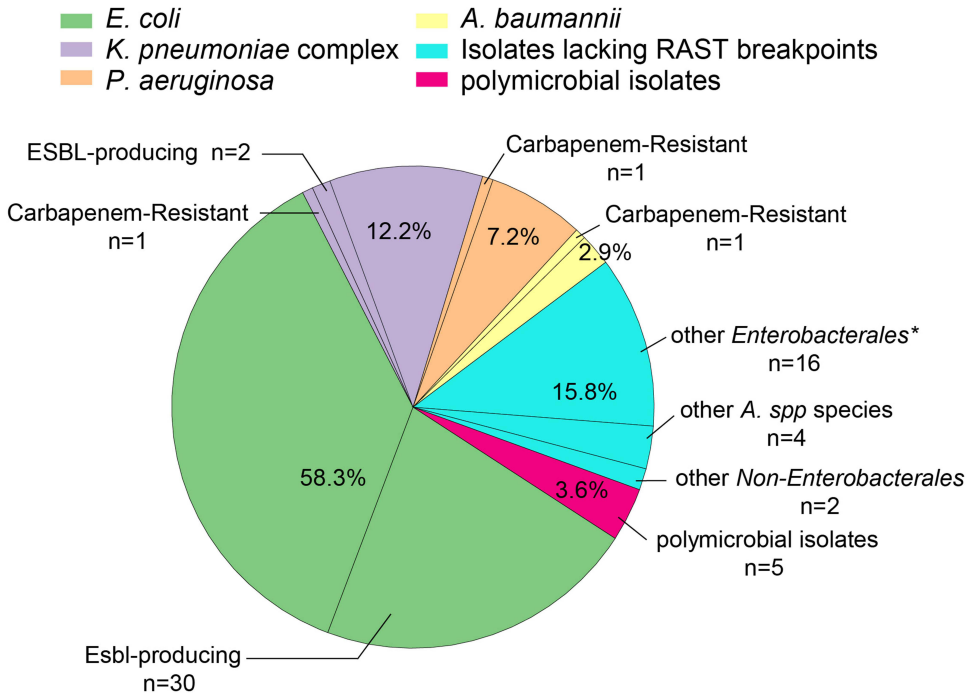

Figure 2 Overview of Gram-negative bacterial isolates tested by RAST and their interpretability based on EUCAST RAST breakpoints. This figure illustrates the distribution of 139 Gram-negative bacterial isolates, including the number of interpretable isolates (112/139) and the categories of organisms that fall within or outside the EUCAST RAST breakpoint criteria. Key resistant phenotypes such as ESBL-producing and carbapenem-resistant isolates are highlighted. *Exclude *E. coli* and *K. pneumoniae* complex.

RAST for *E. coli*

RAST showed excellent performance for *E. coli*. Table 1 summarizes the CA values of various antibiotics after 4 h, 6 h and 16–20 h of incubation, based on 42, 39 and 47 isolates, respectively. The overall CA, calculated as (RAST/AST-concordant cases/total valid tests) ×100%, was 98.9% at 4 hours, 99.5% at 6 hours, and 99.7% at 16–20 hours. Minor errors (mE) were observed for ceftazidime (CAZ) at 4 h (4.3%) and 6 h (2.8%), as well as for tobramycin (TOB) at 4 h (5.1%), 6 h (2.4%), and 16–20 h (2.7%). Major errors (ME) were limited to trimethoprim-sulfamethoxazole (SXT) at 4 h (6.7%), while no ME were observed at 6 h or 16–20 h. No very major errors (VME) occurred for any tested antibiotics across all time points, highlighting the reliability of the method.

