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Usefulness of EUCAST rapid antibiotic susceptibility breakpoints and screening cut-off values directly from blood cultures for the inference of β-lactam resistance mechanisms in Enterobacterales

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Background: Reducing the turnaround time for reporting antimicrobial susceptibility testing (AST) results is important for adjusting empirical treatments and may impact clinical outcomes of septic patients, particularly in settings with high antimicrobial resistance. Disc diffusion could be useful for inferring β-lactam resistance mechanisms.

Objectives: To evaluate the usefulness of EUCAST rapid AST (RAST) disc diffusion breakpoints for the screening of resistance mechanisms (sRAST) and interpretive reading of resistance phenotypes to infer ESBL and carbapenemases production in Enterobacterales.

Methods: Blood cultures were artificially spiked with Enterobacterales clinical isolates with well-characterized β-lactam resistance mechanisms ($n=93$), WT phenotypes ($n=26$) and ATCC strains ($n=8$). AST was performed by disc diffusion directly from blood cultures and inhibition zones were manually measured at 4, 6 and 8 h. To infer the presence of resistance mechanisms, EUCAST RAST breakpoints and screening cut-off values (sRAST) combined with the double-disc synergy test (DDS) for ESBLs or aztreonam susceptibility for carbapenemases detection were used.

Results: DDS together with sRAST detected all ESBL producers as early as at 4 h incubation. Cefotaxime was the antibiotic with the highest discriminatory power. The suspicion of carbapenemase production by sRAST at 8 h was possible in 73% of *Klebsiella pneumoniae* and in 100% of *Escherichia coli* carbapenemase-producing isolates. Phenotypic analysis improves the detection of some low hydrolytic carbapenemases (OXA-48 or KPC-3 mutants).

Conclusions: Early detection of β-lactam resistance mechanisms directly from positive blood cultures was possible using sRAST together with the interpretive reading of antibiotic resistance phenotypes. Some carbapenemase types such as OXA-48 might be difficult to infer. Screening-positive isolates should be confirmed using an alternative technique.

Introduction

Antimicrobial resistance in Enterobacterales is increasing worldwide and represents a threat that affects patient outcomes. According to the 2020 ECDC Surveillance Atlas report, in Spain one out of four (26.8%) *Klebsiella pneumoniae* and 14.1% of *Escherichia coli* invasive isolates are resistant to third-generation cephalosporins while resistance to carbapenems is significantly lower, at 4.7% and 0.4%, respectively.[1] Early selection of an appropriate antibiotic treatment in patients with bacteraemia or sepsis is crucial to improve their survival.[2,3] Consequently, rapid communication of identification and antibiotic resistance phenotypes of bacterial isolates grown in blood cultures may have a great impact on patient management and implies a challenge

for clinical microbiology laboratories.[4,5] In the last decades, a great effort has been made by microbiology laboratories and diagnostic companies to shorten the time of antimicrobial susceptibility testing (AST) results, especially in positive blood cultures.[6] Many of these efforts have been associated with rapid technologies such as PCR-based techniques, MALDI-TOF MS platforms, or combined fluorescent *in situ* hybridization (FISH) with real-time microscopy. Real-time PCR-based techniques, like Xpert® Carba-R (GeneXpert, Cepheid, USA) or BioFire FilmArray® blood culture (bioMérieux, Paris, France), are mainly used today as a multiplex assay. Another useful and widely validated platform is Verigene (Luminex, Texas, USA), a nucleic acid microarray that may detect different genera/species of bacteria and the presence of various resistance genes in less than 2.5 h with high sensitivity (~97%) and specificity (~99.5%). Additionally, Accelerate Pheno (Accelerate diagnostics, Arizona, USA) enables the identification of a large number of bacterial species by FISH and the attainment of complete susceptibility profiles with MIC values in 7 h.[4,7] However, many of these techniques are too expensive for a great number of laboratories and may not give complete information of the expression of the resistance mechanism. Nevertheless, phenotypic methods still make a significant contribution to rapid detection of resistance mechanisms. Such is the case for disc diffusion methodology, a cheap, widely standardized and straightforward method, which even provides results on the same working day.[7] This is important because many microbiology laboratories still do not work 24/7 and only release their results during regular morning and afternoon hours.

