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## EUCAST rapid antimicrobial susceptibility testing (RAST) in blood cultures: validation in 55 European laboratories

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**Objectives:** When bloodstream infections are caused by resistant bacteria, rapid antimicrobial susceptibility testing (RAST) is important for adjustment of therapy. The EUCAST RAST method, directly from positive blood cultures, was validated in a multi-laboratory study in Europe.

**Methods:** RAST was performed in 40 laboratories in northern Europe (NE) and 15 in southern Europe (SE) from clinical blood cultures positive for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* or *Streptococcus pneumoniae*. Categorical results at 4, 6 and 8 h of incubation were compared with results for EUCAST standard 16–20 h disc diffusion. The method, preliminary breakpoints and the performance of the laboratories were evaluated.

**Results:** The total number of isolates was 833/318 in NE/SE. The number of zone diameters that could be read (88%, 96% and 99%) and interpreted (70%, 81% and 85%) increased with incubation time (4, 6 and 8 h). The categorical agreement was acceptable, with total error rates in NE/SE of 2.4%/4.9% at 4 h, 1.1%/3.5% at 6 h and 1.1%/3.3% at 8 h. False susceptibility at 4, 6 and 8 h of incubation was below 0.3% and 1.1% in NE and SE, respectively, and the corresponding percentages for false resistance were below 1.9% and 2.8%. After fine-tuning breakpoints, more zones could be interpreted (73%, 89% and 93%), with only marginally affected error rates.

**Conclusions:** The EUCAST RAST method can be implemented in routine laboratories without major investments. It provides reliable antimicrobial susceptibility testing results for relevant bloodstream infection pathogens after 4–6 h of incubation.

### Introduction

The need for rapid antimicrobial susceptibility testing (RAST) in bloodstream infections is increasing.<sup>1–3</sup> Standard antimicrobial susceptibility testing methods by EUCAST and CLSI require 16–20 h of incubation and the road to the development of methods or devices for RAST is long and winding.<sup>4–8</sup>

Disc diffusion (DD) is easy to use, cheap and dependable when performed in accordance with guidelines.<sup>9,10</sup> It is flexible and rapidly adapted to new antimicrobials. Many laboratories are today acquainted with the EUCAST standardized DD method.<sup>11</sup> However, the call for shorter diagnostic turn-around times has prompted EUCAST to develop a rapid phenotypic antimicrobial susceptibility testing (RAST) method based on DD

directly from blood culture (BC) bottles that can be performed by any laboratory. During the development, BC bottles were spiked with clinical isolates of the most important bloodstream infection pathogens with and without defined resistance mechanisms to agents from all relevant antibiotic classes.<sup>12</sup> This resulted in a method where species were assigned preliminary breakpoints for each of the reading times (4, 6 and 8 h) and where a buffer area [area of technical uncertainty (ATU)] between the susceptible (S) and resistant (R) categories was introduced to reduce the occurrence of false-resistant and false-susceptible results related to technical variation accelerated by the short incubation time (Table S1, available as [Supplementary data](#) at JAC Online).<sup>12</sup>

The aim of this study was to investigate the performance of the EUCAST RAST method in clinical laboratories under everyday diagnostic conditions and to test the accuracy, practicality and robustness of the method. The aim was also to fine-tune the preliminary breakpoints from the RAST development study (version 0),<sup>12</sup> based on the overall results from the development and the two clinical trials. The first trial, performed in northern Europe (NE), was followed by an identical trial in southern Europe (SE), to target countries with higher resistance rates. The results of the trials contributed to the process of determining final EUCAST RAST breakpoints (version 1.0).

## Materials and methods

### Tests performed by local clinical laboratories

The two trials were performed in 55 laboratories in 11 European countries (40 laboratories in 6 countries in NE in the summer of 2017 and 15 laboratories in 5 countries in SE in the summer of 2018; Table 1). A study protocol was distributed to each laboratory (Text S1) and is similar to the RAST methodology document now available on the EUCAST website.<sup>13</sup>

Laboratories were required to perform RAST from one BC positive for a Gram-negative bacterium and one BC positive for a Gram-positive bacterium per weekday, for a period of 30 days inside a defined 3 month span. BCs positive for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* or *Streptococcus pneumoniae* were included. Species identification was performed using the in-house method of the laboratories. Laboratories were instructed to adhere to EUCAST recommendations on disc potencies, antimicrobial susceptibility testing media, agar depth and handling of material.<sup>5,14</sup> Each laboratory listed BC system, antibiotic disc manufacturer and brand of Mueller-Hinton medium used.