Table 1 Summary of RAST Performance for *E. Coli* Compared to MIC Results

Antibiotics	Time/h	RAST		BD MIC			CA (%)	VME/ME/mE (%)
		R	S	R	I	S		
TZP	4	0	17	0	0	47	100(17/17)	0/0/0
	6	0	40	0	0	42	100(40/40)	0/0/0
	16-20	0	39	0	0	39	100(39/39)	0/0/0
CAZ	4	9	14	10	1	36	95.7(22/23)	0/0/4.3
	6	6	30	7	2	33	97.2(35/36)	0/0/2.8
	16-20	5	27	7	1	31	100(32/32)	0/0/0
CZA	4	0	42	0	0	47[a]	100(42/42)	0/0/0
	6	0	42	0	0	42[a]	100(42/42)	0/0/0
	16-20	0	39	0	0	39[a]	100(39/39)	0/0/0
IPM	4	0	40	0	0	47	100(40/40)	0/0/0
	6	0	42	0	0	42	100(42/42)	0/0/0
	16-20	0	39	0	0	39	100(39/39)	0/0/0
MEM	4	0	41	0	0	47	100(41/41)	0/0/0
	6	0	42	0	0	42	100(42/42)	0/0/0
	16-20	0	39	0	0	39	100(39/39)	0/0/0
CIP	4	18	10	22	0	25	100(28/28)	0/0/0
	6	20	17	21	0	21	100(37/37)	0/0/0
	16-20	17	20	19	0	20	100(37/37)	0/0/0
LEV	4	18	12	22	0	25	100(30/30)	0/0/0
	6	20	17	20	0	22	100(37/37)	0/0/0
	16-20	16	19	19	0	20	100(35/35)	0/0/0
GEN	4	8	35	8	0	39	100(43/43)	0/0/0
	6	12	30	12	0	30	100(42/42)	0/0/0
	16-20	8	31	8	0	31	100(39/39)	0/0/0
TOB	4	8	31	9	0	38	94.9(37/39)	0/0/5.1
	6	12	29	12	1	29	97.6(40/41)	0/0/2.4
	16-20	7	30	7	3	29	97.3(36/37)	0/0/2.7
SXT	4	22	14	21	0	26	97.2(35/36)	0/6.7/0
	6	21	21	21	0	21	100(42/42)	0/0/0
	16-20	15	23	16	0	23	100(38/38)	0/0/0
CTX	4	18	24	18[b]	0	29[b]	100(42/42)	0/0/0
	6	17	25	17[b]	0	25[b]	100(42/42)	0/0/0
	16-20	14	25	14[b]	0	25[b]	100(39/39)	0/0/0

Note: n = 47 at 4 h, n = 42 at 6 h, and n = 39 at 16–20 h. The AST results marked with a were obtained using the standard disc diffusion method, while the MIC results marked with b were derived from ceftriaxone data.

Abbreviations: NB, no breakpoint; CA, categorical agreement; VME, very major error; ME, major error; mE, minor error; TZP, piperacillin-tazobactam; CAZ, ceftazidime; CZA, Ceftazidime-avibactam; IPM, Imipenem; MEM, meropenem; CIP, ciprofloxacin; LEV, Levofloxacin; GEN, gentamicin; TOB, Tobramycin; SXT, Trimethoprim-sulfamethoxazole; CTX, Cefotaxime.

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The 6-h reading offered the optimal balance between accuracy and speed. Antibiotics such as piperacillin-tazobactam (TZP), ceftazidime-avibactam (CZA), imipenem (IPM), and meropenem (MEM) achieved consistent 100% CA at all readings. Ceftazidime (CAZ) improved from 95.7% CA at 4 h to 97.2% at 6 h, reaching 100% by 16–20 h. The method also successfully identified all extended-spectrum beta-lactamase (ESBL)-producing strains (37% in all *E. coli*, Figure 2). These results demonstrate the strong clinical utility of RAST, particularly at the 6-h reading, for rapid and accurate susceptibility testing of *E. coli*.

