Contents lists available at ScienceDirect



International Journal of Antimicrobial Agents



journal homepage: www.elsevier.com/locate/ijantimicag

# Direct determination of carbapenem-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa* from positive blood cultures using laser scattering technology \*



Evgeny A. Idelevich <sup>a</sup>, Matthias Hoy <sup>a</sup>, Dennis Knaack <sup>a</sup>, Dennis Görlich <sup>b</sup>, Georg Peters <sup>a</sup>, Matthias Borowski <sup>b,1</sup>, Karsten Becker <sup>a,1,\*</sup>

<sup>a</sup> Institute of Medical Microbiology, University Hospital Münster, Domagkstr. 10, 48149 Münster, Germany
<sup>b</sup> Institute of Biostatistics and Clinical Research, University of Münster, Schmeddingstraße 56, 48149 Münster, Germany

#### ARTICLE INFO

Article history: Received 21 August 2017 Accepted 21 October 2017 Editor: Jean-Marc Rolain

Keywords: Carbapenem resistance Sepsis Bacteraemia Rapid diagnostics Laser scattering Gram-negative rods

### ABSTRACT

Delays in appropriate antimicrobial treatment contribute to increased mortality of septic patients. We aimed to develop a methodology for detection of carbapenem resistance in Gram-negative bacteria directly from positive blood cultures (BCs). Initially, meropenem-resistant Enterobacteriaceae (n = 13) and *Pseudomonas aeruginosa* (n = 32) isolates as well as the same numbers of meropenem-susceptible isolates were used to establish the detection of carbapenem resistance from agar cultures. Growth-based phenotypic detection of meropenem resistance was performed by a laser scattering (LS) method using a BacterioScan<sup>™</sup>216R instrument. A subset of the strain collection consisting of meropenem-susceptible and -resistant isolates (each comprising seven P. aeruginosa and three Klebsiella pneumoniae) was used for determination of carbapenem resistance directly from positive BCs. Lysis/centrifugation and filtration/ dilution methods were investigated for processing of positive BCs. Four different statistical approaches to discriminate between susceptible and resistant bacteria in real-time were applied and were compared regarding their sensitivity and specificity. After 3 h and 4 h of incubation, respectively, detection of carbapenem resistance in Enterobacteriaceae (sensitivity, 100%; specificity, 100%) and P. aeruginosa (sensitivity, 100%; specificity, ≥90%) agar cultures was attainable. Detection of carbapenem resistance directly from positive BCs was achievable with 100% sensitivity and 100% specificity after 4 h and 5 h, respectively, applying lysis/centrifugation and filtration/dilution methods. In conclusion, LS technology combined with lysis/centrifugation and appropriate statistical real-time analyses represents a promising option for rapid detection of carbapenem resistance in Gram-negative rods directly from positive BCs.

© 2017 Elsevier B.V. and International Society of Chemotherapy. All rights reserved.

## 1. Introduction

Sepsis is a life-threatening condition, the prevalence of which is currently increasing [1]. Timely administration of appropriate antimicrobial treatment is essential for survival of sepsis patients [2]. In particular, the alarming increase of carbapenem resistance in Gram-negative bacteria in many countries [3,4] necessitates early knowledge regarding the susceptibility of the pathogen to enable early initiation of adequate antimicrobial therapy. Several studies of bacteraemia caused by carbapenem-resistant *Enterobacteriaceae* 

\* Parts of this work were presented at the ASM Microbe Congress, 16–20 June 2016, Boston, MA [MONDAY-019] and the 68th Annual Meeting of the German Society of Hygiene and Microbiology, 11–14 September 2016, Ulm, Germany [014/DVV].

E-mail address: kbecker@uni-muenster.de (K. Becker).

and *Pseudomonas aeruginosa* have documented that inadequate empirical antimicrobial therapy is associated with increased mortality [5–7]. A very recent study has identified that 30-day-mortality in patients with bacteraemia caused by carbapenem-resistant *Enterobacteriaceae* was as high as 49% [8]. In the same study, the median time from bacteraemia onset until active antimicrobial therapy was 47 h [8]. Such delays in appropriate treatment highlight the need for rapid diagnostics, as rapid detection of carbapenem resistance is critical for early initiation of appropriate antimicrobial treatment.