DD plates were inoculated within 0–24 h of the positive BC signal and BCs were kept in the BC incubator until tested. The agents tested varied with the species (Table 2). All plates were incubated at 35±1°C, Mueller-Hinton in ambient air and Mueller-Hinton with 5% defibrinated horse blood and 20 mg/L β-NAD in 5% CO<sub>2</sub>. The plates were incubated to allow reading of the same plate at 4, 6 and, when possible, 8 h, with re-incubation between readings. All zone diameters were read from the front of the plate with the lid removed. All isolates were stored frozen for later reference testing at the EUCAST Development Laboratory (EDL) in Växjö, Sweden. If a result was not reported, the reason was stated.

### Reference testing

EUCAST standardized DD testing and confirmation of species identification was performed on all isolates.<sup>5,15</sup> Broth microdilution (BMD) was performed on a representative proportion (every fourth *E. coli*, every third *S. aureus* and all isolates of the other species) of the NE isolates.<sup>4,14</sup>

*E. coli* and *K. pneumoniae* isolates resistant to cefotaxime and/or ceftazidime were further tested for ESBL [discs from Mast Diagnostics (Merseyside, UK)] and AmpC [MIC Test Strip (Liofilchem, Roseto degli Abruzzi, Italy)].<sup>16</sup> Isolates resistant to either or both of the cephalosporins but phenotypically negative for ESBL and AmpC and isolates with a meropenem zone <28 mm with the reference method (the carbapenemase screening breakpoint of EUCAST)<sup>16</sup> were sent to the Public Health Agency of Sweden for further characterization of resistance mechanisms using WGS.

All *S. aureus* isolates were screened for methicillin resistance with EUCAST standard DD using cefoxitin.<sup>14,16</sup> The presence of *mecA* or *mecC* in screen-positive isolates was confirmed using standard molecular methods.

### Data analysis

The proportion of readable inhibition zones at each incubation time was calculated for all species/agent combinations. A zone diameter could be: not read (poor growth); read but not categorized (in the ATU); or read and categorized as S or R. For each species, results were interpreted as S, R or ATU using the preliminary breakpoints (breakpoint table version 0) based on data from spiked BC bottles from the development of the RAST methodology.<sup>12</sup> For each participating laboratory, and for each species and agent, categorical errors were calculated using standard DD (EUCAST breakpoint tables, version 8.0, valid at the time of the study) as the reference.<sup>14</sup> Results in the ATU were exempted from error calculations since no categorization was performed. Categorical errors were defined as very major error (VME; RAST = S and reference = R), major error (ME; RAST = R and reference = S) or minor error (mE; RAST = S or R and reference = I).

The results of the analysis of the complete material were later used to fine-tune the breakpoints (EUCAST RAST breakpoints, version 1.0) (Table S1).

The capability of the RAST method to detect ESBL and AmpC in *E. coli* and *K. pneumoniae*, and methicillin resistance in *S. aureus* was evaluated using the RAST clinical breakpoints for cefotaxime, ceftazidime (*E. coli*/*K. pneumoniae*) and cefoxitin (*S. aureus*). Results were also evaluated regarding detection of carbapenemase production in *E. coli* and *K. pneumoniae* following the introduction of RAST screening breakpoints with meropenem in May 2019.<sup>17</sup>

**Table 1.** Locations of participating laboratories and BC systems, Mueller-Hinton media and discs used

| NE or SE (n) | Country (n)  | BC system <sup>a</sup> (n)                                   | Mueller-Hinton manufacturer <sup>a</sup> (n)   | Disc manufacturer <sup>a</sup> (n)   |
|--------------|--|--|--|--|
| NE (40)      | Denmark (3), Finland (3), Iceland (1), Ireland (1), Norway (11), Sweden (21) | BacT/ALERT (bioMérieux) (22), BACTEC (Becton Dickinson) (18) | Becton Dickinson (16), Bio-Rad (1), Fannin L.I.P. (1), Oxoid/Thermo Fisher Scientific (24)                                   | Becton Dickinson (5), Mast Group (1), Oxoid/Thermo Fisher Scientific (32), Rosco Diagnostica (2)                               |
| SE (15)      | France (2), Greece (3), Italy (2), Spain (4), Turkey (4)                     | BacT/ALERT (bioMérieux) (4), BACTEC (Becton Dickinson) (12)  | Agricons Ricerche (1), Becton Dickinson (7), bioMérieux (3), Bio-Rad (2), Liofilchem (1), Oxoid/Thermo Fisher Scientific (2) | Becton Dickinson (5), BioMaxima S.A. (1), Bio-Rad (4), i2a Diagnostics (2), Mast Group (1), Oxoid/Thermo Fisher Scientific (4) |

The number of laboratories is given in parentheses.

