ORIGINAL ARTICLE

Direct blood culturing on solid medium outperforms an automated continuously monitored broth-based blood culture system in terms of time to identification and susceptibility testing

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Abstract

Pathogen identification and antimicrobial susceptibility testing (AST) should be available as soon as possible for patients with bloodstream infections. We investigated whether a lysis-centrifugation (LC) blood culture (BC) method, combined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) identification and Vitek 2 AST, provides a time advantage in comparison with the currently used automated broth-based BC system. Seven bacterial reference strains were added each to 10 mL human blood in final concentrations of 100, 10 and 1 CFU/mL. Inoculated blood was added to the Isolator 10 tube and centrifuged at 3000 *g* for 30 min, then 1.5 mL sediment was distributed onto five 150-mm agar plates. Growth was observed hourly and microcolonies were subjected to MALDI-TOF MS and Vitek 2 as soon as possible. For comparison, seeded blood was introduced into an aerobic BC bottle and incubated in the BACTEC 9240 automated BC system. For all species/concentration combinations except one, successful identification and Vitek 2 inoculation were achieved even before growth detection by BACTEC. The fastest identification and inoculation for AST were achieved with *Escherichia coli* in concentrations of 100 CFU/mL and 10 CFU/mL (after 7 h each, while BACTEC flagged respective samples positive after 9.5 h and 10 h). Use of the LC-BC method allows skipping of incubation in automated BC systems and, used in combination with rapid diagnostics from microcolonies, provides a considerable advantage in time to result. This suggests that the usefulness of direct BC on solid medium should be re-evaluated in the era of rapid microbiology.

Keywords: Antimicrobial susceptibility testing, bacteraemia, bloodstream infections, detection, direct blood culturing, identification, rapid diagnostics, sepsis, solid medium
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Introduction

It is well recognized that sepsis mortality can be considerably lowered if adequate antimicrobial therapy is administered early, ideally in the first hour after onset of symptoms [1]. Obviously, at this very early time point, the causative agent is unknown and the therapy can only be chosen empirically. Nevertheless, rapid microbiological diagnostics still provide significant benefit for patients by shortening the time to appropriate antimicrobial treatment [2,3], reducing mortality [4] and length of hospital stay [3]. Recently, the improvement of clinical outcomes due to rapid diagnostics has been confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) -based identification [5–9]. Considerable advances have been achieved in rapid identification applying direct MALDI-TOF MS to positive blood cultures (BCs) [10] or performing MALDI-TOF MS on a tiny biomass grown very quickly (a few hours) on solid medium after sub-culturing from positive BCs [11–14]. However, incubation of blood samples in automated liquid medium BC systems remains a substantial time-limiting factor. This approach, using continuous

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monitoring for detection of microbial growth, has been the reference standard for detection of microorganisms in blood for the last three decades [15,16]. Before the introduction of these systems, lysis-centrifugation BC (LC-BC) method was commonly used [17–19]. This method implements lysis of blood cells in a whole blood sample and centrifugation of the lysed blood, followed by the removal of supernatant and plating of the sediment onto the solid media [20,21]. We hypothesized that this solid-medium-based direct cultivation method, applied in combination with MALDI-TOF MS may enable faster bacterial identification than automated BC systems. Furthermore, this study aimed to investigate the use of the early available colonies for antimicrobial susceptibility testing (AST).

Materials and methods

Bacterial strains and preparation of spiked blood samples

Seven reference strains of different bacterial species—*Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pneumoniae* (ATCC 49619) and *Haemophilus influenzae* (NCTC 8468)—were cultured on Columbia blood agar (chocolate agar for *H. influenzae*) for 24 h at 36°C. Suspensions with a standardized turbidity of 0.5 McFarland were prepared in 0.85% saline solution (in a preliminary experiment, an expected cell concentration of 0.5 McFarland standard suspension was determined in triplicate for each strain by serial dilution and colony count of plated suspensions). From 0.5 McFarland suspensions, dilution series were prepared and individually calculated volumes (89–350 µL, based on the results of the preliminary experiment) of the appropriate dilution of each strain were added to 10 mL human whole blood to produce final concentrations of 100, 10 and 1 CFU/mL in blood. The real final inoculum size of each suspension used was verified by plating and incubating on tryptic soy agar, Columbia blood agar (for *Streptococcus pneumoniae*) or chocolate agar (for *H. influenzae*).

Human whole blood was obtained from healthy volunteer donors not receiving any systemic medication. The volunteers provided written informed consent before blood donation. Blood was collected in sterile bags (Leukotrap® WB, Pall Medical, Port Washington, NY, USA).