The classical approach for determination of carbapenem resistance from positive blood cultures (BCs) includes overnight subcultivation of isolates on agar followed by overnight antimicrobial susceptibility testing. Hence, this approach takes ca. 2 days from the positivity of a BC bottle, or ca. 3 days from blood sampling [9]. Molecular systems have recently been developed that detect some carbapenemase genes directly from positive BCs within 1–2 h [10,11]. Whilst the PCR approach may be useful for bacteria with impaired growth, the clinical benefit of this information is unfortunately

https://doi.org/10.1016/j.ijantimicag.2017.10.009

0924-8579/© 2017 Elsevier B.V. and International Society of Chemotherapy. All rights reserved.

<sup>\*</sup> Corresponding author. University Hospital Münster, Institute of Medical Microbiology, Domagkstr. 10, 48149, Münster, Germany.

<sup>&</sup>lt;sup>1</sup> These two authors contributed equally to this work.

limited because only selected resistance targets are included. Furthermore, in the absence of a resistance gene, other resistance mechanisms can still lead to failure of carbapenem treatment [12,13]. Particularly in *P. aeruginosa* it is hardly possible to predict susceptibility of an isolate to carbapenems by molecular tests because carbapenem resistance is commonly caused by other mechanisms than carbapenemase production [14,15]. Therefore, there is an urgent need for the development of novel rapid tests for phenotypic detection of carbapenem resistance. Direct resistance detection from relevant clinical samples without a subcultivation step is of particular importance.

In a previous study, we developed the testing procedure and four different statistical approaches for rapid phenotypic detection of important resistance phenotypes in Gram-positive bacteria by laser scattering (LS) technology [16]. In the present proof-of-principle study, we focused on the development of a procedure for resistance detection directly from positive BCs. The aim of this study was to determine the optimal preparation procedure for positive BCs and to investigate the accuracy and rapidity of the LS method for carbapenem resistance detection directly from positive BCs in *Enterobacteriaceae* and *P. aeruginosa*.

#### 2. Materials and methods

#### 2.1. Bacterial strains

Enterobacterial and P. aeruginosa isolates collected within routine diagnostics at the Institute of Medical Microbiology, University Hospital Münster (Münster, Germany) were used for this study. Consecutive meropenem-susceptible and meropenem-resistant isolates were chosen based on the results of routine antimicrobial susceptibility testing using a VITEK<sup>®</sup>2 instrument (bioMérieux, Marcy-l'Étoile, France). Only one isolate per patient was included. In the framework of the study, meropenem susceptibility was confirmed by determination of minimum inhibitory concentrations (MICs) as described below. A total of 45 isolates of meropenemresistant Gram-negative rods were included in the study, consisting of 13 Enterobacteriaceae (7 Klebsiella pneumoniae, 5 Enterobacter cloacae and 1 Serratia marcescens) and 32 P. aeruginosa isolates. Exactly the same numbers of meropenem-susceptible isolates for each species were included as a control group. Identification of isolates was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). Meropenemresistant Enterobacteriaceae isolates were tested for carbapenemase genes by isothermal amplification using a commercial assay (eazyplex<sup>®</sup> SuperBug; Amplex BioSystems GmbH, Giessen, Germany). Pseudomonas aeruginosa isolates were not investigated for the presence of carbapenemase genes because carbapenem resistance in this species is known to be commonly caused by other mechanisms [14,15].

A subset of this strain collection was used for the determination of carbapenem resistance directly from positive BCs. This subset consisted of 10 meropenem-susceptible and 10 meropenemresistant isolates, each comprising 7 *P. aeruginosa* and 3 *K. pneumoniae* isolates.

#### 2.2. Reference antimicrobial susceptibility testing

MICs of meropenem were determined by the broth microdilution method using cation-adjusted Mueller–Hinton broth (CA-MHB) (BD Diagnostics, Heidelberg, Germany) in accordance with Clinical and Laboratory Standards Institute (CLSI) [17] and International Organization for Standardization (ISO) [18] guidelines. Briefly, the final inoculum size was ca.  $5 \times 10^5$  CFU/mL, confirmed by vital cell counting of serial dilutions. The range of meropenem concentrations tested was 0.008–256 mg/L in double dilution steps. Meropenem powder

was purchased from TCI Deutschland GmbH (Eschborn, Germany). The results were read after  $18 \pm 2$  h of incubation at  $35 \pm 1$  °C. All tests were performed in triplicate and the median MIC was taken for analysis. MIC<sub>50</sub>, MIC<sub>90</sub> and MIC ranges were calculated for the groups of organisms. Reference strains *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

# 2.3. Rapid determination of carbapenem resistance from cultures grown on agar

Growth-based detection of meropenem resistance was performed by the LS method using a BacterioScan™216R device (BacterioScan Inc., St Louis, MO) for real-time monitoring of microbial growth. BacterioScan quantifies microbial concentrations in liquid samples by applying proprietary mathematical algorithms to optical measurements. The instrument allows simultaneous measurements of 16 samples containing 2 mL of fluid. The duration and temperature of incubation are adjustable [16].