<sup>a</sup>One laboratory used two BC systems and a few laboratories used Mueller-Hinton agar and antibiotic discs from more than one manufacturer.

**Table 2.** Bacterial isolates, antimicrobial agents and resistance mechanisms included

| Species (number of isolates)   | Antimicrobial agents and disc content                    | Resistant isolates (standard DD) |                      | Resistance mechanisms identified  |   |
|--|--|----------------------------------|----------------------|---|---|
|  |  | NE, n (%)                        | SE, n (%)            | NE  | SE  |
| <i>E. coli</i> (n = 580)<br>(NE n = 430 and SE n = 150)              | piperacillin/tazobactam 30/6 µg                          | 7 (2)                            | 6 (4)                | carbapenemase n = 0, other third-generation cephalosporin resistance n = 24 | carbapenemase n = 1, other third-generation cephalosporin resistance n = 24 |
|  | cefotaxime 5 µg  | 24 (6)                           | 25 (17)              |   |   |
|  | ceftazidime 10 µg  | 17 (4)                           | 21 (14)              |   |   |
|  | imipenem 10 µg <sup>a</sup>                              | 0 (0)                            | 0 (0)                |   |   |
|  | meropenem 10 µg  | 0 (0)                            | 0 (0)                |   |   |
|  | ciprofloxacin 5 µg                                       | 58 (13)                          | 50 (33)              |   |   |
|  | amikacin 30 µg   | 0 (0)                            | 3 (2)                |   |   |
|  | gentamicin 10 µg   | 29 (7)                           | 13 (9)               |   |   |
|  | tobramycin 10 µg   | 25 (6)                           | 17 (11)              |   |   |
|  | <i>K. pneumoniae</i> (n = 134) (NE n = 68 and SE n = 66) | piperacillin/tazobactam 30/6 µg  | 0 (0)                |   |   |
| cefotaxime 5 µg  |  | 4 (6)                            | 35 (53)              |   |   |
| ceftazidime 10 µg  |  | 3 (4)                            | 35 (53)              |   |   |
| imipenem 10 µg <sup>a</sup>  |  | 0 (0)                            | 15 (23)              |   |   |
| meropenem 10 µg  |  | 0 (0)                            | 17 (26)              |   |   |
| ciprofloxacin 5 µg   |  | 10 (15)                          | 35 (53)              |   |   |
| amikacin 30 µg   |  | 0 (0)                            | 13 (20)              |   |   |
| gentamicin 10 µg   |  | 1 (1)                            | 19 (29)              |   |   |
| tobramycin 10 µg   |  | 1 (1)                            | 33 (50)              |   |   |
| <i>P. aeruginosa</i> (n = 69) (NE n = 37 and SE n = 32)              |  | piperacillin/tazobactam 30/6 µg  | 6 (16)               | 4 (13)  |   |
|  | gentamicin 10 µg   | 0 (0)                            | 3 (9)                |   |   |
|  | tobramycin 10 µg   | 0 (0)                            | 3 (9)                |   |   |
|  | ceftazidime 10 µg  | 2 (5)                            | 4 (13)               |   |   |
|  | imipenem 10 µg   | 4 (11)                           | 4 (13)               |   |   |
|  | meropenem 10 µg  | 4 (11)                           | 4 (13)               |   |   |
|  | ciprofloxacin 5 µg                                       | 5 (14)                           | 3 (9)                |   |   |
| <i>S. aureus</i> (n = 337) (NE n = 267 and SE n = 70)                | cefoxitin 30 µg  | 4 (1)                            | 20 (29)              | MRSA n = 4  | MRSA n = 20   |
|  | norfloxacin 10 µg  | 15 (6)                           | 21 (30)              |   |   |
|  | gentamicin 10 µg   | 0 (0)                            | 1 (1)                |   |   |
|  | erythromycin 15 µg <sup>a,b</sup>                        | 5 (2)                            | 20 (29)              |   |   |
|  | clindamycin 2 µg <sup>a,b</sup>                          | 5 (2) <sup>d</sup>               | 19 (27) <sup>d</sup> |   |   |
| <i>S. pneumoniae</i> (n = 31) (NE n = 31 and SE n = 5 <sup>c</sup> ) | oxacillin 1 µg   | 2 (6)                            |                      | benzylpenicillin non-WT isolates (MIC >0.06 mg/L) n = 2                     |   |
|  | norfloxacin 10 µg  | 0 (0)                            |                      |   |   |
|  | erythromycin 15 µg <sup>b</sup>                          | 3 (10)                           |                      |   |   |
|  | clindamycin 2 µg <sup>b</sup>                            | 2 (6)                            |                      |   |   |
|  | trimethoprim/sulfamethoxazole 1.25/23.75 µg              | 2 (6)                            |                      |   |   |