EUCAST has recently developed and validated breakpoints for rapid AST (RAST) directly from positive blood culture bottles, using disc diffusion, in some species (*E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium* and *Acinetobacter baumannii*).[8,9] Additionally, screening cut-off values for the early detection of ESBL and carbapenemases in *E. coli* and *K. pneumoniae* (screening RAST, sRAST) have been published.[10] Nonetheless, the influence of ESBL or carbapenemase type on their detection has not yet been explored. Moreover, the use of interpretive reading approaches (inference of the antimicrobial resistance mechanisms analysing the susceptibility phenotype) has not yet been explored using disc diffusion data obtained with a RAST protocol.[11]

The objective of this work was to evaluate the usefulness of routine rapid EUCAST disc diffusion breakpoints (RAST) for the screening of resistance mechanisms (sRAST) and interpretive reading of antibiotic resistance phenotypes (based on RAST) to infer β-lactam resistance mechanisms in a collection of clinical Enterobacterales isolates.

Methods

Bacterial isolates and spiked blood cultures

A total of 127 Enterobacterales, including 93 clinical isolates with molecularly characterized β-lactam resistance mechanisms, 26 WT clinical

Table 1. Summary of Enterobacterales isolates included in the study and their corresponding resistance mechanism

	β-Lactam resistance mechanism			
Species	ESBL type (*n*)	Carbapenemase type (*n*)	ESBL + carbapenemase type (*n*)	Other (*n*)
Clinical isolates				
E. coli (*n* = 27)	CTX-M (14) SHV (2)	OXA-48 (6) KPC (2) VIM (1)	SHV + OXA-48 (1) CTX-M + KPC (1)	
K. pneumoniae (*n* = 37)	CTX-M (9) SHV[a] (4) CTX-M + SHV[a] (2)	OXA-48 (4) KPC (3) VIM (2)	CTX-M + KPC (5) CTX-M + OXA-48 (4) CTX-M + NDM (1) SHV[a] + KPC (1)	DHA (2)
Other Enterobacterales[b] (*n* = 29)	CTX-M (9) TEM (2) SHV (1)	VIM (7) OXA-48 (4) KPC (3) IMP (1)	CTX-M + OXA-48 (1) CTX-M + KPC (1)	
WT[c] (*n* = 26)				
Reference strains (ATCC reference number)				
E. coli		NDM-1 (2471)		WT (25922) O157 (12900)
K. pneumoniae	SHV-18 (700603)	KPC (1705)		WT (13442)
Klebsiella oxytoca				WT (700324)
E. cloacae				WT (13047)

[a]All SHV included were different from chromosomic broad-spectrum β-lactamase.
[b]*Proteus mirabilis* (*n* = 1), *Providencia stuartii* (*n* = 1), *E. cloacae* (*n* = 16), *K. aerogenes* (*n* = 2), *Klebsiella oxytoca* (*n* = 3), *Citrobacter freundii* (*n* = 4), *Citrobacter koseri* (*n* = 1), *Serratia marcescens* (*n* = 1).
[c]*E. coli* (*n* = 5), *K. pneumoniae* (*n* = 5), *K. oxytoca* (*n* = 2), *P. mirabilis* (*n* = 4), *Proteus vulgaris* (*n* = 1), *S. marcescens* (*n* = 3), *C. freundii* (*n* = 1), *C. koseri* (*n* = 2), *E. cloacae* (*n* = 3).

isolates (without acquired resistance mechanisms), and 8 reference strains (ATCC, USA), were selected for the study (Table 1). Identification of isolates was confirmed by MALDI-TOF (Bruker, MBT Compass reference library, version 2022) before starting the study.