RAST for *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*

For *K. pneumoniae*, RAST demonstrated 100% CA across all incubation periods (4 h, 6 h, 16–20 h) through analysis of 9, 6, and 9 isolates at respective timepoints. Complete antibiotic concordance with standard AST was observed (Table 2),

Table 2 Summary of RAST Performance for *K. Pneumoniae* Compared to MIC Results

Antibiotics	Time/h	RAST		BD MIC			CA (%)	VME/ME/mE (%)
		R	S	R	I	S		
TZP	4	1	7	1	0	8	100(8/8)	0/0/0
	6	0	6	0	0	6	100(6/6)	0/0/0
	16-20	1	8	1	0	8	100(9/9)	0/0/0
CAZ	4	1	5	1	0	8	100(6/6)	0/0/0
	6	0	5	0	0	6	100(5/5)	0/0/0
	16-20	2	6	3	0	6	100(8/8)	0/0/0
CZA	4	0	8	0	0	9[a]	100(8/8)	0/0/0
	6	0	6	0	0	6[a]	100(6/6)	0/0/0
	16-20	0	9	0	0	9[a]	100(9/9)	0/0/0
IPM	4	1	7	1	0	8	100(8/8)	0/0/0
	6	0	6	0	0	6	100(6/6)	0/0/0
	16-20	1	8	1	0	8	100(9/9)	0/0/0
MEM	4	1	7	1	0	8	100(8/8)	0/0/0
	6	0	6	0	0	6	100(6/6)	0/0/0
	16-20	1	8	1	0	8	100(9/9)	0/0/0
CIP	4	1	4	1	0	8	100(5/5)	0/0/0
	6	0	5	0	0	6	100(5/5)	0/0/0
	16-20	2	7	2	0	7	100(9/9)	0/0/0
LEV	4	1	6	1	0	8	100(7/7)	0/0/0
	6	0	5	0	0	6	100(5/5)	0/0/0
	16-20	1	8	1	0	8	100(9/9)	0/0/0
GEN	4	0	8	0	0	9	100(8/8)	0/0/0
	6	0	6	0	0	6	100(6/6)	0/0/0
	16-20	1	8	1	0	8	100(9/9)	0/0/0
TOB	4	0	7	0	0	9	100(7/7)	0/0/0
	6	0	6	0	0	6	100(6/6)	0/0/0
	16-20	0	9	0	0	9	100(9/9)	0/0/0
SXT	4	1	7	1	0	8	100(8/8)	0/0/0
	6	0	6	0	0	6	100(6/6)	0/0/0
	16-20	3	6	3	0	6	100(9/9)	0/0/0
CTX	4	1	6	1[a]	0	8[a]	100(7/7)	0/0/0
	6	0	6	0[a]	0	6[a]	100(6/6)	0/0/0
	16-20	3	6	3[a]	0	6[a]	100(9/9)	0/0/0

Note: n = 9 at 4 h, n = 6 at 6 h, and n = 9 at 16–20 h. The AST results marked with a were obtained using the standard disc diffusion method.

Abbreviations: NB, no breakpoint; CA, categorical agreement; VME, very major error; ME, major error; mE, minor error; TZP, piperacillin-tazobactam; CAZ, ceftazidime; CZA, Ceftazidime-avibactam; IPM, Imipenem; MEM, meropenem; CIP, ciprofloxacin; LEV, Levofloxacin; GEN, gentamicin; TOB, Tobramycin; SXT, Trimethoprim-sulfamethoxazole; CTX, Cefotaxime.

confirming clinical reliability. In *P. aeruginosa*, 7 and 3 isolates were analyzed at 6 h and 16 h, while *A. baumannii* testing included 3 isolates evaluated at both 4 h and 6 h. Similarly, the RAST method for *P. aeruginosa* and *A. baumannii* demonstrated 100% CA, with no apparent ATU or UIZ (Table 3 and Table 4). Besides, the method also successfully identified 2 ESBL-producing and 1 carbapenem-resistant *K. pneumoniae*, 1 carbapenem-resistant *P. aeruginosa* and 1 carbapenem-resistant *A. baumannii*. isolate, as shown in Figure 2.

