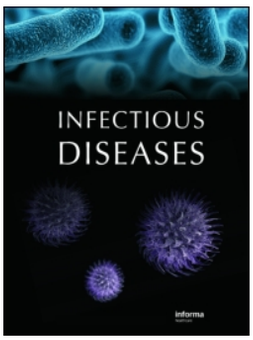

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ORIGINAL ARTICLE

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Rapid detection of antibiotic resistance in positive blood cultures by MALDI-TOF MS and an automated and optimized MBT-ASTRA protocol for *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Introduction: For fast and effective antibiotic therapy of serious infections like sepsis, it is crucial with rapid information about antibiotic susceptibility, especially in a time when the number of infections caused by multi resistant bacteria has escalated in the world.

Methods: Here, we have used a semi-quantitative MALDI-TOF-MS based method for antibiotic resistance detection, MBT-ASTRA™, which is based on the comparison of growth rate of the bacteria cultivated with and without antibiotics. We demonstrate a new protocol where several parameters have been optimized and automated leading to reduced hands-on time and improved capacity to simultaneously analyse multiple clinical samples and antibiotics.

Results: Ninety minutes of incubation at 37 °C with agitation was sufficient to differentiate the susceptible and resistant strains of *E. coli* and *K. pneumoniae*, for the antibiotics cefotaxime, meropenem and ciprofloxacin. In total, 841 positive blood culture analyses of 14 reference strains were performed. The overall sensitivity was 99%, specificity 99% and the accuracy 97%. The assay gave no errors for cefotaxime ($n = 263$) or meropenem ($n = 289$) for sensitive and resistant strains, whilst ciprofloxacin ($n = 289$) gave six (0.7%) major errors (false resistance) and four (0.5%) very major errors (false susceptibility). The intermediate strains showed a larger variety compared to the E-test MIC values.

Conclusions: The hands-on time and the analysis time to detect antibiotic resistance of clinical blood samples can be substantially reduced and the sample capacity can be increased by using automation and this improved protocol.

KEYWORDS
MALDI-TOF-MS
MBT-ASTRA
sepsis
antibiotic susceptibility
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Supplemental data for this article can be accessed here.

Introduction

Sepsis is one of the most serious and urgent infections in clinical practice, and various bacteria, fungi and viruses may be responsible for the infection. The condition is diagnosed on clinical basis according to the third international consensus definition for sepsis and septic shock (Sepsis-3) [1,2]. Bacterial infection is the major cause for sepsis in Sweden and sepsis is a leading cause of death in hospitalized patients, and in Sweden, approximately 70–80/10,000 persons annually since 2015 had sepsis, with a mortality rate of around 20% within 90 d [3–5]. Rapid information about antibiotic susceptibility is crucial when deciding on an appropriate and effective antibiotic therapy for serious infections like sepsis. There is an unmet need for faster susceptibility assays to avoid treatment failure and the need is escalating with the increasing bacterial antibiotic resistance worldwide.

An increasing number of infections are caused by resistant bacteria such as extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, resistant to penicillins, 2nd and 3rd generations cephalosporins, and in some cases also to carbapenems [4,6]. Different nomenclatures have been suggested for classification of ESBLs, and the golden standard in Sweden is the definition by Giske et al. [7], which is used in this study. This definition divides ESBL enzymes into three main groups: $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$. In $ESBL_A$, the most frequently found enzymes are CTX-M followed by TEM and SHV [4]. The genes for these enzymes are horizontally transferrable and their function is inhibited by clavulanic acid. $ESBL_M$ are various ESBLs where the most common type is acquired AmpC and some of the metallo β-lactamases [7]. $ESBL_{CARBA}$ are enzymes conferring carbapenemase activity, such as some metallo β-lactamases and *Klebsiella pneumoniae* carbapenemase (KPC) [7]. The emergence of KPC resistance in *Enterobacteriaceae* has become a substantial clinical problem [8,9]. In 2017 and 2018, annually, around 10,000 cases of $ESBL_A$ and $ESBL_M$ were reported to the Public Health Agency of Sweden by the clinical microbiology laboratories [4,9]. In 2018, 144 new cases (0.14/10,000 inhabitants) of $ESBL_{CARBA}$ were reported, and the clinical findings of $ESBL_{CARBA}$ has tripled since 2014. Most of these strains were acquired abroad (80%) [4,9].