With the aim of making artificially positive blood cultures, aerobic blood culture bottles (BD BACTEC Plus Aerobic/F) were spiked with a suspension of 100–200 cfu/mL in 5 mL of human donor blood (0.5 McFarland diluted 1:1 000 000), following EUCAST methodology,[12] and incubated until they flagged positive in BACTEC FX instruments (Becton Dickinson, USA).

AST

AST was performed by inoculating disc diffusion plates (Mueller–Hinton agar, Oxoid, UK) using 125 ± 25 µL directly from positive blood culture bottles (less than 18 h after being positive) according to RAST EUCAST recommendations.[13] For resistance mechanism screening, 12 antibiotic discs (Oxoid, Hampshire, UK) were placed onto inoculated agar plates: amoxicillin/clavulanate (20–10 µg), piperacillin/tazobactam (30/6 µg), cefoxitin (30 µg), ceftazidime (10 µg), cefotaxime (5 µg), cefepime (30 µg), aztreonam (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), ceftolozane/tazobactam (30/10 µg) and ceftazidime/avibactam (10/4 µg). Discs were placed as follows to allow the observation of synergy, if present: amoxicillin/clavulanate (top) surrounded by cefepime (left), ceftazidime (right) and cefotaxime (bottom). The distance between the discs was 20 mm centre to centre. Plates were incubated at 35 ± 1°C and examined at 4, 6 and 8 h by two independent observers. If discordances (≥1 mm) were found between readings, medium values were calculated, and inhibition diameter was the closest to a whole number. Inhibition zones were manually measured, double-disc synergy (DDS) tests for ESBL detection were recorded, and AST breakpoints for short incubation (RAST) and for the screening of ESBL and/or carbapenemases (sRAST) in *E. coli* and *K. pneumoniae* were applied.[10] In species other than *E. coli* and *K. pneumoniae*, for which no RAST breakpoints or screening cut-off values for blood cultures are available, *K. pneumoniae* screening breakpoints were used, as they were more restrictive.

Interpretive reading to infer resistance phenotypes

Interpretive reading is the inference of a resistance mechanism based on the analysis of the susceptibility phenotype.[11] Accordingly, interpretive reading for ESBL producers was defined as the presence of synergy between a third- or fourth-generation cephalosporin with amoxicillin/clavulanate acid discs and/or absence or decreased inhibition zone, i.e. resistant or within the area of technical uncertainty (ATU) for cefotaxime and/or ceftazidime discs. Moreover, interpretive reading for carbapenemase producers took into account RAST breakpoints for imipenem, meropenem and ceftazidime/avibactam, and standard breakpoints for aztreonam (as no RAST breakpoints are currently available for this antibiotic) (Table 2).[14,15]

Table 2. Phenotypic criteria used to infer most common carbapenemases

Carbapenemase class type	IPM[a]	MEM[a]	ATM[b]	CZA[a]
A	R	R	R	S
B	R	R	S	R
D	S/r	S/r	S	S
Mutated KPC-3	S	S	R	R

IPM, imipenem; MEM, meropenem; ATM, aztreonam; CZA, ceftazidime/avibactam; R, resistant; S, susceptible, r, decreased susceptibility.
[a]EUCAST RAST breakpoints.[14]
[b]Conventional breakpoints.[15]

Results

All inhibition zones from the different isolates could be clearly read at both 6 and 8 h incubation. However, 20% presented difficulties for reading at 4 h as the inhibition zone was not fully defined. When DDS was considered alone, ESBL detection in *E. coli* and *K. pneumoniae* was low after 4 h of incubation and higher after 6 h or 8 h (Table 3). At 4 h, DDS failed to detect 8/16 (50%) ESBL types in *E. coli* and 6/15 (40%) in *K. pneumoniae*. At 6 h, only one CTX-M-15-producing *K. pneumoniae* could not be detected by DDS.