A total of 45 meropenem-resistant and the same number of meropenem-susceptible clinical isolates of Gram-negative rods were used as described above. A volume of 1 mL of meropenem solution in CA-MHB was added to 1 mL of bacterial suspension in CA-MHB to produce a final inoculum of  $5 \times 10^5$  CFU/mL and a final meropenem concentration of 2 mg/L in a 2 mL sample. This breakpoint divides susceptible ( $\leq 2 \text{ mg/L}$ ) and intermediate (4–8 mg/L) or resistant ( $\geq$ 16 mg/L) isolates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [19]. Intermediate and resistant categories were combined and were designated as resistant for the aim of this study. Growth control samples consisted of 2 mL of bacterial suspension in CA-MHB with a final inoculum of  $5 \times 10^5$  CFU/mL without meropenem. All samples were incubated in the BacterioScan instrument for 6 h at 35 °C as described previously [16]. Each sample was automatically measured approximately every 3 min.

## 2.4. Rapid determination of carbapenem resistance directly from positive blood cultures

Ten meropenem-susceptible and ten meropenem-resistant isolates, each comprising seven P. aeruginosa and three K. pneumoniae, were mixed with 10 mL of human whole blood at a final concentration of ca. 10 CFU/mL. The real bacterial concentration in spiked blood samples was controlled by vital cell counting after plating and incubation of serial dilutions of inoculum onto tryptic soy agar (TSA). Inoculated blood was introduced into BD BACTEC<sup>™</sup> Plus Aerobic/F bottles (BD Diagnostics) and was incubated in a BD BACTEC<sup>™</sup> 9240 automated BC system (BD Diagnostics) until positivity signal. Positive BC broth was further processed to prepare samples for measurement by the LS method. Two different processing methods of positive BC bottles were investigated and compared, namely lysis/ centrifugation and filtration/dilution methods. Similar to resistance detection from cultures grown on agar, the inoculum was mixed with meropenem solution to produce final concentrations of 2 mg/L meropenem and ca.  $5 \times 10^5$  CFU/mL. Measurement was performed for 20 h at 35 °C.

#### 2.4.1. Lysis/centrifugation method

For the lysis/centrifugation method, 1 mL of positive BC broth was mixed with 200 µL of lysis buffer (Sepsityper<sup>™</sup> Kit; Bruker, Bremen, Germany), followed by centrifugation. After the supernatant was discarded, 1 mL of washing buffer was added and was again centrifuged as described elsewhere [20]. The pellet was dissolved in CA-MHB, was standardised to a 0.5 McFarland turbidity using a nephelometer (Densimat; bioMérieux) and was diluted 1:100 in CA-MHB. Then, 1 mL of this suspension was mixed with 1 mL of meropenem solution to produce samples with a final inoculum of

ca.  $5 \times 10^5$  CFU/mL and a final meropenem concentration of 2 mg/L. The real microbial concentration of 0.5 McFarland suspensions produced from the pellet was controlled by plating of serial dilutions on TSA and calculation of CFU following overnight incubation. The samples, as well as the growth control samples containing the same bacterial concentration without antibiotic, were measured in BacterioScan cuvettes approximately every 3 min for 20 h at 35 °C.

#### 2.4.2. Filtration/dilution method

In a preliminary experiment, the effect of filtration of a positive BC broth on bacterial concentration was investigated. Four *K. pneumoniae* and three *P. aeruginosa* strains were used. This preliminary experiment was performed using defibrinated sheep blood. Briefly, 10 mL of blood samples spiked with bacteria at a final concentration of 10 CFU/mL were inoculated into BACTEC<sup>TM</sup> Plus Aerobic/F bottles and were incubated in the BACTEC<sup>TM</sup> 9240 automated BC system. Within 5 min after positivity signal, 1 mL of positive BC broth was removed with a syringe and was filtered through a 5 µm syringe filter (Sartorius, Göttingen, Germany). Vital cell count was performed in unfiltered and filtered samples by plating and incubation of serial dilutions on TSA plates to determine the microbial concentration in positive BC broth and the recovery rate of bacteria after filtration.