Table 3 Summary of RAST Performance for *P. Aeruginosa* Compared to MIC Results

Antibiotics	Time/h	RAST		BD MIC			CA (%)	VME/ME/mE (%)
		R	S	R	I	S		
TZP	6	0	7[a]	0	0	7	100(7/7)	0/0/0
	16-20	1	2[a]	1	0	2	100(3/3)	0/0/0
CAZ	6	0	7[a]	0	0	7	100(7/7)	0/0/0
	16-20	1	2[a]	1	0	2	100(3/3)	0/0/0
CZA	6	0	7	0	0	7[b]	100(7/7)	0/0/0
	16-20	0	3	0	0	3[b]	100(3/3)	0/0/0
IPM	6	0	7[a]	0	0	7	100(7/7)	0/0/0
	16-20	1	2[a]	1	0	2	100(3/3)	0/0/0
MEM	6	0	7	0	0	7	100(7/7)	0/0/0
	16-20	1	2	1	0	2	100(3/3)	0/0/0
CIP	6	0	7[a]	0	0	7	100(7/7)	0/0/0
	16-20	0	3[a]	0	0	3	100(3/3)	0/0/0
LEV	6	0	7[a]	0	0	7	100(7/7)	0/0/0
	16-20	0	3[a]	0	0	3	100(3/3)	0/0/0
TOB	6	0	7	0	0	7	100(7/7)	0/0/0
	16-20	0	3	0	0	3	100(3/3)	0/0/0

Note: n = 7 at 6 h, and n = 3 at 16–20 h. The results marked with a were indicated that isolates with zone diameters greater than the ATU interval are reported "Susceptible, increased exposure". The AST results marked with b were obtained using the standard disc diffusion method.

Table 4 Summary of RAST Performance for *A. Baumannii* Compared to MIC Results

Antibiotics	Time/h	RAST		BD MIC			CA (%)	VME/ME/mE (%)
		R	S	R	I	S		
SXT	4	1	2	1	0	2	100(3/3)	0/0/0
	6	1	2	1	0	2	100(3/3)	0/0/0
GEN	4	1	2	1	0	2	100(3/3)	0/0/0
	6	1	2	1	0	2	100(3/3)	0/0/0
IPM	4	1	2	1	0	2	100(3/3)	0/0/0
	6	1	2	1	0	2	100(3/3)	0/0/0
MEM	4	1	2	1	0	2	100(3/3)	0/0/0
	6	1	2	1	0	2	100(3/3)	0/0/0
CIP	4	1	2a	1	0	2	100(3/3)	0/0/0
	6	1	2a	1	0	2	100(3/3)	0/0/0
LEV	4	1	2	1	0	2	100(3/3)	0/0/0
	6	1	2	1	0	2	100(3/3)	0/0/0
TOB	4	1	2	1	0	2	100(3/3)	0/0/0
	6	1	2	1	0	2	100(3/3)	0/0/0

Note: n = 3 at 4 h, and n = 3 at 6 h. The results marked with a were indicated that isolates with zone diameters greater than the ATU interval are reported "Susceptible, increased exposure".

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Table 5 UIZ and ATU Observed for Gram-Negative Bacteria

GN Bacteria	Reading Time 4 h		Reading Time 6 h		Reading Time 16–20 h	
	ATU	UIZ	ATU	UIZ	ATU	UIZ
E. coli n (%)	51(9.8)	85(16.4)	19(4.4)	0(0)	16(3.5)	0(0)
K. pneumoniae n (%)	7(7.1)	12(12.1)	3(3.6)	0(0)	1(1.0)	0(0)
A. baumannii n (%)	0(0)	0(0)	0(0)	0(0)	N/T	N/T
P. aeruginosa n (%)	N/A	N/A	0(0)	0(0)	0(0)	0(0)
Overall n (%)	58(9.1)	97(15.2)	22(3.6)	0(0)	17(3.1)	0(0)

Abbreviations: GN, Gram-negative; ATU, area of technical uncertainty; UIZ, unreadable inhibition zone; N/A, not applicable; N/T, not test.

However, the species benefited from extended incubation times, which enhanced the accuracy of the results, thus confirming the efficacy of RAST in the rapid detection of resistant strains among Gram-negative bacteremia.

