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Evaluation of standardized automated rapid antimicrobial susceptibility testing of Enterobacterales-containing blood cultures: a proof-of-principle study

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Background: Rapid antimicrobial susceptibility testing (RAST) of bacteria causing bloodstream infections is critical for implementation of appropriate antibiotic regimens.

Objectives: We have established a procedure to prepare standardized bacterial inocula for Enterobacterales-containing clinical blood cultures and assessed antimicrobial susceptibility testing (AST) data generated with the WASPLab™ automated reading system.

Methods: A total of 258 blood cultures containing Enterobacterales were examined. Bacteria were enumerated by flow cytometry using the UF-4000 system and adjusted to an inoculum of 10^6 cfu/mL. Disc diffusion plates were automatically streaked, incubated for 6, 8 and 18 h and imaged using the fully automated WASPLab™ system. Growth inhibition zones were compared with those obtained with inocula prepared from primary subcultures following the EUCAST standard method. Due to time-dependent variations of the inhibition zone diameters, early AST readings were interpreted using time-adjusted tentative breakpoints and areas of technical uncertainty.

Results and conclusions: Inhibition zones obtained after 18 h incubation using an inoculum of 10^6 cfu/mL prepared directly from blood cultures were highly concordant with those of the EUCAST standard method based on primary subcultures, with categorical agreement (CA) of 95.8%. After 6 and 8 h incubation, 89.5% and 93.0% of the isolates produced interpretable results, respectively, with CA of >98.5% and very low numbers of clinical categorization errors for both the 6 h and 8 h readings. Overall, with the standardized and automated RAST method, consistent AST data from blood cultures containing Enterobacterales can be generated after 6–8 h of incubation and subsequently confirmed by standard reading of the same plate after 18 h.

Introduction

Bloodstream infections are associated with high mortality rates that are directly related to the timing in administration of the first effective antibiotic(s).[1–5] In patients with suspected sepsis, empirical antibiotic therapy based on clinical and epidemiological data is immediately initiated after blood sampling. Although Gram staining and rapid microbial identification from blood culture bottles by MALDI-TOF MS are helpful to optimize empirical therapy, antimicrobial susceptibility testing (AST) is critical for prescription of targeted antibiotics, especially when considering the increasing incidence of antibiotic resistance.[6–9]

Disc diffusion remains the most widely used method for AST. It is cost-effective, flexible, easy, rapid to perform and allows for detection of synergistic and antagonistic phenomena, heterogeneous resistance and contamination.[10] Depending on the species, disc diffusion requires 16–24 h of incubation before reading of the results. Faster AST methods, such as automated broth microdilution (i.e. VITEK 2, BD Phoenix system, Accelerate Pheno system), are increasingly used in clinical microbiology laboratories,[11,12] although technical limitations and workflow constraints limit the utility of these methods. Other approaches based on MALDI-TOF-MS,[13,14] PCR,[15] nanomechanical sensors,[16] light scattering[17] or colorimetric tests[18] are also being developed for rapid detection of resistance. However, they require expensive equipment and are time-consuming and labour intensive. Clearly, the ease and low cost of disc diffusion represent great advantages that are difficult to match.

One possibility for reducing the delay between the detection of bacterial growth and AST data readings is to shorten the incubation time. Recent studies have shown that early readings of AST

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(after 6–8 h of incubation) provide accurate results for most species–drug combinations.[19–23] However, due to time-dependent variations of the inhibition zone diameters, AST results after short incubation times (6–8 h) cannot be interpreted using the same criteria as those for the standard incubation time (18–24 h). Based on these observations, we have recently conducted a study where we compared early readings of AST with the EUCAST standard method using a fully automated system. On this basis, we have defined time-adjusted tentative clinical breakpoints (CBPs) and areas of technical uncertainty (ATUs), where the clinical interpretation of the growth inhibition zones is uncertain due to poor separation of susceptible and resistant isolates, for interpretation of AST after short incubation times (6–8 h) for Enterobacterales and clinically relevant antibiotics.[24]

For blood cultures with clinically relevant pathogens, an additional means to shorten the time to reportable AST results is to eliminate the need for subculturing for isolation of the pathogen. In this regard, EUCAST has recently released recommendations for rapid AST (RAST) after short incubations (4, 6 and 8 h) performed directly from positive blood cultures.[25,26] The main drawback of the current RAST methodology is the lack of standardized bacterial inocula and the need to repeat RAST with EUCAST standard AST for confirmation.

Standardization of the AST inoculum can be accomplished by various manipulations using a number of methods based on chemical lysis of human blood cells, differential centrifugation or sedimentation, or the use of serum separator tubes. Although encouraging AST results have been obtained with these methods, the significant increase in workload makes them inconvenient for routine diagnostics.[27–29] In principle, fully automated flow cytometer systems allow rapid and accurate determination of bacterial numbers in several types of clinical sample including urine and other body fluids.[30] We here report that bacterial populations in blood cultures containing Enterobacterales can be accurately and rapidly enumerated with the Sysmex UF-4000 fluorescence flow cytometer analyser. Based on the quantification of bacteria in blood cultures by fluorescence flow cytometry, we developed a simple and inexpensive method to standardize inocula for AST.

Using the fully automated WASPLab™ system (Copan Italia),[31] we evaluated 258 blood cultures containing Enterobacterales for the performance of AST with standardized inocula prepared directly from blood cultures after 6, 8 and 18 h of incubation. While the results after 18 h incubation were comparable to those of the EUCAST standard method based on 18 h AST from primary subcultures of blood cultures, AST data at 6 and 8 h incubation were interpreted with time-adapted CBPs and ATUs previously defined for early readings of AST from pure cultures.

Materials and methods

Specimen collection

The study was conducted over a period of 20 months between March 2018 and October 2019 in the clinical microbiology laboratory of the Institute of Medical Microbiology, University of Zurich. Blood culture samples were collected in BACT/ALERT bottles and incubated in the Virtuo microbial detection system (bioMérieux, La Balme-les-Grottes, France) until detection of bacterial growth. Growth-positive bottles were subjected to Gram staining and only those containing Gram-negative monocultures were included in the study ($n = 211$). Isolates of the same species collected from the same patient were considered as duplicates and discarded. In addition, 47 blood cultures spiked with 37 carbapenemase-producing and 10 ESBL-producing Enterobacterales (for details see Table S1, available as Supplementary data at *JAC* Online) were studied. The whole collection comprised 258 Enterobacterales and was used to assess the performance of the standardized automated AST (aAST) method after 6 and 8 h incubations. A part of the collection, referred to as Group 1 (Table S1), was analysed to determine the best inoculum for the standardized aAST method after 18 h incubation.