ESBL detection by DDS in other species (mainly *Enterobacter cloacae*) was challenging and not possible at 4 h in 10/12 (83.3%) isolates, at 6 h in 6/12 (50%) isolates and at 8 h in 5/12 (41.7%) isolates. Four out of 12 ESBL producers (3 *E. cloacae* and 1 *Klebsiella aerogenes*) were not detected using DDS at any timepoint.

All *E. coli* and *K. pneumoniae* ESBL producers could be inferred at 4, 6 and 8 h using cefotaxime and ceftazidime RAST

Table 3. Detection of ESBL-producing Enterobacterales by different phenotypic approaches

		No. of ESBL-producing isolates detected after incubation at different timepoints		
Identification approach	Species (n)	4 h (%)	6 h (%)	8 h (%)
Positive synergy (DDS)	*E. coli* (16)	8 (50)	16 (100)	16 (100)
	K. pneumoniae (15)	10 (67)	14 (93)	15 (100)
	Other species[a] (12)	2 (17)	6 (50)	8 (67)
sRAST (CAZ)	*E. coli* (16)	11 (69)	10 (63)	11 (69)
	K. pneumoniae (15)	14 (93)	14 (93)	13 (87)
	Other species (12)	11 (92)	11 (92)	11 (92)
sRAST (CTX)	*E. coli* (16)	16 (100)	16 (100)	16 (100)
	K. pneumoniae (15)	15 (100)	15 (100)	14 (93)
	Other species (12)	12 (100)	12 (100)	12 (100)
sRAST (CAZ + CTX)	*E. coli* (16)	16 (100)	16 (100)	16 (100)
	K. pneumoniae (15)	15 (100)	15 (100)	14 (93)
	Other species (12)	12 (100)	12 (100)	12 (100)
DDS + RAST (CAZ/CTX)	*E. coli* (16)	16 (100)	16 (100)	16 (100)
	K. pneumoniae (15)	15 (100)	15 (100)	15 (100)
	Other species (12)	12 (100)	12 (100)	12 (100)

CAZ, ceftazidime; CTX, cefotaxime; sRAST: screening cut-off values for the inference of resistance mechanisms.[10]
[a]*E. cloacae* (*n* = 8), *K. aerogenes* (*n* = 2), *P. stuartii* (*n* = 1), *P. mirabilis* (*n* = 1).

Table 4. Detection of carbapenemase-producing isolates using the corresponding screening cut-off values (sRAST) for *E. coli* and *K. pneumoniae*[10]

Identification approach	Species (n)	Number of carbapenemase-producing isolates detected		Type of carbapenemase not detected
		6 h[a] (%)	8 h (%)	Enzymes
Carbapenemase (MEM)	*E. coli* (9)	8 (89)	9 (100)	VIM-2
	K. pneumoniae (9)	7 (78)	8 (89)	OXA-48
	Other species[b] (15)	13 (87)	14 (93)	KPC-3, VIM-2
ESBL + carbapenemase (MEM)	*E. coli* (2)	2 (100)	2 (100)	—
	K. pneumoniae (11)	7 (64)	8 (73)	OXA-48, KPC-3
	E. cloacae, *K. oxytoca*	2 (100)	2 (100)	—

For other species, the criteria for *K. pneumoniae* were used.
MEM, meropenem.
[a]There is no screening cut-off value for 4 h incubation.
[b]*E. cloacae* ($n=7$), *C. freundii* ($n=4$), *K. oxytoca* ($n=2$), *C. koseri* ($n=1$), *S. marcescens* ($n=1$).