RAST for Other *Enterobacterales*

In the 139 Gram-negative bacterial isolates, 16 were identified as other *Enterobacterales* excluding *E. coli* and *K. pneumoniae*, as shown in Figure 2. These included 6 *Serratia marcescens*, 5 *Enterobacter cloacae*, 2 *Citrobacter freundii*, 2 *Enterobacter asburiae*, and 1 *Klebsiella aerogenes*. These isolates could not be directly interpreted based on the EUCAST RAST breakpoints.

However, when applying the RAST breakpoints for *E. coli*, 6 isolates were evaluated at 4 h, 8 at 6 h, and 7 at 16–20 h, achieving an overall CAs of 100% across all time points. This suggests that *E. coli* breakpoints may have potential applicability for other *Enterobacterales*. Nevertheless, further validation with larger sample sizes and diverse species is needed to confirm these preliminary findings and ensure reliable interpretation in clinical practice.

UIZ and ATU

The analysis of UIZ and ATU highlighted the significant impact of incubation time on the interpretability of AST results, particularly for *E. coli* and *K. pneumoniae*, as presented in Table 5. At the 4-hour reading, 16.4% of the *E. coli* isolates and 12.1% of the *K. pneumoniae* isolates exhibited UIZ. The overall ATU rate was 9.1% at 4 hours, decreasing to 3.6% at 6 hours and further to 3.1% at the 16–20-hour reading. This trend clearly demonstrates that extending incubation time improves the reliability of susceptibility testing by reducing the occurrence of ambiguous results.

Furthermore, the ATU values for *E. coli* and *K. pneumoniae* were significantly reduced ($P < 0.05$), while the UIZ values were completely resolved by the six-hour reading (Figure 3). The elimination of UIZ at this point highlights the method's capability to deliver rapid and accurate results for these pathogens. The antibiotics most notably impacted by UIZ and ATU included ceftazidime, levofloxacin, ciprofloxacin, and piperacillin-tazobactam. Overall, these findings highlight the importance of optimizing both incubation and reading times in RAST to minimize technical uncertainties, thereby enhancing the accuracy and clinical utility of RAST for Gram-negative bacteria.

Discussion

The timely administration of appropriate antibiotics is critical for managing bacteremia, as delays significantly increase morbidity and mortality.[17] Several federal drug administration-approved mPCR panels have been commercialized as a technology that can quickly identify the causative bacteria and detect resistance genes for BSIs. However, a major limitation is that negative amplification does not necessarily indicate susceptibility.[18,19] The EUCAST RAST method offers a simpler, faster alternative, allowing AST within 0–18 hours after BCs signal positive. However, before performing the RAST, it is essential for us to know the accurate ID of the isolates. MALDI-TOF MS has been proven to directly identify bacteria in positive BCs.[20–22] However, it has many disadvantages such as time-consuming, complicated operation, and expensive.[22] Unfortunately, due to heavy clinical tasks and insufficient personnel in our laboratory, we are unable to carry out this process. However, the ID of the isolates can be performed using a short-term