Sample processing and incubation

Ten millilitres of inoculated blood was aseptically introduced into an Isolator 10 tube (Wampole Laboratories, Princeton, NJ, USA) containing saponin for blood cell lysis and sodium polyanethol sulphonate as an anticoagulant by piercing the rubber septum with a sterile needle. Immediately after adding the spiked blood sample, tubes were gently inverted several times and centrifuged at 3000 *g* for 30 min. After removal of supernatant, 1.5 mL sediment was vortexed and evenly distributed onto five pre-warmed 150-mm Columbia blood agar plates (chocolate agar for *H. influenzae*). All plates were incubated at 36°C in air with 5% CO_2. Growth was observed hourly and microcolonies were subjected to MALDI-TOF MS for identification and to Vitek 2 for AST as soon as it was deemed possible. Incubation of agar plates was continued up to 24 h and both identification and AST were performed from mature colonies as control.

For comparison, seeded blood was inoculated into an aerobic blood culture bottle (BACTEC™ Plus Aerobic/F; BD Diagnostics, Heidelberg, Germany) and incubated in an automated BC system (BACTEC™ 9240; BD Diagnostics). Time to positivity (i.e. growth detection) was automatically recorded.

All experiments were performed in triplicate on different days and median values were calculated for analysis.

MALDI-TOF MS identification

MALDI-TOF MS using intact cell procedure [11] was performed at the time point at which microcolonies became visible and it was considered after visual inspection that the colony material could be sufficient for investigation. Spectra were acquired using the Microflex LT system (Bruker Daltonics, Bremen, Germany) and analysed by MALDI Biotyper 3.1 (Bruker Daltonics) software. MALDI-TOF MS analysis was performed in triplicate, with tests performed simultaneously on the same target slide. Criteria for successful identification were fulfilled if the score of at least one from three spots was $\geq$2.0 (high confidence identification); however, the time point of achievement of low confidence identification (score $\geq$1.7) as well as achievement of the modified threshold (score $\geq$1.5 and first three identical propositions) were also recorded. The experiment was stopped when intact cell identification was successful. If not, intact cell MALDI-TOF MS was repeated hourly until successful identification (score $\geq$2.0). Also, in the case of failed intact cell identification, it was attempted to increase the score at the same time point by (a) giving additional manual laser shots on the same spots, (b) repeating MALDI-TOF MS analysis using the short on-plate extraction procedure [22], and (c) giving additional manual shots if MALDI-TOF MS with on-plate extraction also failed. For control, intact cell MALDI-TOF MS was also performed at 24 h.

Antimicrobial susceptibility testing

During the hourly inspection of plates, the first time point was determined at which the growth appeared sufficient for preparation of 0.5 McFarland suspension to be used for AST by

Vitek 2 (bioMérieux SA, Marcy l'Étoile, France) instrument with N214 (for *Escherichia coli*), N248 (for *P. aeruginosa*), P632 (for *S. aureus* and *S. epidermidis*), P586 (for *E. faecalis*) and P576 (for *Streptococcus pneumoniae*) cards. Similar to routine diagnostics, no Vitek 2 AST was performed for *H. influenzae*. Unlike the manufacturer's recommendation to prepare 0.5 McFarland suspensions in 3.0 mL saline [23], we used a reduced volume of approximately 1.5 mL, because this reduces the amount of microcolonial biomass needed, which is enough for Vitek 2 AST if no simultaneous Vitek 2 identification is performed. The median AST results for each species/concentration combination were compared with the corresponding results produced by the testing of mature 24-h cultures.

Results

Median time to identification using different confidence thresholds and median time needed for AST (cultivation time until Vitek 2 inoculation, duration of Vitek 2 testing and total time to result) after the processing by the direct LC-BC method are demonstrated in Table 1. For comparison, median time until BACTEC positivity signal (growth detection) is also shown (Table 1).

In 20 of the 21 species/concentration combinations, high confidence species identification (score $\geq$2.0) after LC-BC was achieved even before BACTEC had detected growth in the corresponding samples (the time point at which usually only Gram stain would be possible with automated BC systems). The only exception was *S. epidermidis* at a concentration of 100 CFU/mL, which was identified at 14 h by LC-BC, but BACTEC flagged the bottle positive after 13.9 h. Notably, MALDI-TOF MS identification after LC-BC was achieved after 13 h (i.e. earlier than the BACTEC positive signal) in this strain at this concentration when a score threshold of $\geq$1.7 or a score $\geq$2.0 with on-plate extraction was used (Table 1). On average, for all species at all bacterial concentration levels, high confidence species identification (score $\geq$2.0) was achieved by the intact cell protocol (without extraction) 3.3 h earlier than BACTEC required for growth detection in the corresponding samples. With a score $\geq$2.0 and use of on-plate extraction for some samples, this difference reached 3.5 h. With a low confidence identification threshold of $\geq$1.7, the average difference amounted to 3.5 h and 3.6 h, respectively, when extraction or extraction with additional manual shots was used for some samples. With the modified threshold (score $\geq$1.5 and three identical propositions), the average difference to BACTEC positivity time was 3.6 h.