Since the early days of discovery of bacteria, culture-dependent assays have remained the golden standard for identification of bacteria and antibiotic susceptibility testing. These two tasks are of critical importance as they heavily influence treatment decisions for the patients, but the methods are slow. The identification of the causative pathogens takes at least 5 h, and final antibiotic susceptibility results are generated in 18–36 h. Slow-growing bacteria can take several more days to grow. The standard for MIC determination in routine clinical susceptibility analysis is either disc diffusion or E-test [10]. Thus, the faster the information is available, the faster the patient's treatment can be optimized, potentially saving patient lives, reducing irreversible long-term side effects, as well as minimizing antibiotic toxicity, the risk of developing antibiotic resistance, and use of costly pharmaceuticals [11]. With the automated analysis systems that are commercially available today, such as MicroScan (Siemens Healthcare Diagnostics GmbH, Tarrytown, NY), Phoenix (BD Diagnostic Systems, Cockeysville, MD) or Vitek 2 (bioMérieux, Craponne, France), susceptibility results can be obtained after approximately five to 8 h for fast growing bacteria [12].

Molecular biology methods, such as polymerase chain reaction (PCR), can be useful, but provides only genetic information on the presence or absence of specific resistance-related genes, which not always mirror the complete resistance activity. Additionally, several hundred genes can cause ESBL-production in *Enterobacteriaceae*, and providing PCR-assays for all of these genes are currently expensive and time consuming, a fact that has restricted their general use [13]. The detected gene fragments in PCR-systems might even originate from pathogens already suppressed or killed by antibiotics, and fragments whose origin is unknown [14].

The standard method for species identification of cultured bacteria in clinical laboratories has recently evolved to be matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) based analysis. Further development of semi-quantitative mass spectrometry for antibiotic resistance testing, MBT-ASTRA™ has been described [15–26]. This method measures and calculates the relative growth (RG), e.g. the ratio of the growth of the bacteria cultivated in medium with and without antibiotics. After growth, cells are lysed, an internal standard is added, and analyses are performed with MALDI-TOF MS. The spectra are normalized to the highest peak for a direct correlation between peak area and the amount of bacterial proteins, which correlates to the bacterial growth. For data evaluation, the MBT-ASTRA™ prototype software compares the peak area with and without antibiotics, given that less growth will occur when the bacteria are sensitive compared to the peak area for the bacteria growing

in absence of the antibiotic. This gives information about the RG. So far, several research studies have been conducted and published in order to develop MALDI-TOF MS methods for antibiotic susceptibility analysis [15–26]. However, most of the studies have not considered simultaneous analysis of a variety of antibiotics and a larger number of samples, which is relevant for clinical laboratories.

Here, we demonstrate optimization of several parameters during the preparation and the analysis of positive blood cultures, for the MBT-ASTRA^{TM} method, to reduce the analysis time and the hands-on-time to be able to detect antibiotic resistance faster in the clinic for patients with invasive infections. Our technology is suitable for automatization and expandable to further applications, e.g. simultaneous testing of multiple antibiotics as well as resistance determination directly from clinical samples. Furthermore, a large number of analysis of *Escherichia coli* and *K. pneumoniae* were performed with the new protocol to demonstrate its reproducibility and its clinical use.

Materials and methods

The *E. coli* and *K. pneumoniae* bacterial strains with ESBL resistance of different genotypes were obtained from the Culture Collection, University of Gothenburg (CCUG), Gothenburg, Sweden and Clinical Microbiology, Region Skåne (CMRS), Lund, Sweden (Table 1). All isolates were stored in a medium containing 20% horse serum and 10% glycerol at −25 °C and cultured on Müller Hinton (MH) agar plates at 37 °C for 18–24 h before the final preparation. For direct analysis of bacteria from culture plates, colonies were diluted in sterile 0.85% NaCl to a concentration of 0.5 McFarland (McF). Blood culture bottles (BD Bactec Plus Aerobic/F Culture bottle and BD Bactec Plus Anaerobic Lytic/F Culture bottle) were spiked with a mixture of 100 µl of the bacterial suspension and 10 ml horse blood. The bottles were incubated in an automated BACTEC FX^{TM} blood culture system (Becton Dickinson, Franklin Lakes, NJ) until flagged positive. All preparations and analysis were performed in duplicate.