MALDI-TOF MS identification

Positive blood cultures were subcultured on agar plates prior to identification by MALDI-TOF MS. Samples were prepared by the direct transfer–formic acid method[32] and species identification performed using a Bruker Biotyper MALDI-TOF MS System (Bruker Corporation).

Standard AST

Standard AST was performed from primary subcultures of blood cultures by disc diffusion according to EUCAST guidelines[33] with antibiotic discs (i2a, Perols, France) and Mueller–Hinton agar plates (BD, Franklin Lakes, NJ, USA). The Sirweb/Sirscan system (i2a) was used to measure the inhibition zone diameters.[34]

Preparation of spiked blood cultures

Four millilitres of sterile total human blood were inoculated with 100 cfu/mL of bacteria. The inocula were prepared by diluting a 0.5 McFarland suspension in saline solution. Blood cultures were incubated for 18 h at 37°C.

Enumeration of bacteria in the blood cultures

Bacterial populations in blood cultures were quantified with the fully automated flow cytometry analyser UF-4000 according to the manufacturer's instructions using a body fluid analysis mode (Sysmex Europe GmbH, Germany). In parallel, plate counting was performed to determine the number of viable cells.

aAST with standardized inocula

aAST was performed as previously described.[20] After determining bacterial numbers with the Sysmex UF-4000 system, blood cultures were diluted in 0.9% NaCl to obtain suspensions containing 10^6 and 10^7 cfu/mL. Mueller–Hinton agar plates were automatically inoculated with 60 μL of bacterial suspensions and streaked with the fully automated WASP™ system (Copan Italia S.p.A., Brescia, Italy). Discs impregnated with antibiotics (Oxoid Limited, Basingstoke, UK) were manually placed onto plates using a standard distributor and were reintroduced into the WASP™ system where they were automatically transported to and incubated in a 36 ± 2°C incubator. Pictures of the plates were taken after 6, 8 and 18 h of incubation and growth inhibition diameters were automatically measured by the WASPLab™ reading software. Plate images were inspected by an experienced technician using a light background for plates incubated for 6 and 8 h and a dark background for those incubated for 18 h and, when required (i.e. by failure to detect clear inhibition zones), diameters were adjusted. AST results after 18 h incubation were interpreted according to EUCAST guidelines version 8.0,[33] while those after 6 and 8 h incubation were categorized according to the time-adapted tentative breakpoints and ATUs.[24] Clinical categorization error rates were defined according to ISO 20776-2.[35] Categorical agreement (CA) indicated that the isolates were classified in the same susceptibility category by the two tests, while essential agreement (EA) was defined as $\leq$3 mm difference between the results determined by both tests.[19]

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Results

Sysmex UF-4000 accurately quantifies bacteria in blood samples

Sysmex UF-4000 fully automated flow cytometer allows for quantitative bacterial counts in urines and other body fluids.[30,36] However, quantification of bacteria in blood cultures, which contain massive amounts of human cells, is not included in the manufacturer's specifications. To investigate the impact of blood cells on the accuracy of bacterial enumeration, blood cultures were spiked with *Escherichia coli* ATCC 25922 and diluted in 0.9% NaCl at different ratios and analysed in triplicate. In parallel, samples were plated for counting of viable cells. We found that with 10^{-2}, 10^{-3} and 10^{-4} dilutions, bacterial counts determined by Sysmex UF-4000 were nearly identical to the numbers of viable cells assessed by plating (Figure S1). On the basis of these results and for technical convenience, we chose a 2×10^{-3} dilution (10 μL blood culture in 5 mL of 0.9% NaCl) for determination of bacterial numbers in blood cultures.

Determination of the bacterial numbers in clinical blood cultures

Bacterial numbers in 181 blood cultures as determined by Sysmex UF-4000 were mostly above 5×10^{8} cfu/mL and displayed a very good linear agreement with the bacterial counts as per agar plating (mean of the fold difference ± SD was 1.3 ± 0.98, see Figure 1). Overall, the agreement between the two methods was maintained irrespective of the bacterial load, which ranged between approximately 1×10^{7} and 4×10^{9} cfu/mL.

Determination of the optimal bacterial numbers for aAST

According to EUCAST standard guidelines for AST by disc diffusion,[37] bacterial colonies are suspended in saline solution to obtain a turbidity of 0.5 McFarland, corresponding to approximately 1.5×10^{8} cfu/mL for *E. coli*. A swab is then used for spreading the bacterial suspension over an agar plate. In contrast, in the WASPLab™ system an inoculation loop is dipped into the bacterial suspension and 60 μL thereof is streaked over a rotating agar plate. To determine the inoculum allowing the spreading of equivalent numbers of bacteria on the plates, serial dilutions of blood cultures spiked with *E. coli* ATCC 25922 were used for AST with the WASPLab™ system. The best agreement between the inhibition zone diameters generated by aAST and the EUCAST targets[38] was obtained with suspensions containing 10^{7} and 10^{6} cfu/mL (Table S2, Figure S2). The average mean difference between the inhibition zone diameters and their respective targets was −1.0 ± 0.8 mm and 0.9 ± 0.5 mm, when aAST was performed with suspensions corresponding to 10^{7} and 10^{6} cfu/mL, respectively. Of note, when plates were inoculated directly with positive blood cultures (containing on average approximately 10^{9} cfu/mL), inhibition diameters for all antibiotics were significantly smaller than their respective targets (mean difference −6.2 ± 1 mm), confirming that such high inocula significantly affect AST results. On the basis of