Similarly, Vitek 2 inoculation was possible before the BACTEC positive signal for corresponding samples in 17 of 18 species/concentration combinations. The exception was again *S. epidermidis* at a concentration of 100 CFU/mL. Vitek 2 testing

TABLE 1. Median time to MALDI-TOF MS identification result and median time needed for Vitek 2 antimicrobial susceptibility testing (AST) after the processing by a lysis-centrifugation direct blood culture method, in comparison with the time to BACTEC positivity signal

Organism	Concentration (CFU/mL)	Time to MALDI-TOF MS identification after direct blood culture method (h)			Time to Vitek 2 AST after direct blood culture method (h)			Time to BACTEC positivity signal, h
		High confidence (score ≥2.0)	Low confidence (score ≥1.7)	Modified threshold (score ≥1.5 and three identical propositions)	Time until Vitek 2 inoculation	Duration of Vitek 2 AST	Total time to Vitek 2 result	
Escherichia coli	100	7	7	7	7	7.8	14.8	9.5
	10	7	7	7	7	7.8	14.8	10.0
	1	8	8	8	8	7.8	15.8	11.0
Pseudomonas aeruginosa	100	11	11 (10[b])	11 (10[b])	11	12.3	23.3	13.5
	10	11	11	11	12	13	25.0	15.5
	1	12	12	12	13	14.8	27.8	17.2
Staphylococcus aureus	100	8	8	8	8	10.8	18.8	10.7
	10	8	8	8	8	11.3	19.3	12.7
	1	10 (9[a])	9	9	9	10.8	19.8	14.0
Staphylococcus epidermidis	100	14 (13[a])	13	13	14	11.8	25.8	13.9
	10	15 (14[a])	15 (14[a])	14	14	14	28.0	16.1
	1	15	15	15	17	13	30.0	17.8
Enterococcus faecalis	100	9 (8[a])	8	8	8	11.5	19.5	10.7
	10	9	9	9	9	11.8	20.8	11.9
	1	10	10	10	10	12	22.0	13.0
Streptococcus pneumoniae	100	9	8	8	9	13.8	22.8	10.2
	10	9	9	9	10	13.8	23.8	11.5
	1	12	12	12	12	10.3	22.3	12.4
Haemophilus influenzae	100	11	11	11	—[c]	—	—	16.3
	10	11	11	11	—	—	—	18.6
	1	11	11	11	—	—	—	20.0

[a]Time to identification was reduced by on-plate extraction.
[b]Time to identification was reduced by on-plate extraction followed by additional shots.
[c]No Vitek 2 antimicrobial susceptibility testing is routinely performed for *H. influenzae*.

was started at 14 h in this case, i.e. 6 min after the BACTEC positivity signal. On average for all bacterial concentrations of six species, Vitek 2 AST from colonies cultivated after the LC-BC procedure was started 2.5 h earlier than BACTEC detected bacterial growth in the comparison samples. A complete AST report was ready on average 9.0 h after the BACTEC positivity signal for corresponding samples. An AST profile was generated in all tests without any technical errors or abort runs. For all species/concentration/antibiotic combinations but one, essential agreement (MIC result within plus or minus one of doubling dilution step [24]) was achieved, compared with the testing of standard 24-h cultures. Only in *S. epidermidis* at a concentration of 10 CFU/mL, was the median penicillin MIC for short culture two dilutions lower than the value from the 24-h culture. Categorical agreement (correct categorization as susceptible, intermediate or resistant [24]) was achieved for all species/concentration combinations.

Discussion

Our study demonstrated considerable time saving by direct blood culture on solid medium using the LC-BC method combined with MALDI-TOF MS identification and Vitek 2 AST from the microcolonies grown. In almost all cases, species identification and inoculation of the AST system was possible even before the BACTEC positive signal for corresponding samples. In most cases, successful identification was achieved when microcolonies were visually considered sufficient for identification. Application of extraction, additional shots, or lowered thresholds further reduced the time to identification in only a few cases. In some cases, differences between time to successful identification from solid medium and time to growth detection by BACTEC were particularly pronounced. For instance, after 11 h, *H. influenzae* (1 CFU/mL) was already identified by MALDI-TOF MS subsequent to LC-BC. In contrast, it took a further 9 h until BACTEC detected growth in the corresponding bottle (Table 1). Other examples are differences of 5.2 h and 4.7 h for *P. aeruginosa* (1 CFU/mL) and *S. aureus* (10 CFU/mL) samples, respectively (Table 1).