Bacterial preparation from blood culture bottles

A commonly used method to prepare a purified bacterial pellet is to take two ml of positive blood culture and centrifuge it at 140×*g* for 10 min, discard the pellet and mix the supernatant with 3 ml 0.85% NaCl and centrifuge at 300×*g* for another 10 min [27]. The bacterial pellet is thereafter ready to use. In this study, several modifications of the pellet preparation method were evaluated in order to reduce time without loss of quality of the bacterial pellet. Addition of 0.25% Triton X-100, 5% Saponin, MALDI Sepsityper kit 50 Lysis buffer (Bruker, Bremen, Germany) or 0.4 M Caps 0.6% Brij in the washing steps was analysed, and as a substitute for the washing steps using Serum Separator Tubes (SST; Becton, Dickinson and Company, Franklin Lakes, NJ) (data not shown). The following method gave the best outcome and did not interfere with the McFarland measurement: 1 ml blood from the aerobic blood culture bottles was added to 200 µl 5% Saponin solution and 1 ml of the mixture was then added to 200 µl MALDI sepsityper KIT 50 Lysis buffer. No Saponin solution was added to the anaerobic bottles. The mixtures were centrifuged at 13,000×*g* for 2 min. The supernatant was removed, 1 ml sterile water was added and the tube was thoroughly mixed. After an additional centrifugation at 13,000×*g* for 2 min, the supernatant was removed and

Table 1. Bacterial strains used their resistance genes and MIC values for cefotaxime (CTX), meropenem (MER) and ciprofloxacin (CIP).

				MIC by E-test		
Source	Number	Species	Resistance genes	CTX (mg/l)	MER (mg/l)	CIP (mg/l)
CCUG	10785	*K. pneumoniae*	–	0.015	0.015	0.002
CCUG	8619400	*E. coli*	–	0.06	0.03	0.015
CCUG	58538	*E. coli*	MOX	128	0.06	0.03
CCUG	58543	*E. coli*	CMY-2	64	0.03	0.06
CCUG	58547	*K. pneumoniae*	VIM	256	>32	>32
CCUG	59351	*E. coli*	CTX-M 15	>256	0.015	>32
CCUG	59357	*E. coli*	SHV12/5A	3	0.008	0.03
CCUG	59360	*K. pneumoniae*	SHV12/5A	2	0.015	0.03
CMRS	756	*E. coli*	SHV	2	0.008	0.015
CMRS	101076	*K. pneumoniae*	NDM, CTX-M1(15)	32	4	>32
CMRS	549078	*E. coli*	DHA	2	0.015	0.12
CMRS	518178	*K. pneumoniae*	DHA, CTX-M1(15)	>256	0.015	0.5
CMRS	101067	*K. pneumoniae*	KPC	>256	>32	>32
CMRS	500182	*K. pneumoniae*	KPC, CTX-M1(15), CMY-2	>256	>32	>32

the pellet was mixed with cation-adjusted MH broth (CAMH; Sigma-Aldrich, St. Louis, MO) to 0.5 McF.

Incubation time and antibiotic concentrations

Based on our and previous findings for the MBT-ASTRA™ assay, the incubation time for the bacterial strains, with and without antibiotics added to the medium, was set to 90 min [18]. For titration experiments to determine the antibiotic breakpoint concentrations, fresh bacterial isolates were incubated at 0.5 McF in 200 µl CAMH either in microtubes or a 96-well plate at 37 °C for 90 min with agitation, with and without cefotaxime, meropenem or ciprofloxacin at concentrations ranging from 0.5 to 128 mg/l. Based on these results, 32 mg/l cefotaxime, 16 mg/l meropenem and 4 mg/l ciprofloxacin were selected and used in the MBT-ASTRA assay for this study.