<sup>a</sup>No tentative breakpoints available or not evaluated in the trial.

<sup>b</sup>The erythromycin and clindamycin discs were placed at a distance of 12–20 mm from edge to edge for staphylococci and 12–16 mm from edge to edge for streptococci to detect inducible clindamycin resistance.

<sup>c</sup>The five *S. pneumoniae* isolates from SE were excluded from the evaluation due to the low number.

<sup>d</sup>Including isolates with inducible resistance.

### Quality control (QC)

The EUCAST standard QC procedure<sup>18</sup> was performed both by the participating laboratories and the EDL to control the media and discs used.

Furthermore, the RAST QC procedure was performed by each laboratory up to five times per QC strain: *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and

*S. pneumoniae* ATCC 49619. BC bottles were inoculated with 1 mL from a 100–200 cfu/mL solution (suspension adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and then diluted 1:1 000 000) together with 5 mL of blood. DD according to the RAST method was performed following a positive signal of the BC. QC data were submitted to the EDL together with the RAST results.

## Results

In general, laboratories reported few problems adhering to the protocol. Some laboratories could not read results at 8 h because of limited opening hours.

The total number of isolates included in NE/SE was 833/318 (Table 3). Of these, 430/150 were *E. coli*, 68/66 were *K. pneumoniae*, 37/32 were *P. aeruginosa*, 267/170 were *S. aureus* and 31/5 were *S. pneumoniae*. The *S. pneumoniae* isolates from SE were excluded from the evaluation due to the low number. Resistance rates for each species using reference antimicrobial susceptibility testing are shown in Table 2.

The median time for inoculation of DD plates after a positive BC signal (recorded for 84% of the bottles) was 7 h 36 min (range = 0 h 3 min–25 h 32 min) and 99.2% of these isolates were handled within the instructed time limit of 24 h.

After 4 h of incubation, *E. coli* and *K. pneumoniae* grew well. The proportion of readable zones of the total number of completed tests in NE/SE was 91%/90% for *E. coli* and 97%/89% for *K. pneumoniae*. Although Gram-positive bacteria generally required a longer time to results, zones for *S. aureus* could be read after 4 h in 72%/50% of the completed tests in NE/SE. For *S. pneumoniae* in NE, zones could be read after 4 h in 54% of the completed tests.

*P. aeruginosa*, as shown in the initial study, did not grow after 4 h but could mostly be read after 6 h (88/63% in NE/SE) (Table S2).

In total, the proportion of readable zones after 4, 6 and 8 h of incubation was 88%, 96% and 99%, respectively (Table 4).

Aggregated zone diameter distributions were compared with those obtained using spiked bottles during the development of the

EUCAST RAST method<sup>12</sup> and there were no systematic differences between the distributions from the spiked bottles and the multi-laboratory trials.

The different BC systems (bioMérieux, *n* = 26; Becton Dickinson, *n* = 30; one laboratory used both) and manufacturers of media (*n* = 7) and discs (*n* = 7) used are listed in Table 1. No systematic differences related to BC system or antimicrobial susceptibility testing materials were detected (data not shown).

### Results with preliminary RAST breakpoints (version 0)

The proportion of results in the ATU decreased with incubation time in both trials (NE/SE): 21%/19% after 4 h, 17%/15% after 6 h and 14%/13% after 8 h of incubation (Table 3).

As expected, the number of resistant isolates was higher in the SE trial but this had only a limited effect on the frequency of categorical errors (Tables 2 and 3). False susceptibility (VME) at 4, 6 and 8 h of incubation was below 0.3% and 1.1% in NE and SE, respectively, and the corresponding percentages for false resistance (ME) were below 1.9% and 2.8% (Table 3). For results related to each individual species, see Tables S2 and S3 and Figures S1 to S5.