Another preliminary experiment investigated the effect of filtrate dilution on the quality of LS measurements. Briefly, 10 mL of sterile human blood was introduced into a BACTEC<sup>™</sup> Plus Aerobic/F bottle, followed by manual shaking of the bottle. Then, 2 mL of broth was drawn into a syringe, followed by filtration through a 5 µm syringe filter (Sartorius). The filtrate was diluted with CA-MHB at 1:1, 1:10, 1:20, 1:100, 1:200 and 1:1000. Then, 2 mL samples of each dilution, as well as a control CA-MHB sample without blood, were filled into BacterioScan cuvettes and were measured for 10 h at 35 °C.

In the main experiment, two 2 mL samples were drawn with a syringe from each positive BC bottle. One sample was filtered through a 5  $\mu$ m syringe filter (Sartorius), whilst the other sample was left unfiltered. Similar to the preliminary experiments, vital cell count was performed in unfiltered and filtered samples by plating and incubation of serial dilutions on TSA plates to determine the microbial concentration in positive BC broth and the recovery rate of bacteria after filtration. Based on results from the preliminary experiment, ca.  $1-5 \times 10^8$  CFU/mL was expected in the filtrate of a positive BC broth. Hence, the filtrate samples were diluted 1:100 with CA-MHB prior to inoculation of cuvettes.

#### 2.5. Statistical analysis

Testing from cultures grown on agar as well as testing directly from positive BCs was accomplished by determining a series of cutoffs (i.e. one cut-off for each minute of measurement) to discriminate between susceptible and resistant bacteria. Cut-off determination was done by receiver operator curve (ROC) analysis. As previously suggested [16], four different statistical approaches (a–d) were applied to obtain the data for the ROC analyses. In approach (a), only concentrations from samples with antibiotics were used for ROC analyses. In approach (b), ROC analyses were done using the ratio of concentrations with and without antibiotics. Approach (c) was similar to (a), but here the concentrations were replaced by slopes of bacterial growth. These slopes were estimated by real-time regression [21] and could therefore also be derived during LS. In approach (d), the ratio of slopes was used.

The accuracy of these approaches to discriminate between resistance and susceptibility was assessed by sensitivity (the probability to correctly determine resistance) and specificity (the probability to correctly determine susceptibility) with respective 95% confidence intervals (CIs) at each minute of measurement. Data from broth microdilution were used as the reference method for determining sensitivities and specificities. In addition, the categorical agreement (CA) of each approach was calculated as recommended by ISO 20776-2 guideline [22] as well as the incubation time needed to achieve a CA of  $\geq$ 90%. Since determination of cut-offs and estimation of sensitivity and specificity were performed using the same data, probably leading to biased estimates, leave-one-out cross-validation was also used to estimate sensitivities and specificities (with 95% CIs). All analyses were carried out using R 3.3.2. [23].

#### 3. Results

MIC<sub>50</sub>, MIC<sub>90</sub> and MIC ranges of meropenem-resistant and meropenem-susceptible *Enterobacteriaceae* and *P. aeruginosa* isolates used in this study, as determined by the broth microdilution reference method, are shown in Table 1. Among meropenemresistant *Enterobacteriaceae* isolates, seven were OXA-48-positive (all *K. pneumoniae*) and in six isolates (five *E. cloacae* and one *S. marcescens*) no carbapenemase genes were detected.

Whilst the growth characteristics of three species within the *Enterobacteriaceae* group (*K. pneumoniae, E. cloacae* and *S. marcescens*) were quite homogeneous, growth rates were obviously different between *Enterobacteriaceae* and *P. aeruginosa*. Therefore, these two groups of bacteria were evaluated separately for testing from grown colonies. However, for testing directly from positive BCs, all species were analysed in a pooled evaluation owing to the limited number of isolates in this subset.