Table 5. Phenotypic results, using RAST criteria,[14,15] of carbapenemase and ESBL + carbapenemase-producing isolates not detected by screening cut-off values (sRAST)[10]

			IPM		MEM		ATM		CZA	
			6 h	8 h	6 h	8 h	6 h	8 h	6 h	8 h
Carbapenemase (MEM)	*E. coli*	VIM-2	S	**ATU**	S	S	S	S	S	S
	K. pneumoniae	OXA-48 (1)	**ATU**	S	S	S	S	S	S	S
		OXA-48 (2)	S	S	S	S	S	S	S	S
	E. cloacae	KPC-3	S	S	S	S	R	R	S	S
		VIM-2	S	S	S	S	R	R	**ATU**	S
ESBL + carbapenemase (MEM)	*K. pneumoniae*	OXA-48 (1)	S	S	S	S	R	R	S	S
		OXA-48 (2)	S	S	S	S	R	R	S	S
		KPC-3 (1)	S	S	S	S	R	R	S	**ATU**
		KPC-3 (2)	S	S	S	S	R	R	**R**	**R**

Antibiotic and hour combinations that may raise suspicion of the presence of a carbapenemase are indicated by bold type.
IPM, imipenem; MEM, meropenem; ATM, aztreonam; CZA, ceftazidime/avibactam; R, resistant; S, susceptible.

breakpoints together (as recommended by EUCAST), with the exception of one *K. pneumoniae* isolate harbouring SHV-12 that failed to be detected at 8 h by 1 mm above the screening cut-off value (<16 mm). If only ceftazidime RAST breakpoints were used for the analysis, a lower number of ESBL producers could be suspected (Table 3) than when using cefotaxime breakpoints alone. The detection of all ESBL producers in all species was possible when both approaches were used together (RAST cefotaxime and ceftazidime and DDS results).

As for the carbapenemase detection by sRAST (meropenem <20 mm in *E. coli* or <18 mm in *K. pneumoniae*) (Table 4), we were able to recognize all carbapenemase producers except one VIM-2 in *E. coli* and two OXA-48-producing *K. pneumoniae* at 6 h. This might be due to low hydrolytic activity of OXA-48. Additionally, in *E. cloacae*, only one KPC-3 and one VIM-2 could not be detected, but in both cases inhibition zones were close to the screening cut-off value (1 mm of difference). Interestingly, in three out of these five isolates, phenotypic analysis allowed us to suspect the presence of a carbapenemase (Table 5) as inhibition diameters of carbapenems other than meropenem were included in ATU ranges. All but one OXA-48-producing *K. pneumoniae* isolate could be suspected using imipenem RAST breakpoints and phenotypic analysis (Table 2).

Regarding the group of ESBL and carbapenemase producers, at 6 h we could not detect 2/6 KPC-3 (33%) and 2/4 OXA-48 (50%) in *K. pneumoniae* using sRAST. In the first case, failure was due to the presence of mutated KPC-3 conferring resistance to ceftazidime/avibactam and collateral susceptibility to meropenem. Phenotypic analysis was able to infer the presence of a mutated KPC carbapenemase, as this takes other carbapenem antibiotics into account and not only meropenem susceptibility.

Among other resistance mechanisms, two cephamycinase-producing *K. pneumoniae* (DHA) and one *K. oxytoca* hyperproducing K1 were detected as possible ESBL producers using sRAST, which would require further screening (data not shown).

All WT isolates exhibited non-resistant or decreased susceptibility phenotypes using either RAST or sRAST criteria.

Discussion

During the last three decades, many clinical microbiology laboratories around the world have performed disc diffusion AST directly from blood culture bottles using 16–20 h incubation standardized breakpoints for clinical interpretation. However, it was not until recently that several studies have emphasized the need to standardize the methodology for discriminating between resistant and WT populations within a short period of time.[16,17] Therefore, breakpoints for RAST from positive blood cultures and cut-off values for screening of resistance mechanisms have been recently validated by EUCAST.[8,9] These studies observed how the diameter of the inhibition zone changes over incubation time, and the usefulness of introducing an ATU to attenuate the variations inherent to the technique.