### *E. coli* and *K. pneumoniae*

In general, incorrect categorization was rare, partly because the piperacillin/tazobactam ATU could buffer a large part of uncertain results.

Most of the VMEs were related to ciprofloxacin but when comparing the preliminary ciprofloxacin RAST breakpoints (version 0)

**Table 3.** Theoretical and actual numbers of tests aggregated for all species, the proportions of tests that could be read and interpreted as S or R (using breakpoint table version 0) after 4, 6 and 8 h and the categorical errors with RAST versus standard DD by EUCAST breakpoint tables version 8.0 at each reading time

|  | Incubation time (h) |           |           |           |           |           |
|--|---------------------|-----------|-----------|-----------|-----------|-----------|
|  | 4                   |           | 6         |           | 8         |           |
|  | NE                  | SE        | NE        | SE        | NE        | SE        |
| Theoretical number of tests <sup>a</sup>                                 | 4932                | 2092      | 5199      | 2162      | 5199      | 2162      |
| Number of completed tests <sup>b</sup>                                   | 4571                | 1827      | 5089      | 2121      | 4542      | 2113      |
| Readable zones <sup>c</sup> (% of completed tests)                       | 4034 (88)           | 1590 (87) | 4943 (97) | 1978 (93) | 4477 (99) | 2084 (99) |
| Results calculated on readable zones (%)                                 |                     |           |           |           |           |           |
| not interpreted as S or R (ATU)  | 21                  | 19        | 17        | 15        | 14        | 13        |
| interpreted as S   | 74                  | 62        | 80        | 68        | 82        | 70        |
| interpreted as R   | 4.8                 | 19        | 3.8       | 17        | 3.7       | 16        |
| Errors calculated on the total number of zones interpreted as S or R (%) |                     |           |           |           |           |           |
| mEs  | 0.3                 | 1.5       | 0.1       | 1.4       | 0.2       | 1.4       |
| MEs  | 1.9                 | 2.8       | 0.8       | 1.2       | 0.6       | 0.8       |
| VMEs   | 0.1                 | 0.5       | 0.2       | 0.9       | 0.3       | 1.1       |
| total errors   | 2.4                 | 4.9       | 1.1       | 3.5       | 1.1       | 3.3       |

Total number of isolates included: NE *n* = 833 and SE *n* = 318.

Minor error (mE; RAST = S or R and reference method = I); major error (ME; RAST = R and reference method = S); very major error (VME; RAST = S and reference method = R).

<sup>a</sup>Total number of possible isolate/agent combinations. The lower number of tests at 4 h is explained by the absence of norfloxacin breakpoints for *S. aureus*.

<sup>b</sup>Number of completed tests after excluding missing data (e.g. disc dropped).

<sup>c</sup>Number of tests with readable inhibition zones.

**Table 4.** Theoretical and actual numbers of tests, the proportions of tests that could be read and interpreted as S or R after 4, 6 and 8 h and the categorical errors with RAST versus standard DD by EUCAST breakpoint tables version 8.0 at each reading time for all species in NE + SE [RAST breakpoint table version 0 (v. 0) and version 1 (v. 1.0)]

|  | Incubation time (h) |           |             |        |             |        |
|--|---------------------|-----------|-------------|--------|-------------|--------|
|  | 4                   |           | 6           |        | 8           |        |
| Breakpoint table   | v. 0                | v. 1.0    | v. 0/v. 1.0 |        | v. 0/v. 1.0 |        |
| Theoretical number of tests <sup>a</sup>                                 | 7024                | 7361      | 7361        |        | 7361        |        |
| Number of completed tests <sup>b</sup>                                   | 6398                | 6718      | 7210        |        | 6655        |        |
| Readable zones <sup>c</sup> (% of completed tests)                       | 5624 (88)           | 5811 (87) | 6921 (96)   |        | 6561 (99)   |        |
| Results calculated on readable zones (%)                                 |                     |           |             |        |             |        |
| breakpoint table   | v. 0                | v. 1.0    | v. 0        | v. 1.0 | v. 0        | v. 1.0 |
| not interpreted as S or R (ATU)  | 20                  | 16        | 16          | 7.5    | 14          | 5.7    |
| interpreted as S   | 71                  | 75        | 76          | 84     | 78          | 86     |
| interpreted as R   | 8.8                 | 8.8       | 7.6         | 8.5    | 7.7         | 8.6    |
| Errors calculated on the total number of zones interpreted as S or R (%) |                     |           |             |        |             |        |
| breakpoint table   | v. 0                | v. 1.0    | v. 0        | v. 1.0 | v. 0        | v. 1.0 |
| mEs  | 0.7                 | 0.6       | 0.5         | 0.6    | 0.6         | 0.8    |
| MEs  | 2.2                 | 2.1       | 0.9         | 1.1    | 0.7         | 0.9    |
| VMEs   | 0.2                 | 0.2       | 0.4         | 0.4    | 0.6         | 0.5    |
| total errors   | 3.1                 | 3.0       | 1.8         | 2.1    | 1.8         | 2.2    |

Total number of isolates included:  $n = 1151$ .