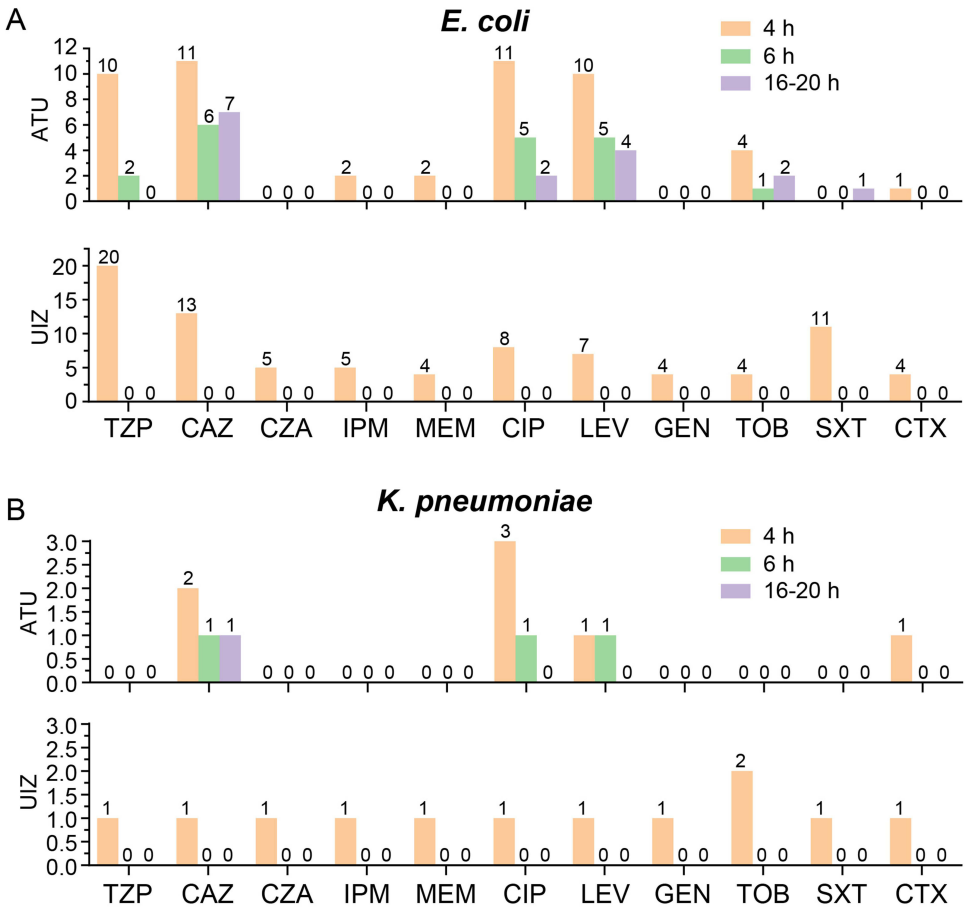

Figure 3 Distribution of ATU /UIZ for *E. coli* and *K. pneumoniae* at different RAST reading times (**A**) Counts of ATU and UIZ events for *E. coli* across all tested antibiotics at 4 h, 6 h and 16–20 h reading times. (**B**) Counts of ATU and UIZ events for *K. pneumoniae* across all tested antibiotics at 4 h, 6 h and 16–20 h reading times.

incubation method, and then combined with RAST results at 4h and/or 6h to interpret susceptibility. When the intended shorter incubation is not possible to achieve, incubation can be extended to 16–20 h for RAST. Then, the ID of the isolates can be performed using conventional methods.

In this study, we successfully integrated RAST into clinical workflows, reducing turnaround time by 16–24 hours without significant additional manpower or costs. Compared to CLSI methods, the EUCAST RAST protocol was more convenient, achieving categorical agreement (CA) rates of 98.9% at 4 h, 99.5% at 6 h, and 99.7% at 16–20 h for *E. coli*. These results align with those of Bianco et al[10] (CA ranged from 98% at 4 h to 99.4% at 16–20 h) and Cherkaoui et al[23] (CA > 95%), demonstrating the practicality of a 6 h reading for routine use. The RAST showed excellent results with low error percentage (mE, ME, VME) meeting the performance standards of the US Food and Drug Administration (FDA) (ME < 3%, VME < 1.5%)[24] (except for Trimethoprim-sulfamethoxazole at the 4-h reading in *E. coli*). However, it should be noted that the absolute number of errors for many of these was only one or two per category but exceeded the allowed proportion. In addition, more than 80% of the RASTs were eventually interpreted, and the proportion would be even higher if other *Enterobacterales* were added, which highlights the significance of our work.