Extraction in microtubes

After 90 min incubation at 37 °C, the suspensions were centrifuged at 13,000×*g* for 2 min. The supernatants were discarded, the pellets were resolved in 150 µl pure water and centrifuged for two more minutes at the same speed. The pellets were resolved with 100 µl 70% ethanol and centrifuged for 2 min at 13,000×*g*. The supernatants were then discarded and the pellets were left to air dry for 5 min. After drying, the pellets were subjected to 10 µl 70% formic acid for 3 min, then 10 µl acetonitrile, containing an internal protein standard (1:50 vol:vol; RNase B Bruker, Bremen, Germany), was added and the samples were further centrifuged at 13,000×*g* for 2 min. In quadruple, 1 µl of the extracted solution was added on a MALDI target plate, and 1 µl of saturated HCCA (alpha-cyano-4-hydroxycinnamic acid; Bruker, Bremen, Germany) in matrix solution (50% acetonitrile, 2.5% trifluoroacetic acid) was added before analysed [18,22].

Extraction in 96-well filter membrane plates

Five microliter 0.4 M Caps 0.6% Brij was added to the bacterial suspensions that had been grown for 90 min at 37 °C, and the samples were further incubated at room temperature for 3 min in order to avoid clogging of 96-well filter membranes. The suspensions were transferred to a 96-well 0.45 µm filter membrane plate (Pall Corporation, Port Washington, NY), and either centrifuged at 1500×*g* for 10 min or subjected to 68 psi pressure for 6–12 min until dry, using a Waters Positive Pressure-96 Processor (Waters, Milford, MA). To each well, 200 µl pure water was added, and then centrifuged for 10 min or subjected to pressure. Next, 200 µl 70% ethanol was added and centrifuged for another 10 min or subjected to pressure. Thereafter, 25 µl 70% formic acid was added, and incubated for 3 min. Next, 25 µl acetonitrile containing the internal protein standard (Bruker, Bremen, Germany) was added, and the plate was centrifuged at 1500×*g* for 5 min with a collector plate underneath. In quadruple, 1 µl of the extracted solution was added on a MALDI target plate, and 1 µl of HCCA matrix solution was overlaid.

Pre analysis step automated

The described pre-analysis steps were performed by either experienced staff, or with the Freedom EVO® (Tecan, Männedorf, Switzerland) robotic system. The automated system prepared 96-well culture plate with bacteria and antibiotics, incubated at 37 °C with continuous shaking (200 rpm), transferred the culture to 96-well filter plate, did all pipetting steps to filter plate, transferred the extraction to MALDI-target plate and the final HCCA matrix solution was added. The automated method was less labour intensive, and it took around 2 h for 96 samples to be prepared, compared to the manual steps that required around 4 h. No differences in the results of the analysis were seen between the two different methods.

MALDI-TOF MS analysis and MBT-ASTRA™

MALDI-TOF MS measurements were performed with a microflex LT/SH bench-top MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with a 60 Hz nitrogen laser, recording the *m/z* range between 2000 and 20,000 Da using standard settings for specie identification except that all peaks were considered for spectra evaluation instead of excluding the highest peak. An external calibration standard (Bacterial Test Standard, BTS; Bruker, Bremen, Germany) was used for instrument calibration. The resulting spectra were uploaded to the MBT-ASTRA™ web-based prototype by Idelevich et al. novel dedicated prototype software (MBT MASTeR, Bruker Daltonik, Bremen, Germany) [18–20]. The software, normalizes the peaks, performs peak picking, and determines the area under the curve (AUC) of each spectrum. Relative growth was calculated as the ratio of the AUC in the presence over the AUC in the absence of antibiotics. An RG cut-off value of 0.4 was used to distinguish between susceptible and resistant isolates.