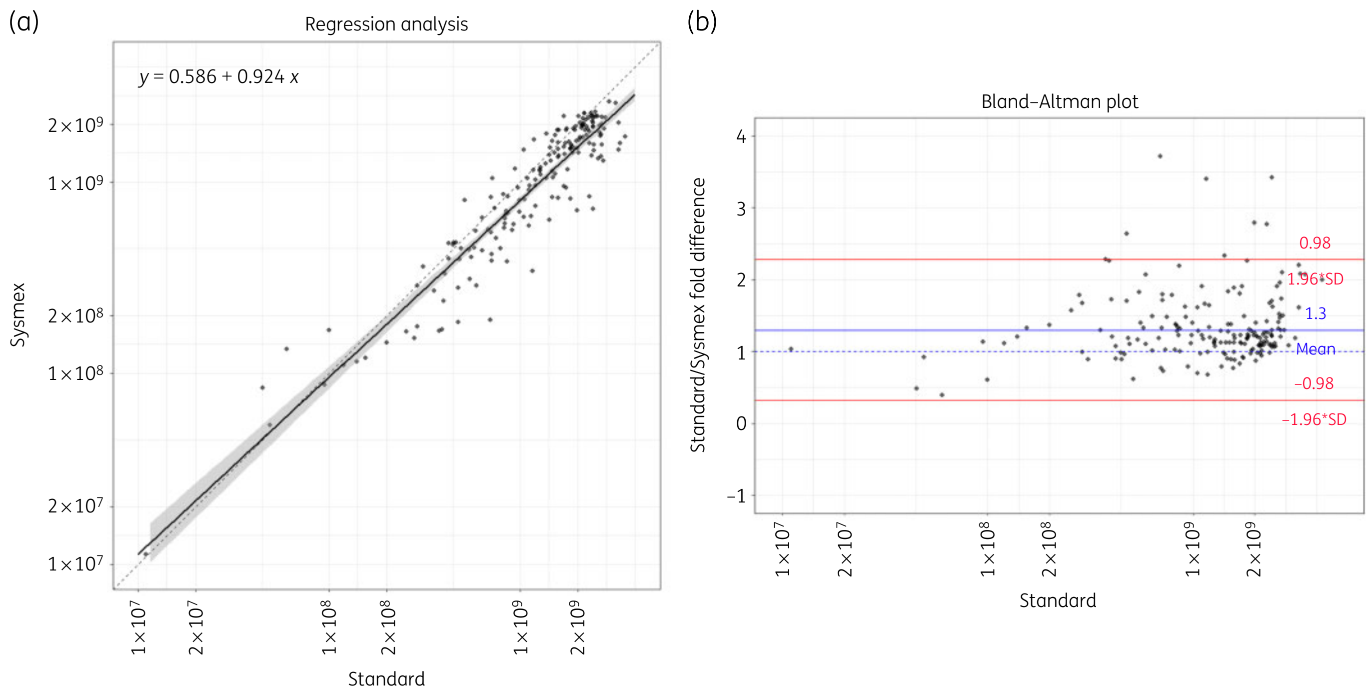

Figure 1. Enumeration of bacteria in blood cultures. Correlation between the bacterial numbers in 181 blood culture samples containing Enterobacterales determined by plate counting (Standard) and the Sysmex UF-4000 flow cytometry analyser (Sysmex). (a) Linear regression analysis. The dotted 45° line represents 100% EA. The 95% CI is depicted in grey. (b) Bland–Altman plot. Dots represent the fold difference between bacterial counts determined by the standard method and by Sysmex plotted against the values obtained by the standard method. Identity is indicated by the dotted blue line. The continuous blue line indicates the mean of the fold difference between the measurements obtained with the two methods. The red lines indicate the 95% limits of agreement between measurements.

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these findings, we selected 10^7 and 10^6 cfu/mL to perform RAST with clinical samples.

Based on the enumerations by Sysmex, 181 blood cultures were diluted with 0.9% NaCl to achieve average suspensions of 10^6 and 10^7 cfu/mL and used for aAST, with the results analysed after 18 h of incubation. An EA between the EUCAST standard method and aAST was obtained in 88.8% of the cases with the 10^6 cfu/mL inoculum and in 80.3% with the 10^7 cfu/mL inoculum (Tables S3 and S4 and Figure S3). The mean differences between EUCAST standard and aAST performed with the 10^6 and 10^7 cfu/mL inocula were -0.2 ± 2.2 and 1.0 ± 2.6 mm, respectively. The difference between EUCAST and aAST was significantly higher with the 10^7 cfu/mL than with the 10^6 cfu/mL inoculum for the susceptible strains (average mean of 1.0 ± 2.6 versus -0.2 ± 2.2 mm, respectively), while the difference was nearly identical for the resistant and 'susceptible by increased exposure' (for simplicity hereafter termed as 'intermediate') populations, indicating that the inoculum size mostly affected the results of the susceptible strains. (Tables S3 and S4). Overall, with a standard incubation time of 18 h the best agreement with the EUCAST method was achieved with bacterial suspensions containing 10^6 cfu/mL.

aAST at 18 h using a standard inoculum prepared directly from blood cultures versus the EUCAST standard method based on primary subcultures

The aAST results of 258 positive blood samples prepared with an inoculum of 10^6 cfu/mL were first analysed after the standard 18 h incubation time. Altogether, of the 3806 tested organism–drug combinations, the mean difference between the inhibition zones obtained with the aAST after 18 h and those with the EUCAST method was 0 ± 2.3 mm (Table 1). Overall CA was 95.8%, ranging from 89.5% for piperacillin/tazobactam to 98.8% for cefpodoxime. The percentages of minor errors (mEs), major errors (MEs) and very major errors (vMEs) were 2.3%, 1.6% and 2.5%, respectively. The highest numbers of mEs were observed for piperacillin/tazobactam (23/258), imipenem (15/250) and cefepime (15/258). For amoxicillin/clavulanic acid and ertapenem, relatively high numbers of MEs (11/258 and 10/258, respectively) and vMEs (12/258 and 2/258, respectively) were found. All clinical categorization errors corresponded to isolates with values very close to the CBPs (Figures 2 and 3). In summary, these data revealed that standardized aAST directly from positive blood cultures can generate reliable 18 h AST results for Enterobacterales and several clinically relevant antibiotics.

Based on this premise, we subsequently analysed the aAST results at 6 and 8 h using the tentative breakpoints and ATUs we proposed for the reading of aAST at early incubation times using inocula prepared from pure cultures of Enterobacterales.[24]

Reading at 6 and 8 h

The results obtained when applying time-adjusted tentative breakpoints and ATUs (Table S5) to the 6 h readings are summarized in Table 2 and depicted in Figures 2 and 3. Overall, of the 3806 species–drug combinations tested, 3405 (89.5%) were interpretable and classified as susceptible or resistant, while 401 (10.5%) fell within the ATUs. For susceptible and resistant strains, AST results were interpretable in 91.9% and 87.5% of the cases, respectively. Due to the overlap of ATUs and intermediate zones (Figure 3), intermediate strains were interpretable in only a few cases. Of the 3405 interpretable results, 3365 (98.8%) were correctly categorized as susceptible or resistant, with mE, ME and vME rates of 0.4%, 0.5% and 1.2%, respectively. The results of aAST at 8 h, applying the tentative early reading breakpoints and ATUs, are presented in Table 3 and depicted in Figures 2 and 3. Compared with the 6 h reading, the number of interpretable results was significantly higher (93.0% versus 89.5%). Importantly, the proportion of strains correctly categorized as resistant or susceptible (98.4%) as well the rates of mE, ME and vME (0.8%, 0.6% and 1.6%, respectively) remained nearly the same. Overall, due to the better separation of the susceptible and resistant strains, reading aAST after 8 h incubation generated a higher number of interpretable results (Figure 3).