Minor error (mE; RAST = S or R and reference method = I); major error (ME; RAST = R and reference method = S); very major error (VME; RAST = S and reference method = R).

<sup>a</sup>Total number of possible isolate/agent combinations. The lower number of tests at 4 h (breakpoint table v. 0) is explained by the absence of norfloxacin breakpoints for *S. aureus*.

<sup>b</sup>Number of completed tests after excluding missing data (e.g. disc dropped).

<sup>c</sup>Number of tests with readable inhibition zones.

with the 2019 reference breakpoint of EUCAST<sup>19</sup> most VMEs ( $n = 20/26$ ) disappeared, indicating that the reference method in this case was partly responsible for the incorrect interpretations. See further notes in the section entitled 'Additional considerations'.

In *K. pneumoniae*, mEs with meropenem caused the majority of incorrect interpretations (Table S3 and Figures S1 and S2).

ESBL-producing *E. coli* and *K. pneumoniae* were fairly frequent in both NE ( $n = 28$ ) and SE ( $n = 34$ ). There were no carbapenem-resistant isolates in NE but 26 were detected in SE (Table 2). RAST correctly identified all isolates with ESBL provided cefotaxime and ceftazidime zones were readable and could be categorized.

Of the 26 carbapenem-resistant isolates, the 6 h meropenem screening breakpoint failed to detect one KPC-3-positive isolate and one OXA-244-positive isolate.<sup>17</sup> All carbapenem-resistant isolates that could be read at 8 h were identified, using the screening breakpoint.

### *P. aeruginosa*

Only results after 6 and 8 h were evaluated since zones were not readable at 4 h (Table S2). At 6 h some MEs, mainly related to ceftazidime, were observed. The few VMEs were not related to any specific agent (Table S3 and Figure S3).

### *S. aureus*

There were no breakpoints for norfloxacin at 4 h of incubation in the preliminary breakpoint table (version 0), leaving only gentamicin and ceftazidime for evaluation. The high levels of MEs in both NE and SE were mostly with gentamicin. The proportion of categorical errors decreased with increasing incubation time. Errors were still prominent in SE after both 6 and 8 h of incubation (Table S2). However, 2 of the 15 SE laboratories accounted for 80% (21/26) of these errors and the VMEs were almost exclusively related to the norfloxacin screen disc (Table S3 and Figure S4).

Among the *S. aureus* there were 24 MRSA isolates, 4 in NE and 20 in SE (Table 2). All were resistant to ceftazidime using standardized DD and *mecA* positive by PCR. All readable zones correctly identified isolates as methicillin resistant after 4 h. At 6 and 8 h of incubation one isolate was misinterpreted as methicillin susceptible.

### *S. pneumoniae*

Early reading was difficult in *S. pneumoniae*, with 54%, 78% and 91% readability after 4, 6 and 8 h of incubation, respectively. Unlike other species in the trial, the proportion of incorrect interpretations increased with incubation time: 3.2%, 4.3% and 8.5% after 4, 6 and 8 h, respectively (Table S2). Incorrect interpretations (almost exclusively MEs) were not related to any specific antimicrobial agent (Table S3 and Figure S5).

### Results with final EUCAST RAST breakpoint table (version 1.0)

Based on the aggregated results of the first study<sup>12</sup> and the NE and SE trials, the initial, preliminary breakpoints (version 0) were adjusted to form EUCAST RAST breakpoint table version 1.0 (Table S1).<sup>20</sup>

The adjustments performed reduced the width of several ATUs, most prominently for piperacillin/tazobactam in *E. coli* and *K. pneumoniae* and for clindamycin in *S. pneumoniae*. Breakpoints were also added for norfloxacin in *S. aureus* after 4 h of incubation. With these changes, more results could be categorized and reported, while error rates were only marginally affected (Table 4).