Reference method for MIC determination

The minimal inhibiting concentration (MIC) values for each bacterial strain were determined by E-test gradient strip (bioMerieux, Marcy-l'Étoile, France) for the different antibiotics. In brief, E-test was placed on Muller–Hinton agar plates that had been inoculated with a 0.5 McF suspension of the strain. The plate was incubated at 37 °C for 16 h before the MIC value was read. The MIC value was used to classify the strain as susceptible, intermediate or resistant strains to a specific antibiotic according to EUCAST breakpoint [10].

Data evaluation

The MBT-ASTRA RG values was compared to the E-test MIC values used in the clinical laboratory. Comparisons were expressed as agreement, major error (false resistance) or very major error (false susceptibility) [28]. Sensitivity was defined as number of true resistant isolates (resistant by both MBT-ASTRA assay and MIC) over total resistant isolates (resistant only by MIC). Specificity was defined as the number of true susceptible isolates (susceptible by both MBT-ASTRA assay and MIC) over total number of susceptible (susceptible only by MIC) isolates. Accuracy was defined as the number of true resistant and true susceptible replicates over the total number of replicates.

Results

Analysis optimization

Three key results emerged from the analysis optimization phase. One, the new protocol for bacterial preparation of positive blood culture bottles reduced the hands-on time from 45–20 min for a 96-well plate. Second, 90 min of culture at 37 °C with agitation, was sufficient to see the difference between susceptible and resistant strains of *E. coli* and *K. pneumoniae*. The third important result was that the short culture and the post culture steps could be automated, and the total analysis time was reduced with approximately 2 h.

Data evaluation

E-test results in comparison to MBT-ASTRA-results

The assay was performed with blood culture bottles spiked with 14 well characterized strains, some carrying known resistance genes (Table 1). The MIC values obtained from the E-tests showed that the strains susceptible for cefotaxime varied between 0.015 and 0.12 mg/l, whereas three strains were classified as intermediate with MIC values of 2 mg/l (Figure 1) [10]. The resistant strains MIC values varied between 3 mg/l and >256 mg/l. The MIC values for the strains susceptible to meropenem varied between 0.008 and 0.25 mg/l, the intermediate at 4 mg/l, and the resistant strains had MIC values >32 mg/l. For ciprofloxacin, the MIC values for the susceptible strains varied between 0.002 and 0.5 mg/l and the resistant strains had MIC values >32 mg/l. The RG values from the MBT-ASTRA analysis showed substantial variations for the intermediate strains, and for ciprofloxacin in some susceptible strains and in one resistant strain (Figure 1).

Spiked blood culture bottles of 14 reference strains

In total, 898 analyses of the 14 reference strains were performed (Figure 2). The intermediate reference strains were not included in the data evaluation, which were based on 841 analyses, with major errors (false resistance) at six (0.7%) occasions, and very major errors (false susceptibility) at four (0.5%) occasions (Table 2). The