Since ATUs mostly coincide or encompass the intermediate zones, rates of interpretable results were, on average, higher for antibiotics without a defined intermediate zone, i.e. cefuroxime, cefpodoxime, ceftriaxone and ertapenem, than for those for which Enterobacterales have intermediate phenotypes. Moreover, for the β-lactam/inhibitor combinations amoxicillin/clavulanic acid and piperacillin/tazobactam, the significant overlap of susceptible and resistant populations within the ATUs (Figure 3b and c) caused high numbers of uncertain isolates (28.3% and 19.4% for the 6 h timepoint, respectively). In addition, we note that only 47.1% and 52.9% of the imipenem-resistant isolates were interpretable at 6 h and 8 h, respectively. However, this did not result in vMEs, as the remaining resistant isolates fell within the ATU (Figure 3). These findings were consistent with previously reported results of rapid aAST from pure cultures.[24]

Detection of CPE and ESBL producers at 6 and 8 h

All 28 ESBL producers had inhibition zone diameters for cefpodoxime well below the tentative screening cut-off diameter of <20 mm (Table 4), allowing for the detection of ESBLs in all these strains. Moreover, synergy between cephalosporins (cefepime and/or ceftriaxone) and the β-lactamase inhibitor clavulanic acid became visible after just 6 h of incubation, thus promptly confirming the presence of ESBLs in all suspected isolates.

All tested carbapenemase-producing Enterobacterales (CPE) presented inhibition zone diameters for meropenem below the tentative screening cut-off diameter <25 mm after just 6 h incubation (Table 5). Thus, carbapenemase production was correctly suspected at the 6 h reading in all CPE.

Discussion

The number of bacterial cells in positive blood cultures can vary considerably (from 5×10^7 to 4×10^9 cfu/mL, Figure 1), mostly depending on when the culture is taken out of the system and analysed. Since the great majority of samples became positive during the night, they remained in the incubator for several additional hours, allowing cultures to reach a plateau phase. As a consequence, bacterial numbers were often equal to or above 10^9 cfu/mL. Based on the assumption that, in diagnostic laboratories, the bulk of blood cultures become positive overnight, the Comité de l'Antibiogramme de la Société Française de

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Table 1. Comparative analysis of aAST at 18 h using a 10^6 cfu/mL inoculum directly from blood cultures with that of EUCAST standard method based on primary subcultures of blood cultures ($n = 258$)

Antibiotic	Total	EA (%) ±≤3 mm	CA	Clinical categorization error			Δmean EUCAST − aAST 18 h ± SD (mm)				Clinical categorization[a] (%)		
				mE (%)	ME (%)	vME (%)	all	S	I	R	S	I	R
Amoxicillin/ clavulanic acid	258	236 (91.5)	235 (91.1)		11	12	−0.6±2.1	−0.5±2.1		−0.6±2.1	95 (36.8)		163 (63.2)
Piperacillin/ tazobactam	258	232 (89.9)	231 (89.5)	23	3	1	0.1±2.2	0.1±2.2	0.5±2.8	−0.1±2.2	175 (67.8)	15 (5.8)	68 (26.4)
Cefuroxime	249	238 (95.6)	244 (98.0)		3	2	0.2±1.5	0.4±1.6		−0.2±1.1	144 (57.8)		105 (42.2)
Cefpodoxime	257	245 (95.3)	254 (98.8)		1	2	0.0±1.5	0.1±1.8		−0.1±1.0	151 (58.8)		106 (41.2)
Ceftriaxone	258	224 (86.8)	254 (98.4)	4			−0.5±2.2	−0.6±2.3		−0.4±1.9	161 (62.4)		97 (37.6)
Cefepime	258	223 (86.4)	242 (93.8)	15	1		−0.3±2.4	−0.4±2.3	−2.3±3.9	0±2.2.0	169 (65.5)	6 (2.3)	83 (32.2)
Ertapenem	258	201 (77.9)	246 (95.3)		10	2	0.7±3.0	0.6±2.7		1.0±3.7	201 (77.9)		57 (22.1)
Imipenem	250	205 (82.0)	235 (94.0)	15			−1.0±2.5	−0.8±2.5	−0.7±1.7	−2.5±2.8	220 (88.0)	13 (5.2)	17 (6.8)
Meropenem	258	220 (85.3)	246 (95.3)	12			0.4±2.5	0.5±2.5	−0.1±2.6	−0.6±2.4	224 (86.8)	14 (5.4)	20 (7.8)
Nalidixic acid	251	227 (90.4)	244 (97.2)	7			−0.2±1.9	−0.2±2.3	−2.0±2.1	−0.1±1.1	145 (57.8)	9 (3.6)	97 (38.6)
Ciprofloxacin	244	192 (78.7)	231 (94.7)	12	1		0.0±2.7	−0.1±3.2	−0.1±3.4	0.1±1.2	156 (63.9)	7 (2.9)	81 (33.2)
Norfloxacin	251	215 (85.7)	244 (97.2)		5	2	−0.1±2.6	−0.1±3.0		0.0±1.5	162 (64.5)		89 (35.5)
Amikacin	246	233 (94.7)	240 (97.6)		1	5	0.1±1.8	0.1±1.8		−0.4±1.2	224 (91.1)		22 (8.9)
Gentamicin	255	235 (92.2)	251 (98.4)		3	1	0.4±1.9	0.6±2.0		−0.3±1.3	200 (78.4)		55 (21.6)
Tobramycin	255	231 (90.6)	250 (98.0)		4	1	0.3±2.1	0.4±2.3		−0.1±1.3	186 (72.9)		69 (27.1)
Total	3806	3357 (88.2)	3647 (95.8)	88 (2.3)	43 (1.6)	28 (2.5)	0±2.3	0±2.3	−0.6±2.7	−0.2±1.9	2613 (68.7)	64 (1.7)	1129 (29.7)

S, susceptible; I, susceptible by increased exposure; R, resistant.
[a]Categorization is based on the EUCAST methodology.