The time advantage of LC-BC in comparison with BACTEC was more pronounced in samples with lower bacterial concentrations (Table 1). This trend was observed for both time until identification and time until Vitek 2 inoculation. Rapid diagnostics of 'low-CFU' samples appear particularly important because most bacteraemias represent low inoculum infections [25]. It additionally demonstrates the feasibility of early use of small amounts of microcolonies for diagnostics and high recovery rates with LC-BC. The recovery rate after 24 h of growth was 92–96% in preliminary experiments (data not shown).

Use of the LC-BC method allows the incubation in automated BC systems to be skipped by direct blood culturing, which provides a time advantage. Here, we did not sub-culture positive BC bottles to measure the incubation times necessary for identification and inoculation for AST in the comparison group. However, these times have been thoroughly investigated in our previous studies [11,26] and by other authors [12–14]. Both time to identification and time to Vitek 2 inoculation were longer in the present study than was demonstrated for very briefly incubated cultures on solid medium after sub-cultivation from positive BC bottles [11]. This is most probably a result of the differences in the inoculum size being introduced onto the agar. In incubated BC bottles, high inocula are reached, which after sub-culturing rapidly result in visible bacterial growth, described as lawn, haze, or veil, after several hours [11–14]. In contrast, individual microcolonies are observed as a result of the direct cultivation on agar after LC-BC because of a low (real) inoculum size. These separate microcolonies obviously need more time than bacterial lawn until they become visible for the investigator and can be used for analysis. However, the fact that species identification and inoculation in the AST system were possible even before the BACTEC positivity signal in almost all cases, shows that it is still advantageous to start incubation directly on solid medium and not in liquid medium. In the latter case, detection of growth by the automated BC system is only a starting point for sub-culture onto agar and subsequent incubation. In this study, AST from microcolonies directly cultured on solid medium generated a final result on average 9.0 h after the BACTEC positivity signal for corresponding samples processed in this automated system. According to a previous study, the total time from positive BC sub-culture (at positivity signal) to AST result was 33–34 h with a standard procedure using the automated BC system [26]. It included standard 24-h cultivation time until Vitek 2 inoculation and duration of Vitek 2 AST. Notably, the total time to result is considerably shorter if the method of briefly incubated solid medium cultures is used for positive BCs [26,27].

With the advent of automated BC incubators, the use of an Isolator system has been largely abandoned. Comparative studies demonstrated earlier detection of microbial growth or reduced processing time by the automated continuous monitoring systems [17,18,28,29]. However, even back then, some authors recognized that (a) detection with the Isolator could have been earlier if one had observed the plates more frequently [17,18], and (b) the time to availability of isolated colonies is more important than the time to detection [19], because at the latter time point only Gram stain is possible with automated BC systems, and the broth has to be sub-cultured on solid medium to gain colonies for identification and AST. In contrast, the Isolator directly delivers colonies on solid medium, which are

immediately used for identification and AST, addressing the need for faster diagnostics. Further advantages of direct blood culturing on solid medium include that: (a) quantification of bacterial load is possible; (b) Gram staining can be omitted because species identification is done by MALDI-TOF MS even more easily and quickly once the colony has become visible; (c) false-positive signals of automated systems (in the absence of any microbial growth) are avoided; and (d) release and better detection of intracellular bacteria due to blood lysis (lysis reagents are also included in some media for automated systems).

We do not generally advocate the switch from automated BC systems to the Isolator system because of existing technical and logistical drawbacks. The most reported shortcomings of an LC-BC approach are the increased workload in the laboratory (the need for centrifugation and manual distribution of sediment) and the possibility of contamination [17,18,28]. The laboratories using this system should establish the optimal specimen processing and feasible intervals for observation of agar plates within the laboratory's operating hours. We believe that technical improvements of direct blood culturing on solid media such as easier blood processing (feasible at a patient's bed), exploitation of storage and transport times as well as contamination-free operation (E.A. Idelevich and K. Becker, pending patent application) will make this method most suitable for the modern diagnosis of bloodstream infections. Compatibility with 'intelligent' incubators will further accelerate and optimize diagnosis through early automatic growth detection by imaging technology as well as automatic transfer of microcolonies to the identification and AST systems.

In conclusion, direct blood cultivation on solid medium enables much earlier identification and AST than a liquid-based automated BC system. This finding is striking and suggests that the usefulness of direct blood cultivation on solid medium should be re-evaluated in the era of rapid microbiology.

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Transparency declaration

EAI and KB are the inventors of the pending patent application 'Apparatus and method for processing of body fluids', owned by the University of Münster. Other authors declare no conflicts of interest.

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