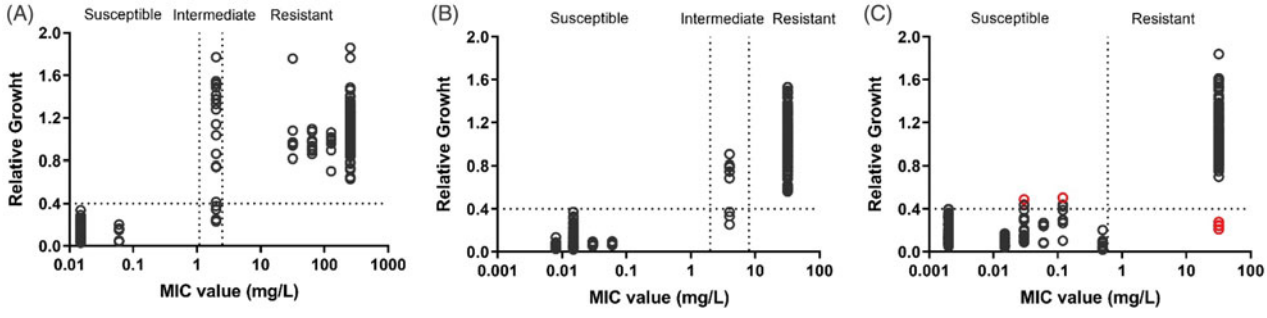

Figure 1. MIC values (Table 1) obtained from E-test (*x*-axis) plotted against the relative growth from the MBT-ASTRA analysis (*y*-axis) of (A) cefotaxime ($n = 263$), (B) meropenem ($n = 289$) and (C) ciprofloxacin ($n = 289$). The ratio of the AUCs in the presence and absence of antibiotics provides the relative growth. The susceptibility/resistance threshold was set at a relative growth value of 0.4 (horizontal dotted line). The vertical dotted line separates antibiotic susceptible, intermediate and resistant strains according to their MIC-values as classified by EUCAST [10]. Circles out of the dotted lines represent errors given from the MBT-ASTRA assay (red online).

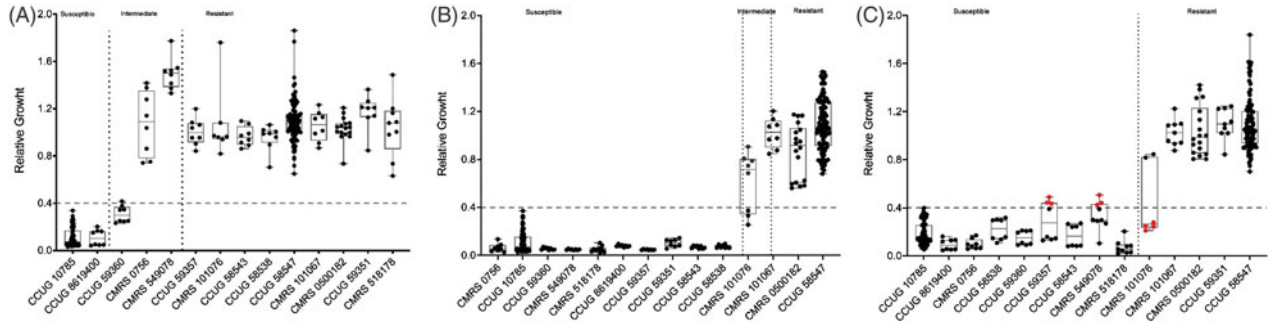

Figure 2. Blood culture bottles spiked with 14 different reference strains. The strains are arranged from left to right in order of increasing MIC value (Table 1). (A) Cefotaxime, (B) meropenem and (C) ciprofloxacin. The ratio of the AUCs in the presence and absence of antibiotic provides the relative growth. The susceptibility/resistance threshold was set at a relative growth value at 0.4 for the MBT-ASTRA analysis. Data are displayed with box and whisker plots, the medians are indicated by centrelines, the minima and maxima by whiskers, the 25th and 75th percentiles by boxes. Circles out of the dotted lines represent errors given from the MBT-ASTRA assay (red online).

Table 2. Susceptibility analysis from blood cultures, performed with MBT-ASTRA compared with routine diagnosis using E-tests.

	Isolate resistant by E-test		Isolates susceptible by E-test							
Antibiotic	Susceptible by MBT-ASTRA	Resistant by MBT-ASTRA	Susceptible by MBT-ASTRA	Resistant by MBT-ASTRA	Total replicates	Very major error rate	Major error rate	Sensitivity	Specificity	Overall accuracy
Cefotaxime	0	172	91	0	263	0.0%	0.0%	100%	100%	100%
Meropenem	0	127	162	0	289	0.0%	0.0%	100%	100%	100%
Ciprofloxacin	4	136	143	6	289	1.4%	2.1%	97%	96%	97%
Total	4	435	396	6	841	0.5%	0.7%	99%	99%	97%

overall sensitivity was 99%, specificity 99% (ranging from 96% to 100%) and the accuracy 97%.

Reproducibility of data points of two reference strains

A reproducibility analysis was performed with blood culture bottles spiked with the reference strains CCUG 10785 and CCUG 58547 in order to evaluate all the data obtained from the MBT-ASTRA. In total, 2205 data points were evaluated, with major errors (false resistance) at 21 (1.0%) data points, and very major errors (false susceptibility) at five (0.2%) data points (Table 3; Figure 3).