The ATU represents an area where the separation between susceptibility categories is poor. When a result is inside the ATU it cannot be interpreted. Extended incubation improved the interpretability of RAST results by reducing ATU and UIZ. ATU rates declined from 9.1% at 4h to 3.1% at 16–20h, while UIZ was eliminated by 6h. The results at 16–20 h readings were lower than those reported by Jonasson et al,[9] with the average proportion of ATU being 6% across all species. This proportion was also lower than the 5.2% reported by Bianco et al.[10] The introduction of the prolonged incubation (16–20 h) will increase the usefulness of the EUCAST RAST method in clinical laboratories with limited opening hours. Species-specific differences were noted; for example, *P. aeruginosa* showed no growth at 4h,

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whereas *E. coli* and *K. pneumoniae* exhibited higher UIZ rates (16.4% and 12.1%, respectively) that resolved completely with sufficient incubation. Antibiotics such as ceftazidime, levofloxacin, ciprofloxacin, and piperacillin-tazobactam were most affected by these limitations, underscoring the importance of optimized reading times for reliable AST results.

RAST demonstrated high accuracy in detecting ESBL-producing and carbapenem-resistant Gram-negative bacteria. Among the isolates tested, 37.0% of *E. coli* and 11.8% of *K. pneumoniae* were ESBL-producing, while carbapenem resistance was identified in one isolate each of *K. pneumoniae, P. aeruginosa*, and *A. baumannii*. In our study, EUCAST RAST demonstrated 100% CA in determining microbial susceptibility to ceftazidime/avibactam, consistent with the findings reported by Bianco et al.[10] More MDR or Carbapenemase-producing isolates were still needed (for further validation). These findings highlight the clinical utility of RAST in guiding timely, targeted therapy and implementing effective infection control measures. The EUCAST RAST breakpoint only includes *E. coli, K. pneumoniae, Pseudomonas aeruginosa, A. baumannii* in gram-negative bacteria.[8] The difference is that the CLSI document includes all *Enterobacterales, P. aeruginosa* and *Acinetobacter spp*.[7] Additionally, our data suggest that *E. coli* RAST breakpoints could be applied to other *Enterobacterales* with 100% CA and no observed errors, which has not been reported in earlier studies.[25,26] Recent multicenter studies have demonstrated that tentative breakpoints proposed for *Enterobacterales* other than *E. coli/K. pneumoniae* showed overall performances comparable to those observed for *E. coli/K. pneumoniae* (overall CA 98.3%).[10] The accuracy of RAST may be compromised when applied to certain slow-growing Gram-negative bacteria. For example, in the present study, a few strains demonstrated negligible growth at 4 h. False susceptibility or resistance rates may vary depending on the bacterial species. The rates in ceftazidime and tobramycin for *E. coli* were relatively higher, which is consistent with the previously published data.[10,27]

Despite its promising results, this study has some limitations. Firstly, this study was conducted at a single center rather than across multiple centers, which limits the generalizability of the findings. Secondly, the sample size for certain species was relatively small, and there was limited diversity among multidrug-resistant organisms. Thirdly, the study did not include molecular characterization of MDR isolates, such as carbapenemase gene profiling. However, the overall reliability and practicality of the RAST method offer a solid foundation for future research and the potential for expanding clinical guidelines. By significantly reducing diagnostic turnaround times, RAST has the potential to improve clinical workflows and enhance antimicrobial stewardship, especially in resource-limited settings.

Conclusions

This study demonstrates that the EUCAST RAST method, combining short-term incubation ID with RAST reading at 6 h or conventional incubation ID with RAST at 16–20 h, offers a reliable and efficient approach for Gram-negative bacilli directly from positive blood cultures. Tentative breakpoints proposed for other *Enterobacterales* showed satisfactory performances. Despite minor limitations such as ATU, UIZ, and a small sample size, RAST achieved high accuracy, benefiting over 80% of Gram-negative isolates while significantly reducing turnaround time without requiring substantial additional resources. Future studies with larger, more diverse datasets are needed to validate its application, particularly for multidrug-resistant organisms.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval of Studies and Consent for Publication

This study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of the First Affiliated Hospital of Yangtze University, Hubei Province, China (Approval No. LL2023124). The research involved a retrospective analysis of clinical data, ensuring that patient privacy was strictly protected throughout the study. Written informed consent was obtained from all participants, or their legal guardians where applicable, allowing for the use of their medical information in this publication.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this article.

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