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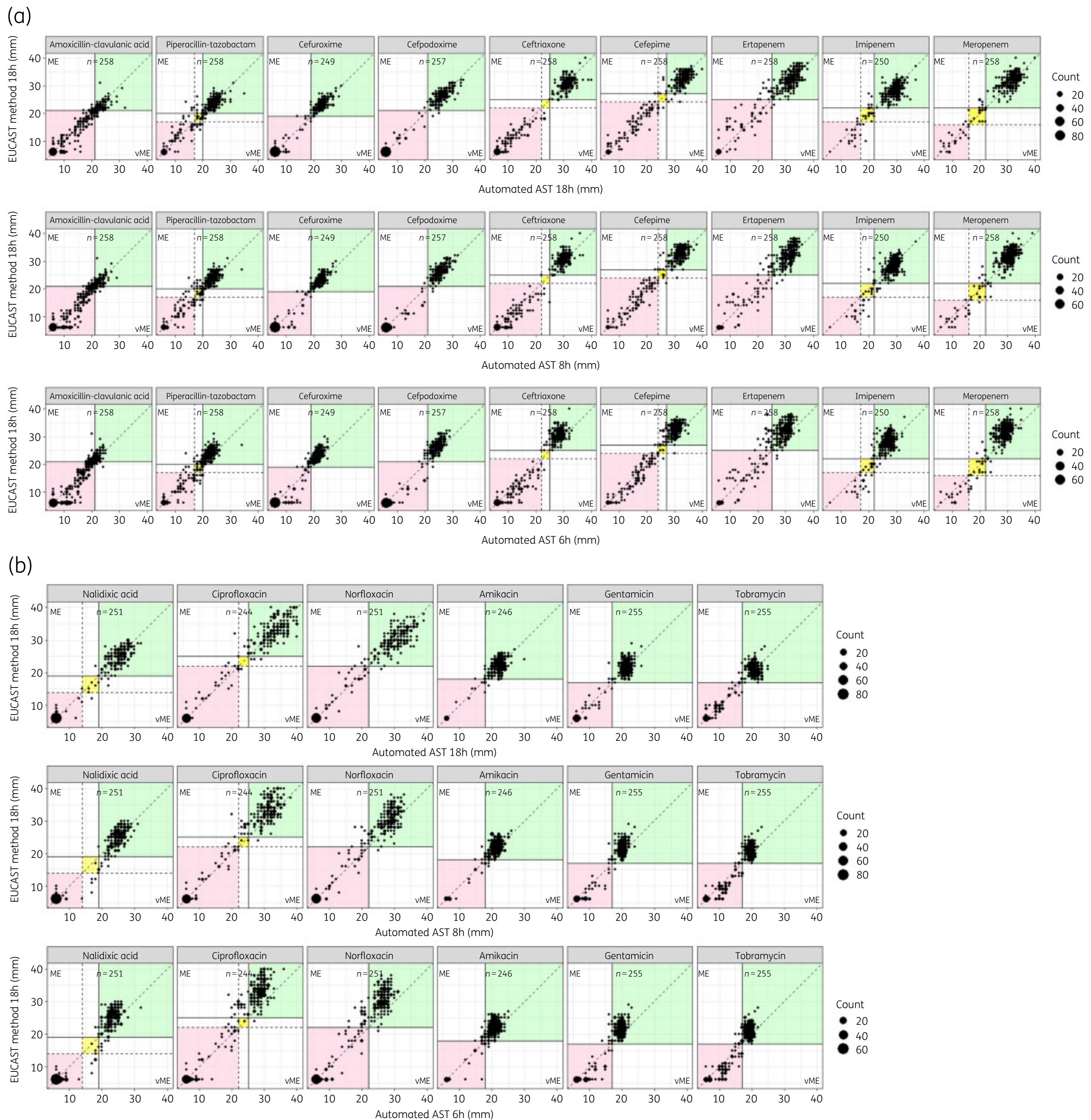

Figure 2. Correlation between inhibition zone diameters determined by the standard EUCAST method based on primary subcultures of positive blood cultures (*y*-axis) and by aAST using blood cultures directly and an inoculum size of 10^6 cfu/mL after 18 h, 8 h and 6 h (*x*-axis) for (a) β-lactams and (b) quinolones and aminoglycosides. The size of the dots reflects the number of isolates. The black continuous and dashed lines indicate the upper and lower EUCAST CBPs, respectively. The coloured areas indicate CA: susceptible (green), intermediate (yellow) and resistant (red). The upper left white quadrant indicates ME, the lower right white quadrant indicates vME. The dashed black diagonal lines indicate identity between the results obtained with the two methods. The number of observations is indicated at the top centre of each figure.

Microbiologie and BSAC suggest using standard dilutions for direct AST of blood cultures. However, this approach does not take into account blood cultures that become positive during the workday and are immediately analysed by the operator. 'One size fits all' is seemingly inadequate for preparation of standardized inocula for blood cultures.

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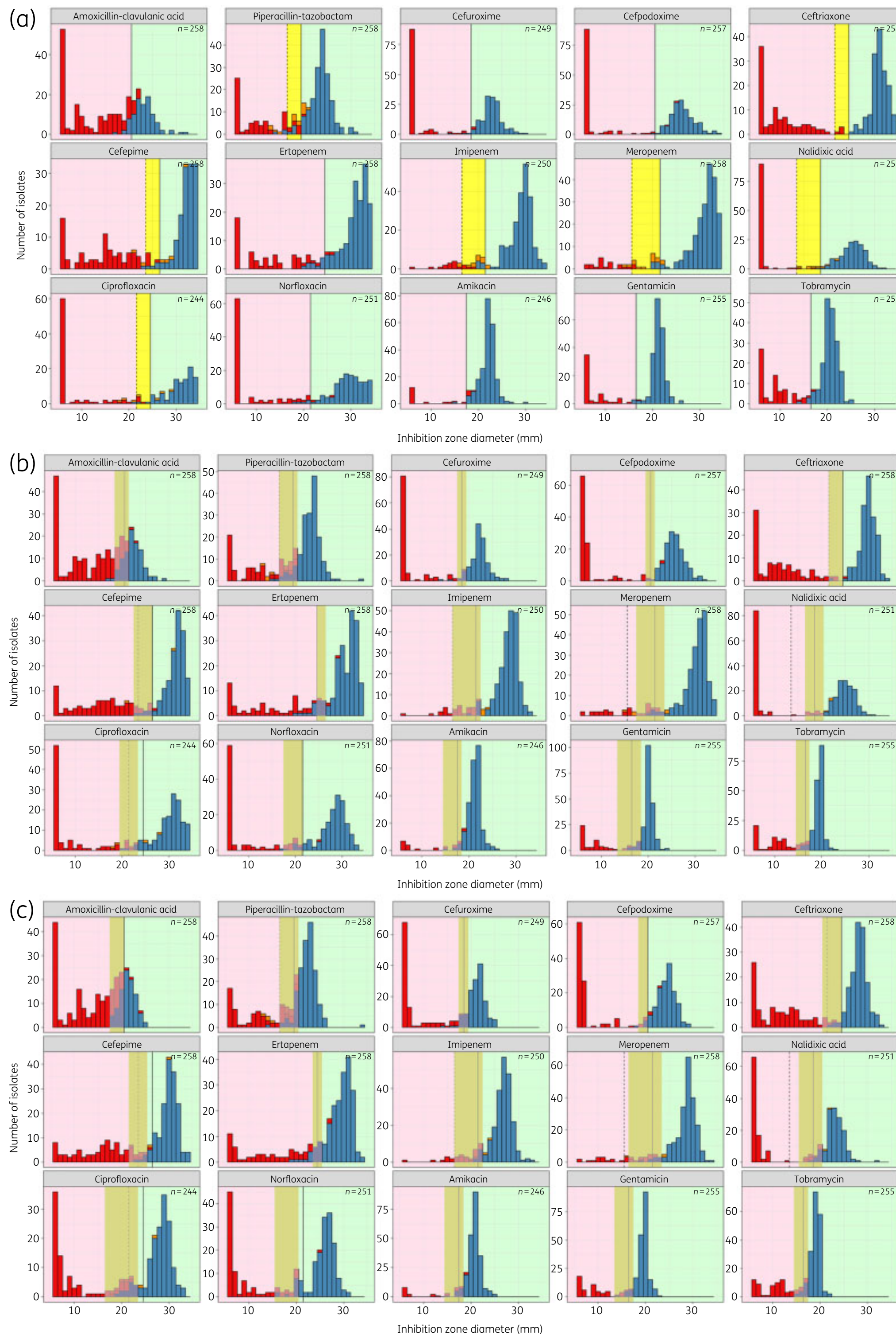