Discussion

Out of the different bacterial preparation methods for positive blood cultures investigated in this study, the most convenient and rapid method was to use the SST-tubes and direct centrifugation to obtain a purified bacterial pellet. Unfortunately, the background interference was not to our satisfaction, since high McFarland values could be seen in preparations from negative blood cultures with this method. Funke and Funke-Kissling [29] found that the use of SST-tubes did not lead to an increased rate of contamination, but it could possibly lead to interference with blood components that are not completely removed. In the region served by the clinical microbiology laboratory in Lund, the BD Bactec Plus Anaerobic Lytic/F Culture bottle is the standard anaerobic blood culture media, and they are not suitable for the SST method since the blood components are lysed. This leads to no separation of the blood components with the SST centrifugation method. Thus, the final protocol for bacterial preparation used in this study are based on Schubert et al. [30] lysis protocol for the MALDI Sepsityper kit, with the addition of saponin to the aerobic bottles. The anaerobic bottles already contains saponin, hence the blood preparation becomes equivalent for both aerobic and anaerobic bottles.

With our optimized protocol, we demonstrate that an incubation time of 90 min can detect antibiotic resistance in *E. coli* and *K. pneumoniae*. Other findings suggest timeframes of 60 min to 4 h, depending on the bacteria species, chosen antibiotics, and its concentrations [18–22,31,32]. The antibiotic concentrations for the final analysis of the blood cultures were in our study set to 32 mg/l cefotaxime, 16 mg/l meropenem and 4 mg/l ciprofloxacin. In contrast, Jung et al. [22] found that 2 mg/l cefotaxime and 1 mg/l ciprofloxacin were sufficient, but with two and a half to three hours incubation time. Lange et al. [18] suggest 8 mg/l meropenem with an incubation time of 1–2 h and Sparbier et al. [19] suggest incubation with 20 mg/l cefotaxime and 8 mg/l meropenem for 2 h, and 4 mg/l ciprofloxacin for 3 h. The MBT-ASTRA^{TM} method is relatively easy to adjust for

Table 3. Reproducibility analysis performed with the reference strains CCUG 10785 and CCUG 58547.

	CCUG 10785			CCUG 58547			
Antibiotic	Major error rate (%)	Very major error rate (%)	Agreement rate (%)	Major error rate (%)	Very major error rate (%)	Agreement rate (%)	Accuracy
Cefotaxime	2 (0.6%)	0 (0%)	329 (99.4%)	0 (0%)	1 (0.3%)	394 (99.7%)	99.6%
Meropenem	6 (1.7%)	0 (0%)	344 (98.3%)	0 (0%)	0 (0%)	402 (100%)	99.2%
Ciprofloxacin	13 (3.9%)	0 (0%)	319 (96.1%)	0 (0%)	4 (1.0%)	391 (99.0%)	97.7%