#### Errors per laboratory

When interpreting results using the final EUCAST RAST version 1.0 breakpoint table, 93% ( $n = 36/40$ ) of NE laboratories and 73% (11/15) of SE laboratories showed errors of less than 5% (VMEs, MEs and mEs).

The somewhat poorer result among laboratories in SE was primarily explained by 4 of the 15 laboratories accounting for 56% of errors.

#### Standard DD versus BMD

BMD was performed on a subset of the isolates. Error rates for the RAST method were generally not affected by whether BMD or standard DD was used as the reference (Table S4).

#### QC

The three levels of QC performed are presented in Tables S5 to S7. Participating laboratories submitted the results from standard DD testing (Table S5) and for the RAST procedure (Table S6). The RAST QC results, together with the results from the initial study,<sup>12</sup> formed the basis for defining the targets and ranges for RAST QC recommended by EUCAST for the implementation of RAST in clinical laboratories.<sup>20</sup> The QC results obtained by the reference laboratory are presented in Table S7.

### Discussion

Many attempts have been made to shorten the time required for reporting antimicrobial susceptibility testing results. Both in-house RAST and commercially available methods have been developed. Genotypic methods are expensive, do not quantitate susceptibility, detect only known resistance and are so far incapable of delivering comprehensive susceptibility patterns. This is why phenotypic methods are still preferred, at least for fast-growing bacteria.

New phenotypic antimicrobial susceptibility testing methods are being developed, such as nanotechnologies, flow cytometry and microfluid single-cell analysis<sup>2,21,22</sup> as well as those based on measuring antibiotic degradation or residual viable organisms by spectrophotometry (MALDI-TOF MS) after short incubation in the presence of an antimicrobial.<sup>23–25</sup> Still, a standardized rapid method, with results in 4–6 h after BC positivity, has been lacking.

However, direct antimicrobial susceptibility testing with reading of inhibition zones after 4–8 h of incubation has been used since the 1980s.<sup>26,27</sup> Also, attempts have been made to standardize an

inoculum from 4–6 h subcultures or from positive BC broth<sup>28–30</sup> but without shortened incubation time, early reading and adapted breakpoints.

In a recently published initiative from CLSI to create guidelines for RAST, only 80% of the antimicrobial susceptibility tests could be read after 6 h of incubation and categorical agreements (58.9%–73.2%) were low and related to BC manufacturer, with more errors related to BacT/ALERT bottles.<sup>31</sup> CLSI did not attempt to create breakpoints suited to the shortened reading time. Instead they accepted the errors obtained when using their standard breakpoints.

Reading plates after short incubation is complicated by thin (although confluent) and sometimes hazy growth with poorly demarcated zone edges. Furthermore, zone diameters change during incubation and with short incubation the margin between susceptible and resistant populations is less pronounced. In general, inhibition zone sizes for susceptible isolates increase over time, while zone diameters for resistant isolates tend to decrease.<sup>26,31,32</sup> Based on this knowledge, it is crucial to develop breakpoints that are not only specific to species but also to each incubation time.

Our study aimed to investigate the performance of the EUCAST RAST method directly from positive BC bottles in routine clinical microbiology laboratories across Europe without major training or investments in expensive devices. None of the participating laboratories had practised the EUCAST RAST methodology prior to receiving the protocol. Targeting laboratories in different parts of Europe offered an opportunity to investigate the differences in outcome between laboratories with high and low resistance levels. We expected there to be more multiresistance in laboratories around the Mediterranean and that this could result in more errors. However, in *K. pneumoniae*, where there was significantly more resistance in SE than in NE, errors were only moderately higher (mainly mEs) in SE laboratories than in NE laboratories.

The higher error rates that were obtained in four of the SE laboratories were not explained by resistance rates, resistance mechanisms or QC results out of range. By excluding those laboratories, SE and NE showed similar error rates and the differences in SE error rates between *S. aureus* and the Gram-negative bacteria disappeared.

Since we could not find an obvious explanation for a higher error rate in SE laboratories we assume that difficult-to-read zone diameters were causing the problems, which may also explain the high error rate observed after 8 h of incubation in *S. pneumoniae*, where one isolate was not readable until after 8 h of incubation and then interpreted as pan resistant (all antimicrobials measured as having no zones at all), a result not likely in NE. Excluding this isolate resulted in an error rate at 8 h similar to that at 4 and 6 h of incubation.