Figure 3. Distribution of the inhibition zone diameters determined by standardized aAST using blood cultures directly and an inoculum size of 10^6 cfu/mL after (a) 18 h, (b) 8 h and (c) 6 h compared with clinical categorization based on results of the EUCAST standard method. The black continuous and dashed vertical lines indicate the upper and lower EUCAST CBPs, respectively. The coloured areas indicate the clinical categories based on EUCAST CBPs for the 18 h readings and RAST CBPs for the 6 and 8 h readings: susceptible (green), intermediate (yellow) and resistant (red). The grey areas indicate the time-adapted tentative ATUs. The number of observations is indicated at the top right of each figure. The colour of every strain reflects the classification based on results of the 18 h standard EUCAST methodology: resistant (red), intermediate (orange) and susceptible (blue).

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Table 2. Comparative analysis of aAST at 6 h using a 10^6 cfu/mL inoculum prepared directly from blood cultures and time-adapted tentative CBPs and ATUs with that of EUCAST standard methodology (18 h) based on primary subcultures of blood cultures ($n = 258$)

			Interpretable				Clinical categorization error[a]			
Antibiotic	Total	Uncertain (%)	all (%)	S (%)	I (%)	R (%)	CA (%)	mE (%)	ME (%)	vME (%)
Amoxicillin/clavulanic acid	258	73 (28.3)	185 (71.7)	66 (69.5)		119 (73.0)	182 (98.4)			3
Piperacillin/tazobactam	258	50 (19.4)	208 (80.6)	151 (86.3)	4 (26.7)	53 (77.9)	203 (97.6)	4	1	
Ceftriaxone	258	11 (4.3)	247 (95.7)	156 (96.9)		91 (93.8)	247 (100)			
Cefpodoxime	257	8 (3.1)	249 (96.9)	147 (97.4)		102 (96.2)	245 (98.4)		1	3
Cefuroxime	249	18 (7.2)	231 (92.8)	127 (88.2)		104 (99.0)	228 (98.7)		3	
Cefepime	258	18 (7.0)	240 (93.0)	164 (97.0)	2 (33.3)	74 (89.2)	237 (98.8)	2		1
Ertapenem	258	14 (5.4)	244 (94.6)	187 (93.0)		57 (100)	234 (95.9)		8	2
Imipenem	250	29 (11.6)	221 (88.4)	211 (95.9)	2 (15.4)	8 (47.1)	219 (99.1)	2		
Meropenem	258	23 (8.9)	235 (91.1)	215 (96.0)	3 (21.4)	17 (85.0)	232 (98.7)	3		
Nalidixic acid	251	23 (9.2)	228 (90.8)	133 (91.7)	2 (22.2)	93 (95.9)	226 (99.1)	2		
Ciprofloxacin	244	30 (12.3)	214 (87.7)	142 (91.0)	2 (28.6)	70 (86.4)	212 (99.1)	2		
Norfloxacin	251	23 (9.2)	228 (90.8)	151 (93.2)		77 (86.5)	227 (99.6)			1
Amikacin	246	20 (8.1)	226 (91.9)	211 (94.2)		15 (68.2)	224 (99.1)			2
Gentamicin	255	36 (14.1)	219 (85.9)	171 (85.5)		48 (87.3)	219 (100)			
Tobramycin	255	25 (9.8)	230 (90.2)	170 (91.4)		60 (87.0)	230 (100)			
Total	3806	401 (10.5)	3405 (89.5)	2402 (91.9)	15 (23.4)	988 (87.5)	3365 (98.8)	15 (0.4)	13 (0.5)	12 (1.2)

S, susceptible; I, susceptible by increased exposure; R, resistant.
[a]Categorization is based on the EUCAST methodology and data refer to the number of interpretable strain/antibiotic combinations.

Table 3. Comparative analysis of aAST at 8 h using a 10^6 cfu/mL inoculum prepared directly from blood cultures and time-adapted tentative CBPs and ATUs with that of EUCAST standard methodology (18 h) using primary subcultures of blood cultures ($n = 258$)