#### 3.1. Detection of carbapenem resistance from agar cultures

The accuracy of the LS method for detection of carbapenem resistance in *Enterobacteriaceae* from agar cultures was best in combination with statistical approach (b) (Fig. 1).  $CA \ge 90\%$  (recommended accuracy acceptance limit [22]) was achieved after 99 min with this approach and the estimated sensitivity and specificity were both 100% (95% CI 75–100%) after 180 min. However, it should be noted that approach (b) was only slightly better than approach (d), as can be seen in Supplementary Table S1, which lists the sensitivity and specificity estimates of all four statistical approaches.

Table 1

Meropenem MIC<sub>50</sub>, MIC<sub>90</sub> and MIC range of meropenem-resistant and meropenem-susceptible *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates used in the study as determined by broth microdilution reference method.

| MIC (mg/L)        | Isolates for testing from cultures grown on agar |                          |                      |                          | Isolates for testing directly from |                          |
|-------------------|--|--------------------------|----------------------|--------------------------|------------------------------------|--------------------------|
|                   | Enterobacteriaceae                               |                          | P. aeruginosa        |                          | positive blood cultures            |                          |
|                   | Resistant $(n = 13)$                             | Susceptible ( $n = 13$ ) | Resistant $(n = 32)$ | Susceptible ( $n = 32$ ) | Resistant $(n = 9)$                | Susceptible ( $n = 10$ ) |
| MIC <sub>50</sub> | 8  | 0.016                    | 16                   | 0.25                     | 16                                 | 0.25                     |
| MIC <sub>90</sub> | 16   | 0.12                     | 64                   | 1                        | 16                                 | 0.5                      |
| Range             | 8-16   | 0.016-0.12               | 4-64                 | 0.03-2                   | 8-64                               | 0.016-1                  |

MIC, minimum inhibitory concentration; MIC<sub>50/90</sub>, MIC required to inhibit 50% and 90% of the isolates, respectively.



Fig. 1. Estimated sensitivities and specificities to discriminate between resistant and susceptible bacteria (per hours of measurement). Each diagram shows the results of the respective best working statistical discrimination approach; the estimated sensitivities and specificities of all approaches (a–d) are given in Supplementary Tables S1–S4.

For *P. aeruginosa* cultures grown on agar, statistical approach (b) was best again (Fig. 1). After 207 min the CA was  $\geq$ 90% and after 240 min the estimated sensitivity was 100% (95% CI 89–100%) and the estimated specificity was  $\geq$ 90% (95% CI  $\geq$ 75% to  $\geq$ 98%). However, the sensitivity and specificity estimates of approach (b) were very similar to that of approach (d) after 240 min (Supplementary Table S2).

## 3.2. Detection of carbapenem resistance directly from positive blood cultures

One resistant *P. aeruginosa* isolate was excluded from the experiments on resistance detection directly from positive BCs because it signalled positive only after 55.6 h of incubation in BACTEC.

#### 3.2.1. Lysis/centrifugation method

The mean real concentration of bacteria in spiked blood samples prior to incubation in BACTEC was 17.5 CFU/mL (range 9–27 CFU/mL), as confirmed by the vital counts in this series of experiments. The real final bacterial concentration in the test, resulting from a 0.5 McFarland suspension produced from the pellet after treatment by lysis/centrifugation method, was  $2.3 \times 10^6$  CFU/mL on average (range  $1.3 \times 10^6$ – $3.7 \times 10^6$  CFU/mL).

Detection of carbapenem resistance directly from positive BCs using the lysis/centrifugation procedure worked best in combination with statistical approach (d) (Fig. 1). CA  $\geq$  90% was achieved after 147 min and the sensitivity and specificity were estimated to be 100% (95% CI  $\geq$ 66–100%) after 240 min. However, the accuracy of approach (d) was very similar to that of approach (c) (Supplementary Table S3) and was only slightly better than that of approaches (a) and (b).

#### 3.2.2. Filtration/dilution method

Whilst the bacterial concentration in positive BC broth amounted on average to  $1.54\times10^8$  CFU/mL in the preliminary experiment, the concentration of bacteria after filtration through a 5  $\mu m$  filter was  $1.56\times10^8$  CFU/mL. Thus, no cell loss due to filtration was shown.

It was demonstrated in another preliminary experiment that blood components have a detrimental effect on the quality of LS measurements. Sterile samples containing blood showed falsely high values of bacterial concentrations (Supplementary Fig. S1). This negative effect of interference with optical measurements gradually decreased with serial dilution of blood samples. However, even after 1:200 and 1:1000 dilutions the calculated concentration values were still higher than for CA-MHB control without blood (Supplementary Fig. S1). The falsely elevated cell concentrations decreased over time of incubation owing to sedimentation of blood components. Therefore, the differentiation between resistant and susceptible isolates would still be possible.