There was no systematic difference between BC bottles from different manufacturers in our study, which is contrary to the findings of the CLSI study.<sup>31</sup> During the RAST development, it was shown that the impact of removing a positive BC bottle from the incubator immediately or several hours after the positive signal was small.<sup>12</sup> It has previously been shown that continued incubation of bottles beyond a positive signal does not significantly affect the bacterial concentration in the broth.<sup>25,29</sup>

The following four factors explain the high level of categorical agreement compared with previous trials:

- (i) The use of species-specific breakpoints avoids the splitting of WT distributions (distributions of MICs for organisms without phenotypically detectable resistance mechanisms) and thereby increases reproducibility of susceptibility categorization.<sup>33–37</sup> Because of the poorer separation between WT and non-WT distributions with shortened incubation, distinguishing between susceptible and resistant isolates becomes even more difficult.
- (ii) The use of incubation-time-specific breakpoints takes into account the incomplete antibiotic diffusion in the agar after short incubation and the progress of zone diameter development over time.
- (iii) Introduction of the ATU as a buffer between the S and R breakpoints prevents unreliable results and thus avoids many VMEs and MEs since it absorbs the increased variation inherent in the RAST methodology (poorly controlled inoculum, incomplete separation between WT and non-WT populations, difficulties in reading poorly demarcated zone edges). Consequently several ATUs are wider at 4 h compared with 6 and 8 h of incubation.
- (iv) Breakpoints when finalized in the EUCAST RAST breakpoint table version 1.0 took into account unavoidable inter-laboratory variation.

To evaluate the clinical impact of obtaining antimicrobial susceptibility testing results in 4, 6 or 8 h was beyond the scope of this study. Clinical benefits of rapid species identification and shortened time to antimicrobial susceptibility testing results, such as increased proportion of optimally treated bloodstream infections, decreased hospital length of stay, decreased rate of transfer to ICU and decreased in-hospital mortality, have been shown previously.<sup>2,38–40</sup> Shortened turn-around time of laboratory reports combined with the introduction of antimicrobial stewardship teams further reduce ICU length of stay and 30 day mortality.<sup>41–43</sup>

The EUCAST RAST method is already in use in many laboratories. Two recently published studies evaluated the method with reduced median 'time to report' up to 17 h and acceptable results regarding categorical agreement when compared with their standard methods. However, instead of proper reference methods, they used commercial methods without reference status.<sup>44,45</sup>

In order to guide laboratories on how to streamline BC handling and reduce turn-around times for the benefit of the patient we recommend the section 'Tips and tricks' in [Text S2](#).

It is important to emphasize that this method was developed to allow RAST results to obviate the need for confirmatory, secondary standard antimicrobial susceptibility testing. However, in instances where a result is in the ATU after 8 h of incubation, a standard overnight antimicrobial susceptibility test is needed. Neither all antibiotics relevant for the treatment of bloodstream infection nor all species have so far received RAST breakpoints and RAST breakpoints should not be extrapolated to other antibiotics or species. EUCAST continues to evaluate new antibiotics and species, and the EUCAST RAST breakpoint table is gradually expanding.

## Conclusions

We have shown that EUCAST RAST directly from positive BC bottles can be mastered by routine clinical microbiology laboratories without major training or investment in expensive devices. The cheap

and flexible method performed as intended when tried in a total of 55 laboratories in NE and SE. Our results show that, for most positive BCs, a valid RAST result with an overall categorical agreement of 97% can be obtained after 4–6 h, depending on species, when using the current RAST breakpoints from EUCAST. The successful introduction of the RAST method in routine microbiology enables rapid evaluation of empirical antibiotic treatment in bloodstream infections.

## Additional considerations

Since this study was performed, EUCAST has published RAST breakpoints for *S. aureus* and clindamycin, including a method for detection of inducible clindamycin resistance.<sup>46</sup>

The results in this study were evaluated against EUCAST breakpoint tables version 8.0 (2018). In EUCAST breakpoint tables version 9.0 (2019)<sup>19</sup> the zone diameter breakpoints for Enterobacterales and ciprofloxacin have been adjusted, reducing the number of ciprofloxacin VMEs in this study (RAST breakpoint version 0) from 13 to 4 and 13 to 2 in *E. coli* and *K. pneumoniae*, respectively.

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## Transparency declarations

None to declare.

## Author contributions

E.J., E.M. and G.K. planned the study. E.J. and A.Å. performed the standardized antimicrobial susceptibility testing. All authors analysed and evaluated the results as well as contributed to writing the manuscript.

## Supplementary data

Tables S1 to S7, Texts S1 and S2 and Figures S1 to S5 are available as Supplementary data at JAC Online

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