			Interpretable				Clinical categorization error[a]			
Antibiotic	Total	Uncertain (%)	all (%)	S (%)	I (%)	R (%)	CA (%)	mE (%)	ME (%)	vME (%)
Amoxicillin/clavulanic acid	258	45 (17.4)	213 (82.6)	79 (83.2)		134 (82.2)	205 (96.2)		1	7
Piperacillin/tazobactam	258	41 (15.9)	217 (84.1)	159 (90.9)	4 (26.7)	54 (79.4)	211 (97.2)	4	2	
Cefuroxime	249	11 (4.4)	238 (95.6)	134 (93.1)		104 (99.0)	236 (99.2)		2	
Cefpodoxime	257	3 (1.2)	254 (98.8)	149 (98.7)		105 (99.1)	252 (99.2)			2
Ceftriaxone	258	4 (1.6)	254 (98.4)	160 (99.4)		94 (96.9)	253 (99.6)			1
Cefepime	258	14 (5.4)	244 (94.6)	163 (96.4)	2 (33.3)	79 (95.2)	241 (98.8)	2		1
Ertapenem	258	13 (5.0)	245 (95.0)	189 (94.0)		56 (98.2)	234 (95.5)		9	2
Imipenem	250	25 (10)	225 (90.0)	212 (96.4)	4 (30.8)	9 (52.9)	221 (98.2)	4		
Meropenem	258	18 (7.0)	240 (93.0)	216 (96.4)	5 (35.7)	19 (95.0)	235 (97.9)	5		
Nalidixic acid	251	11 (4.4)	240 (95.6)	144 (99.3)	3 (33.3)	93 (95.9)	237 (98.8)	3		
Ciprofloxacin	244	12 (4.9)	232 (95.1)	151 (96.8)	4 (57.1)	77 (95.1)	223 (96.1)	9		
Norfloxacin	251	17 (6.8)	234 (93.2)	155 (95.7)		79 (88.8)	232 (99.1)		1	1
Amikacin	246	14 (5.7)	232 (94.3)	216 (96.4)		16 (72.7)	230 (99.1)			2
Gentamicin	255	20 (7.8)	235 (92.2)	185 (92.5)		50 (90.9)	235 (100)			
Tobramycin	255	19 (7.5)	236 (92.5)	176 (94.6)		60 (87.0)	236 (100)			
Total	3806	267 (7.0)	3539 (93.0)	2488 (95.2)	22 (34.4)	1029 (91.1)	3481 (98.4)	27 (0.8)	15 (0.6)	16 (1.6)

S, susceptible; I, susceptible by increased exposure; R, resistant.
[a]Categorization is based on the EUCAST methodology and data refer to the number of interpretable strain/antibiotic combinations.

EUCAST has recently proposed a methodology for RAST from blood cultures based on direct plating of 100–150 μL on Mueller–Hinton or Mueller–Hinton fastidious agar plates with reading after 4, 6 and 8 h of incubation using specifically adapted breakpoints.[25,39] Although this method represents an improvement, since it significantly reduces the time between culture positivity and AST results, it comes with some limitations. Due to unstandardized inocula, which are most often considerably higher

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Table 4. Screening performance of cefpodoxime for ESBL detection ($n = 28$)

	aAST 6 h				aAST 8 h				aAST 18 h				EUCAST 18 h			
IZD (mm) ≤	total obs.	ESBL	ESBL/ total ESBL (%)	ESBL/ total obs.[a] (%)	total obs.	ESBL	ESBL/ total ESBL (%)	ESBL/ total obs.[a] (%)	total obs.	ESBL	ESBL/ total ESBL (%)	ESBL/ total obs.[a] (%)	total obs.	ESBL	ESBL/ total ESBL (%)	ESBL/ total obs.[a] (%)
6	40	19	67.9	47.5	49	23	82.1	46.9	55	26	92.9	47.3	56	26	92.9	46.4
7	53	25	89.3	47.2	55	26	92.9	47.3	56	26	92.9	46.4	56	26	92.9	46.4
9	54	25	89.3	46.3	56	26	92.9	46.4	56	26	92.9	46.4	57	26	92.9	45.6
10					56	26	92.9	46.4	58	27	96.4	46.6				
11	55	26	92.9	47.3					61	28	100	45.9	62	27	96.4	43.5
12	57	26	92.9	45.6	57	26	92.9	45.6					63	28	100	44.4
13					60	27	96.4	45	64	28	100	43.8	63	28	100	44.4
14	62	28	100	45.2	62	28	100	45.2					65	28	100	43.1
15													66	28	100	42.4
16					63	28	100	44.4	65	28	100	43.1				
17	63	28	100	44.4												
18	64	28	100	43.8					65	28	100	43.1				
19	65	28	100	43.1	66	28	100	42.4	66	28	100	42.4	68	28	100	41.2
20	70	28	100	40.0	67	28	100	41.8	67	28	100	41.8	69	28	100	40.6
21	79	28	100	35.4	68	28	100	41.2	68	28	100	41.2	71	28	100	39.4
22	90	28	100	31.1	76	28	100	36.8	75	28	100	37.3	74	28	100	37.8
23	114	28	100	24.6	89	28	100	31.5	83	28	100	33.7	85	28	100	32.9
24	141	28	100	19.9	112	28	100	25	100	28	100	28	98	28	100	28.6
25	178	28	100	15.7	143	28	100	19.6	127	28	100	22	118	28	100	23.7
26	199	28	100	14.1	172	28	100	16.3	156	28	100	17.9	144	28	100	19.4
27	213	28	100	13.1	192	28	100	14.6	177	28	100	15.8	174	28	100	16.1
28	216	28	100	13.0	204	28	100	13.7	191	28	100	14.7	192	28	100	14.6
29	218	28	100	12.8	212	28	100	13.2	200	28	100	14	204	28	100	13.7
30					214	28	100	13.1	210	28	100	13.3	209	28	100	13.4
31					217	28	100	12.9	213	28	100	13.1	216	28	100	13
32					218	28	100	12.8	214	28	100	13.1	218	28	100	12.8
33									217	28	100	12.9				
34									218	28	100	12.8				

The dashed line indicates the tentative cut-off (cefpodoxime <20 mm) for ESBL screening for 6 and 8 h readings and the EUCAST cut-off (cefpodoxime <21 mm) for ESBL screening for 18 h readings.
IZD, inhibition zone diameter; obs., observations.
[a]Total observations without CPE.

than those of the standard method, the inhibition diameters are smaller. In addition, because of the shorter incubation times, large overlapping areas of resistant and susceptible isolates allow only categorization of clinical isolates with highly distinct phenotypes (very susceptible or very resistant). As a consequence, EUCAST has set new discriminative breakpoints and introduced ATUs where AST results cannot be interpreted. If results cannot be reported after 4 h of incubation, plates must be reincubated and reading performed at a later timepoint (6 or 8 h). Most discomfortingly, the proposed method comes with a considerable increase in workload since, on top of RAST, an additional EUCAST standard disc diffusion AST must be performed to confirm the RAST data.