In the main experiments with the filtration/dilution method, the average concentration of bacteria in inoculated blood samples was 8.8 CFU/mL (range 3.7–11.7 CFU/mL). The mean incubation time in the BACTEC automated BC system until positive signal amounted to 10.3 h and 14.9 h for *K. pneumoniae* and *P. aeruginosa* isolates, respectively. The mean time between positivity signal and processing of positive BC broth was 1.8 h.

The average concentration of *K. pneumoniae* cells in positive BC broth before and after filtration through a 5  $\mu$ m filter was 3.6 × 10<sup>8</sup> CFU/mL and 3.7 × 10<sup>8</sup> CFU/mL, respectively. For *P. aeruginosa*, the concentrations amounted to 2.1 × 10<sup>8</sup> CFU/mL and 1.7 × 10<sup>8</sup> CFU/mL before and after filtration, respectively. Thus, there was no considerable cell loss due to filtration.

Because of the unfavourable optical effect of remaining blood components, a reliable distinction between resistant and susceptible isolates was possible at a later time point with the filtration/ dilution method compared with the lysis/centrifugation method. The statistical approach (c) delivered the best results here (Fig. 1), with estimated sensitivity and specificity of 100% (95% CI  $\geq$ 66–100%) after 300 min (see Supplementary Table S4), and with a CA of  $\geq$ 90% after 224 min.

It is important to note that all sensitivity and specificity values from Supplementary Tables S1–S4 were estimated by four different approaches using the training data set, i.e. the data set that was also used to develop the statistical decision rules. Hence, these sensitivity and specificity estimates cannot be considered to be unbiased. However, the sensitivity and specificity estimates obtained by leave-one-out cross-validation (Supplementary Tables S5–S8) were very similar to those presented in Supplementary Tables S1–S4, therefore indicating their reliability.

#### 4. Discussion

This study focused on methodology development for rapid detection of carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* from positive BCs. Rapid diagnostics in this field are urgently required to better address the high mortality of sepsis patients aggravated by increasing multidrug resistance observed in Gram-negative bacteria. Preparation of blood-containing samples for antimicrobial susceptibility testing is challenging because of the need for inoculum standardisation and the risk of measurement disturbances, the latter particular hampering optical methods.

Blood components remaining after the filtration/dilution procedure caused disturbance of optical measurements, which considerably hindered evaluation. Further dilution may eliminate the problem but provides inoculum counts that are lower than recommended for standard susceptibility testing. We therefore decided to use a 1:100 dilution of positive BC broth (actual 1:200 dilution of the positive broth after addition of antibiotic solution) and rejected further dilution because of the risk of an unfavourable effect on antimicrobial susceptibility testing owing to low inoculum. Dilution is simple and it is generally possible to use this method despite the disturbance effect on optical management. Whilst the concentration curves of susceptible isolates with antibiotics or sterile controls are descending in the course of time owing to sedimentation of blood components, curves for samples containing resistant bacteria or growth controls rise. At some time point, the growth of uninhibited bacteria overgrowths the descending measurement noise and the actual growth becomes detectable. Hence, the easy procedure of filtration/dilution is still able to provide good estimation of susceptibility or resistance (Supplementary Fig. S1). However, it would extend the time to results because the growth of resistant bacteria first needs to overcome the background measurement noise in order to be detected.

Processing of positive BCs with lysis/centrifugation prior to testing by the LS method resulted in clean samples, as was previously shown in a study investigating antimicrobial susceptibly testing of yeasts directly from positive BCs [20]. Colony counting of the standardised 0.5 McFarland suspensions produced by lysis/centrifugation revealed that microbial concentrations after the lysis/centrifugation procedure were high enough to enable preparation of a standardised 0.5 McFarland suspension and that bacteria remained viable during the selective lysis of blood cells, which is a requirement for antimicrobial susceptibly testing. An additional advantage of the lysis/ centrifugation method is that the time from positivity of a BC bottle until BC processing as well as the variations of microbial concentration in positive BC bottles at the time point of processing do not relevantly affect the final inoculum size. This is due to the fact that the inoculum for antimicrobial susceptibly testing is adjusted by preparation of a standardised 0.5 McFarland suspension and subsequent dilution. Thus, lysis/centrifugation represents, in our opinion, a preferable processing method for positive BCs even though it is more time consuming. The benefit of the filtration/dilution method is its easiness, but the reliable results of testing are available somewhat later. Interestingly, filtration of positive BCs did not have the desired effect itself, since the optical measurements were still disturbed after filtration. It was dilution that gradually improved the measurement quality at higher titres.