In this work, the usefulness of the EUCAST screening cut-off values for the screening of resistance mechanisms in spiked blood cultures has been tested using a bacterial collection with well-characterized resistance mechanisms. Moreover, the ability to suspect β-lactam resistance mechanisms has been compared with the interpretive reading of the antibiotic susceptibility phenotype based on the short incubation breakpoints (RAST). ESBL detection in *K. pneumoniae* and *E. coli* was possible by DDS from 6 h incubation using manual reading. However, due to AmpC hyperproduction among other species, only 67% of isolates were correctly considered as ESBL producers.

When using sRAST cut-offs, resistance may be overestimated if it is not checked with a second technique, as recommended by EUCAST. This is because other β-lactam resistance mechanisms (different from ESBLs or carbapenemases) can reduce inhibition zones to third-generation cephalosporins, such as plasmidic cephamycinases.

On the other hand, detection of resistance mechanisms by phenotypic approaches may have the drawback of missing low hydrolytic or low-expressed enzymes, such as OXA-48-type carbapenemases.[18] Combining phenotypic methods (such as sRAST) with other phenotypic or genotypic methods in patients with risk factors or high suspicion of multidrug resistance may be optimal.

In our study we were unable to suspect the presence of nine carbapenemase enzymes (four OXA-48, three KPC and two VIM) in three species (*E. coli*, *K. pneumoniae* and *E. cloacae*) at any timepoint. Therefore, some carbapenemase-producing isolates were not accurately detected at 6 h using only current sRAST, and interpretive reading using other antibiotics (e.g. imipenem, ceftazidime/avibactam) to infer resistance mechanisms should be included. The same occurred with an SHV-12-producing *K. pneumoniae*, finally detected by DDS.

In most cases, differences of only 1 mm can lead to errors in interpretation thus it is important to perform a thorough analysis of phenotypic interpretation. As described by Åkerlund and colleagues, the inhibition zones of strains with resistance mechanisms tend to decrease with incubation over time. These authors suggested that if an automated reading method was used, it could be detected more precisely and in a short lapse of time.[9]

Early detection of β-lactam resistance mechanism permits preliminary AST results to be available in the same shift and consequently antibiotic empirical treatments may be quickly adjusted. The clinical impact of early AST results has been explored by different studies and authors. Berinson and colleagues found that 30 day mortality was reduced, although the difference was not significant.[19] Other studies conducted using Accelerate Pheno showed a positive impact in time to definitive therapy, shorter duration of antibiotic therapy, and shorter hospital length of stay, especially coupled with an antimicrobial stewardship programme.[20,21]

The main drawbacks of our work are that screening cut-off values are not yet available for some Enterobacterales species and those for *K. pneumoniae* were used instead. More data would be required to establish specific breakpoints for these species. Also, our collection of isolates does not include many strains with infrequent resistance mechanisms (e.g. plasmidic cephamycinases). Moreover, it reflects isolates from a single-centre study. Lastly, manual reading of inhibition zones can lead to errors in interpretation with consequent over- or under-estimation of resistance. Nevertheless, we were able to demonstrate that combining RAST, sRAST and interpretive reading of susceptibility phenotypes can enhance the detection of β-lactam resistance mechanisms in Enterobacterales directly from positive blood cultures.

Conclusions

EUCAST sRAST cut-off values allow the suspicion of ESBL and carbapenemase production in *E. coli*, *K. pneumoniae* and, to some extent, in other Enterobacterales. Based on the results from this study, their application together with the interpretation of antibiotic susceptibility phenotypes by a clinical microbiologist would enable early detection of resistance mechanisms and potentially early adjustment of empirical treatments. Finally, disc diffusion is an easy technique and is useful for the early and confident communication of antibiotic resistance mechanisms from positive blood cultures.

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Transparency declarations

The authors declare no conflicts of interest.

Data availability

The raw data supporting the results of this study will be made available upon reasonable request from the corresponding author.

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