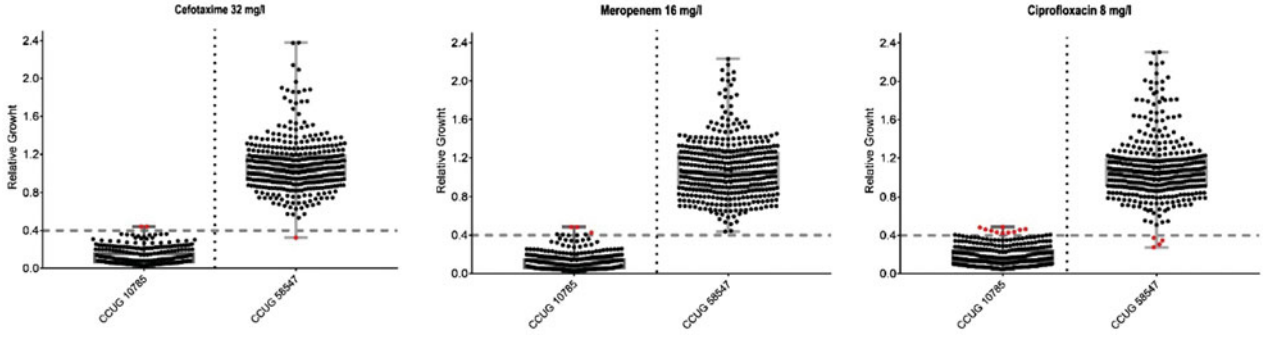

Figure 3. Reproducibility of the relative growth values of (A) cefotaxime, (B) meropenem and (C) ciprofloxacin with the reference strains CCUG 10785 and CCUG 58547. The ratio of the AUCs in the presence and absence of antibiotic provides the relative growth. The susceptibility/resistance threshold was set at a relative growth value of 0.4. Circles out of the dotted lines represent errors given from the MBT-ASTRA assay (red online).

incubation times and a variety of other antibiotics, and in the present study, the incubation time managed to be reduced to 90 min for *E. coli* and *K. pneumoniae*. This study focused on *Enterobacteriaceae*, which explains the selected antibiotics for rapid susceptibility results. This is a limitation, but the method could be adjusted for other species and gram positive bacteria as well. Unpublished data from present study show that the incubation time for gram positive bacteria, will be prolonged at least from 180–300 min depending on the concentrations and antibiotics selected.

The post incubation steps with the washing and elution steps are time-consuming and quite laborious. When the number of samples is substantial, the 96-well filter membrane plates saved more than an hour, even though the centrifugation time increased compared to the microtube method. Over all for 96 wells, the 2 h pre analysis saving time is a positive addition to the method, since it was partly performed by a laboratory robot, the Freedom EVO® automatic system. In addition, the filter plate eliminates the risk of manually pipetting away the bacterial pellet, which reduces the number of false sensitive results.

The MBT-ASTRA™ software requires MALDI-TOF MS measurements in quadruplicates for the susceptibility analysis. This approach is rather time consuming and Jung et al. [20,22] suggested that triplicate or duplicate analysis might be sufficient. However, a considerable variability was seen in our results and we would not recommend a reduction in number of repeated measurements in order to save time. An alternative way to speed up the analysis further is to analyse the samples with a faster MALDI-TOF MS instrument with a 200 or 1000 Hz laser instead of a 60 Hz laser.

When blood culture spiked with 14 different reference strains was analysed with the assay it gave no errors for cefotaxime ($n = 263$) or meropenem ($n = 289$), whilst ciprofloxacin ($n = 289$) gave six major errors and four very major errors, which gave an overall accuracy of 97%. The intermediate strains showed a large variety of data points, other than that, no correlation between the RG value and the E-test MIC was observed, which also are concluded in other studies [18,19,21]. Lange et al. [18] suggest that the inability to correlate the MBT-ASTRA analysis to the MIC values obtained by the E-test might depend on the relative short antibiotic incubation time or varied antibiotic concentrations. To confirm or reject these correlations, further studies with this focus are required.

The reproducibility of the assay on blood cultures spiked with two different reference strains, one susceptible and one resistant, showed an accuracy of 97.7% for ciprofloxacin, 99.2% for meropenem and 99.6% for cefotaxime. In the reproducibility test, 2205 data points in total were used, approximately 90 replicates for each strain and antibiotic. In a study by Maxson et al. [21], 40 replicates of blood cultures spiked with ciprofloxacin 4 mg/l incubated for 2 h, were evaluated; they found a five-percentage error rate, compared to our two-percentage error rate. However, the reproducibility test

in this study was performed with two reference strains with a cutoff value of 0.4, whilst they used four reference strains and a cutoff value of 0.5. The reproducibility test in our study was performed with reference strains well within the safe MIC-values for susceptibility and resistance. Further reproducibility studies of strains with a larger variety of MIC-values would be useful when evaluating the suitability for the MBT-ASTRA™ method in a clinical setting. Nonetheless, the new protocol presented in this study substantially reduced the hands-on-time and the total analysis time to obtain a rapid preliminary result regarding antibiotic resistance. This can lead to faster effective antibiotic treatment and it can be of critical importance if it is a serious infection like sepsis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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