The accuracy of differentiation between susceptible and resistant isolates directly from positive BCs and the time needed for such differentiation were comparable with the testing from grown agar cultures. However, such a direct comparison is limited by the fact that Enterobacteriaceae and P. aeruginosa isolates tested directly from positive BCs were analysed as one group. We expect the results for BC experiments with Enterobacteriaceae to be better if this group is evaluated separately. This is due to more rapid growth of Enterobacteriaceae compared with non-fermenters such as P. aeruginosa. Owing to the particularly early response of susceptible K. pneumoniae isolates to meropenem, visual differentiation between resistant and susceptible isolates was possible already after ca. 1.5 h for this species (data not shown). However, more isolates are needed to prove this assumption. It is noteworthy that not only resistant but also even intermediate isolates (MIC 4-8 mg/L) were correctly detected as non-susceptible during testing both grown cultures and positive BCs by the LS method.

Rapid discrimination between resistance and susceptibility was feasible by combining microbiological with statistical methods. Four statistical approaches were applied and the results of the best working method are presented in Fig. 1. However, the differences between the four approaches were rather small and even the worst working approaches still provided remarkably high sensitivities and specificities (Supplementary Tables S1–S8). Since cross-validation yielded similar sensitivities and specificities, we expect that our approaches would deliver as accurate and fast results for future samples. Moreover, we assume that refining the statistical approaches can further improve the accuracy and rapidity of the methodology.

The potential advantage of the presented technology for clinical diagnostics includes the availability of cuvettes that can be individually filled with an antibiotic of interest to accomplish ondemand runs. Together with the compact and closed design of the instrument, the technology is well suited for single tests that ideally could take place as point-of-care diagnostics. Pre-filling of antimicrobials by the manufacturer, e.g. in lyophilised form, would contribute to the simplicity and convenience of application. Several antibiotics concentrations, e.g. for determination of MICs or for differentiation between intermediate and resistant categories, as well as several different antibiotics can be tested simultaneously. However, one limitation of the system in its current form is that only 16 samples can be simultaneously tested, which precludes the automated testing of multiple isolates to a large number of antibiotics.

In conclusion, the LS method combined with lysis/centrifugation is promising for rapid determination of carbapenem resistance in Gram-negative rods directly from positive BCs. The time to result is particularly short for *Enterobacteriaceae*. The performance of this method warrants further investigation applying a wider geographic, genotypic and phenotypic diversity of isolates.

#### Acknowledgments

The authors are thankful to Barbara Grünastel and Damayanti Kaiser for excellent technical assistance.

*Funding*: The BacterioScan<sup>™</sup>216R device and consumables were provided for the study by BacterioScan Inc. (St Louis, MO).

*Competing interests*: EAI has received congress travel support from BacterioScan Inc. All other authors declare no competing interests. *Ethical approval*: Not required.

#### Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2017.10.009.

#### Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2012. Crit Care Med 2013;41:580–637.