We here demonstrated that bacterial numbers in blood cultures containing Enterobacterales can be reliably determined with a simple and rapid (less than 15 min) method using flow cytometry. This allowed for consistent standardization of the inoculum and the attainment of 18 h AST results, prepared directly from blood cultures, which are highly consistent with those obtained with the 18 h EUCAST standard method based on previous preparation of subcultures (Figure 2a and b, Table 1). Most importantly, we found that the use of standardized inocula allows for early (6 and 8 h) reading of AST data. Since the standardized bacterial inocula used are significantly lower than those of the EUCAST RAST method, it is important to note that bacterial growth is in general not visible at 4 h of incubation and for this reason was not investigated. Through the application of time-adapted tentative breakpoints and ATUs, early readings at 6 and 8 h produced interpretable results for 89.5% and 93.0% of the isolates, respectively. Note that, per definition, ATUs encompass the intermediate zones, thus strains tested as intermediate in the standard 18 h incubation are classified as ATUs in 6 and 8 h readings. Application of time-adapted breakpoints resulted in CA of >98.4% compared with

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Table 5. Screening performance of meropenem for CPE detection ($n = 39$)

IZD (mm) ≤	aAST 6 h total obs.	aAST 6 h CPE	aAST 6 h CPE/total CPE (%)	aAST 6 h CPE/total obs. (%)	aAST 8 h total obs.	aAST 8 h CPE	aAST 8 h CPE/total CPE (%)	aAST 8 h CPE/total obs. (%)	aAST 18 h total obs.	aAST 18 h CPE	aAST 18 h CPE/total CPE (%)	aAST 18 h CPE/total obs. (%)	EUCAST 18 h total obs.	EUCAST 18 h CPE	EUCAST 18 h CPE/total CPE (%)	EUCAST 18 h CPE/total obs. (%)
6	2	2	5.1	100	2	2	5.1	100	2	2	5.1	100	4	4	10.3	100
7									4	4	10.3	100				
8	3	3	7.7	100	4	4	10.3	100	5	5	12.8	100	6	6	15.4	100
9	4	4	10.3	100	6	6	15.4	100	10	10	25.6	100	9	9	23.1	100
10	7	7	17.9	100	8	8	20.5	100	11	11	28.2	100	12	12	30.8	100
11	10	10	25.6	100	11	11	28.2	100	14	14	35.9	100	14	14	35.9	100
12	11	11	28.2	100	13	13	33.3	100					16	16	41	100
13	13	13	33.3	100					16	16	41	100	18	18	46.2	100
14									18	18	46.2	100	19	19	48.7	100
15	14	14	35.9	100	16	16	41	100	20	20	51.3	100	20	20	51.3	100
16	18	18	46.2	100	20	20	51.3	100	24	24	61.5	100	22	22	56.4	100
17	21	21	53.8	100	22	22	56.4	100	25	25	64.1	100	27	27	69.2	100
18	22	22	56.4	100	23	23	59	100	26	26	66.7	100	28	28	71.8	100
19	25	24	61.5	96	24	24	61.5	100								
20	28	27	69.2	96.4	30	29	74.4	96.7	33	32	82.1	97	29	29	74.4	100
21	33	31	79.5	93.9	34	32	82.1	94.1	38	36	92.3	94.7	34	33	84.6	97.1
22	38	35	89.7	92.1	38	35	89.7	92.1	42	39	100	92.9	38	36	92.3	94.7
23	41	37	94.9	90.2	40	36	92.3	90	43	39	100	90.7	39	37	94.9	94.9
24	46	39	100	84.8	43	39	100	90.7					42	37	94.9	88.1
25	57	39	100	68.4	47	39	100	83	46	39	100	84.8	46	39	100	84.8
26	69	39	100	56.5	53	39	100	73.6	50	39	100	78	49	39	100	79.6
27	84	39	100	46.4	58	39	100	67.2	58	39	100	67.2	53	39	100	73.6
28	118	39	100	33.1	71	39	100	54.9	66	39	100	59.1	62	39	100	62.9
29	183	39	100	21.3	94	39	100	41.5	81	39	100	48.1	77	39	100	50.6
30	223	39	100	17.5	128	39	100	30.5	104	39	100	37.5	102	39	100	38.2
31	246	39	100	15.9	176	39	100	22.2	133	39	100	29.3	128	39	100	30.5
32	253	39	100	15.4	228	39	100	17.1	180	39	100	21.7	155	39	100	25.2
33	254	39	100	15.4	245	39	100	15.9	221	39	100	17.6	197	39	100	19.8
34	255	39	100	15.3	253	39	100	15.4	246	39	100	15.9	225	39	100	17.3
35	258	39	100	15.1	257	39	100	15.2	250	39	100	15.6	244	39	100	16
36									254	39	100	15.4	254	39	100	15.4
37									256	39	100	15.2	255	39	100	15.3
38													256	39	100	15.2
39					258	39	100	15.1								
40									257	39	100	15.2	258	39	100	15.1
43									258	39	100	15.1				

The dashed line across the 6 and 8 h columns indicates the tentative cut-off (meropenem <25 mm) for CPE screening for 6 and 8 h readings. For 18 h readings of aAST and EUCAST standard methodology, the EUCAST cut-off meropenem <28 mm is indicated with a dashed line across the 18 h columns. Isolates with 25–27 mm zones only need to be investigated for carbapenemase production if they are resistant to piperacillin/tazobactam and/or temocillin. Investigation for the presence of carbapenemases is always warranted if a zone diameter for meropenem is <25 mm (indicated with a dotted line).
IZD, inhibition zone diameter; obs., observations.

standard 18 h EUCAST testing based on primary subcultures of blood cultures. Clinical categorization errors were low for both 6 and 8 h readings, with mE, ME and vME rates of 0.4%, 0.5% and 1.2% and 0.8%, 0.6% and 1.6%, respectively. In addition, defining cut-off screening diameters for cefpodoxime and meropenem allowed for early detection of ESBL and carbapenemase production. Finally, the high congruence of the 18 h AST data prepared directly from blood cultures with that of the standard 18 h EUCAST procedure prepared for primary subcultures allows reading after short (6–8 h) and standard (18 h) incubation time for confirmation of RAST to be done on the same plate without the need for an additional AST.

As a limitation, this study has been performed with 211 Enterobacterales-positive blood cultures collected at the Institute

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of Medical Microbiology of the University of Zurich during 2018–19, representing a local prevalence. Although 47 blood cultures spiked with ESBL and carbapenemase producers were included in the analysis, some clinically relevant resistance mechanisms remained under-represented. Thus, further studies including large numbers of resistant isolates are required to assess the robustness of the method. In addition, as a proof-of-concept study, we focused our attention on antibiotics commonly employed for first-line treatment of bloodstream infection with Enterobacterales, not including antibiotics such as trimethoprim/sulfamethoxazole or penicillins.

In conclusion, we have established an accurate method to perform RAST of Enterobacterales-containing positive blood cultures circumventing the need for prior subculture. The developed procedure allows the separation of susceptible from resistant populations with great confidence. In addition, AST data can be read after short and standard incubation times from the same plate, circumventing the need to perform EUCAST standard disc diffusion AST and thus making this procedure highly suitable for diagnostic laboratories.

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Transparency declarations

The Institute of Medical Microbiology, University of Zurich and Copan Italia S.p.A. cooperate in the development of aAST, E.C.B. is a former consultant of Copan Italia. All other authors: none to declare.

Supplementary data

Tables S1 to S5 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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