- [2] Gradel KO, Jensen US, Schonheyder HC, Ostergaard C, Knudsen JD, Wehberg S, et al. Impact of appropriate empirical antibiotic treatment on recurrence and mortality in patients with bacteraemia: a population-based cohort study. BMC Infect Dis 2017;17:122.
- [3] Magiorakos AP, Suetens C, Monnet DL, Gagliotti C, Heuer OE. The rise of carbapenem resistance in Europe: just the tip of the iceberg? Antimicrob Resist Infect Control 2013;2:6.
- [4] Thaden JT, Lewis SS, Hazen KC, Huslage K, Fowler VG Jr, Moehring RW, et al. Rising rates of carbapenem-resistant Enterobacteriaceae in community hospitals: a mixed-methods review of epidemiology and microbiology practices in a network of community hospitals in the southeastern United States. Infect Control Hosp Epidemiol 2014;35:978–83.
- [5] Girometti N, Lewis RE, Giannella M, Ambretti S, Bartoletti M, Tedeschi S, et al. *Klebsiella pneumoniae* bloodstream infection: epidemiology and impact of inappropriate empirical therapy. Medicine (Baltimore) 2014;93:298–309.
- [6] Tumbarello M, Repetto E, Trecarichi EM, Bernardini C, De Pascale G, Parisini A, et al. Multidrug-resistant *Pseudomonas aeruginosa* bloodstream infections: risk factors and mortality. Epidemiol Infect 2011;139:1740–9.
- [7] Tumbarello M, Viale P, Viscoli C, Trecarichi EM, Tumietto F, Marchese A, et al. Predictors of mortality in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: importance of combination therapy. Clin Infect Dis 2012;55:943–50.
- [8] Satlin MJ, Chen L, Patel G, Gomez-Simmonds A, Weston G, Kim AC, et al. Multicenter clinical and molecular epidemiological analysis of bacteremia due to carbapenem-resistant Enterobacteriaceae (CRE) in the CRE epicenter of the USA. Antimicrob Agents Chemother 2017;61. e02349-16 [pii].
- [9] Kerremans JJ, Verboom P, Stijnen T, Hakkaart-van Roijen L, Goessens W, Verbrugh HA, et al. Rapid identification and antimicrobial susceptibility testing reduce antibiotic use and accelerate pathogen-directed antibiotic use. J Antimicrob Chemother 2008;61:428–35.
- [10] Banerjee R, Teng CB, Cunningham SA, Ihde SM, Steckelberg JM, Moriarty JP, et al. Randomized trial of rapid multiplex polymerase chain reaction-based blood culture identification and susceptibility testing. Clin Infect Dis 2015;61:1071–80.
- [11] Dodemont M, de Mendonca R, Nonhoff C, Roisin S, Denis O. Performance of the Verigene Gram-negative blood culture assay for rapid detection of bacteria and resistance determinants. J Clin Microbiol 2014;52:3085–7.
- [12] Idelevich EA, Becker K. Identification and susceptibility testing from shortly incubated cultures accelerate blood culture diagnostics at no cost. Clin Infect Dis 2016;62:268–9.

- [13] Culbreath K, Petti CA. Balancing enthusiasm for innovative technologies with optimizing value: an approach to adopt new laboratory tests for infectious diseases using bloodstream infections as exemplar. Open Forum Infect Dis 2015;2:ofv075.
- [14] Chalhoub H, Saenz Y, Rodriguez-Villalobos H, Denis O, Kahl BC, Tulkens PM, et al. High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations. Int J Antimicrob Agents 2016;48:740–3.
- [15] Riera E, Cabot G, Mulet X, Garcia-Castillo M, Del CR, Juan C, et al. Pseudomonas aeruginosa carbapenem resistance mechanisms in Spain: impact on the activity of imipenem, meropenem and doripenem. J Antimicrob Chemother 2011;66:2022–7.
- [16] Idelevich EA, Hoy M, Görlich D, Knaack D, Grünastel B, Peters G, et al. Rapid phenotypic detection of microbial resistance in Gram-positive bacteria by a real-time laser scattering method. Front Microbiol 2017;8: 1064.
- [17] Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—tenth edition. Wayne, PA: CLSI; 2015. Document M07-A10.
- [18] International Organization for Standardization. ISO 20776-1. Clinical laboratory testing and in vitro diagnostic test systems—susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices—part 1: reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. Geneva, Switzerland: International Organization for Standardization; 2006.
- [19] European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0; 2017. Available from http://www.eucast.org [Accessed 20 December 2017].
- [20] Idelevich EA, Grunewald CM, Wüllenweber J, Becker K. Rapid identification and susceptibility testing of *Candida* spp. from positive blood cultures by combination of direct MALDI-TOF mass spectrometry and direct inoculation of Vitek 2. PLoS ONE 2014;9:e114834.
- [21] Borowski M, Fried R. Online signal extraction by robust regression in moving windows with data-adaptive width selection. Stat Comput 2014;24:597– 613.
- [22] International Organization for Standardization. ISO 20776-2. Clinical laboratory testing and in vitro diagnostic test systems—susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices—part 2: evaluation of performance of antimicrobial susceptibility test devices. Geneva, Switzerland: International Organization for Standardization; 2007.
- [23] R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2017. Available from: http://www.R-project.org. [Accessed 20 December 2017].