MALDI-TOF as a Tool for Rapid Species Identification from Blood Culture Vials
MALDI-TOF as a Tool for Rapid Species Identification from Blood Culture Vials

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Abstract
Early adequate antimicrobial therapy improves outcome in septicemia. Rapid species identification (ID) from blood cultures is thus important. Matrix-assisted laser desorption-ionisation time of flight (MALDI-TOF) can provide species ID within an hour from blood culture (BC) vials. The present study aimed at comparing the performance and hands on time of two methods for preparing samples from BC vials prior to species ID using MALDI-TOF analysis: sample preparation with Sepsityper™ kit and samples smeared from inoculated agar plates after 1-5 hours incubation. Aerobic and anaerobic BC vials, BacT/Alert and BacTEC, were inoculated in triplicates with five reference strains. Viable count was applied as a control of growth in the BC vials. Thereafter two methods were tested for species ID: sample preparation with the Sepsityper™ kit (Bruker Daltonics) followed by Ethanol/Formic Acid Extraction and MALDI-TOF analysis as well as a plating method where 100µl of BC-suspension were inoculated on chocolate agar and incubated in CO₂ in 1, 2, 3, 4 and 5 h followed by MALDI-TOF analysis without extraction.

Using the Sepsityper™ all species were identified with ID score >2.0 from BacTEC, while none from the BacT/Alert vials. The time to result using the plating method was quite similar for BacT/Alert and BacTEC: *Escherichia coli* could be detected after 2h, *Enterococcus faecalis* and *Pseudomonas aeruginosa* after 3h. *Staphylococcus aureus* and *Streptococcus agalactiae* could not be identified. Sample preparation with the Sepsityper™ kit followed by MALDI-TOF analysis allows a rapid ID from BacTEC vials with a hands on time of one hour for 12 samples. However, ID from the plating method is less laborious and seems as a promising alternative.
SAMMANFATTNING

Snabb artbestämning av bakterier i blododlingar är av stor vikt för patientens behandling vid sepsis. Nyligen har Matrix Assisted Laser Desorption Ionization-Time of flight (MALDI-TOF) rapporterats kunna genomföra artbestämning på ca 1 timme. Syftet med denna studie var att beräkna tidsvinst vid direkt artbestämning från blododlingsflaskor med MALDI-TOF i jämförelse med när bakterier odlas på agarplattor följd av MALDI-TOF.

I två fabrikat av blododlingsflaskor (BacTEC och BacT/Alert) inokulerades fem referensstammar. Blododlingsflaskorna inkuberades därefter i 35-37 °C i 18 timmar. Provbehandling med Sepsityper™ kit utfördes, följt av MALDI-TOF analys. Inokulat från blododlingsflaskorna odlades samtidigt på agarplattor för kontroll av växt med viable count och för försök till artbestämning med MALDI-TOF efter 1-5 timmars inkubering. Med Sepsityper™ kunde samtliga referensstammar identifieras med ID score > 2.0 från BacTEC men ingen från BacT/Alert. Försök till artbestämning från agarplattor resulterade i samstämmiga resultat från BacTEC och BacT/Alert: *E. coli* kunde identifieras efter 2 timmar, *E. faecalis* och *P. aeruginosa* efter 3 timmar medan *S. aureus* och *S. agalactiae* ej kunde identifieras. Försöken visar att Sepsityper™ tillåter en snabb artbestämning från BacTEC med en arbetstid på ca 1 timme för 12 prover. Artbestämning direkt från agarplatta innebär mindre arbetsinsats (10 minuter för 12 prover) och verkar vara ett lovande alternativ.
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INTRODUCTION

The invasion of bacteria into the sterile bloodstream may lead to clinical sepsicaemia with the typical symptoms of fever, chills and sometimes abdominal pain or other localised symptoms. This can develop into the life threatening condition septic shock (1). Septic shock is a drastic response from the immune system to the bacterial invasion, leading to multiple organ failure and in several cases death (2). It is thus important to administer an early and appropriate empirical antimicrobial therapy to lower the risk of death. In patients with septic shock treated with appropriate empirical therapy the mortality is around 20 % while for patients treated with inappropriate antibiotics it is as high as 34 %. To guide the treatment it is important to have a rapid and reliable identification of the causing microorganism (3).

The most common bacterial genera causing bacteraemia at Växjö Central Hospital are staphylococci, Enterobacteriaceae and streptococci. Coagulase Negative Staphylococcus (CoNS) is the most common finding in blood cultures at Växjö Central Hospital (Sundqvist M, MD, Växjö Central Hospital, personal communication). These are however very often judged as a contamination due to their abundance on the skin and their relatively low pathogenic features and are not included in the present study (4). At Växjö Central Hospital the most common pathogen found in blood cultures, after CoNS, are Escherichia coli, a gram negative facultative anaerobic rod, causing intra- and extraintestinal infections, especially urinary tract infections. The third most common species is Staphylococcus aureus (table I), a facultative anaerobic, gram-positive coccus that occurs in irregular grape-like clusters when Gram stained. Septicaemia caused by S. aureus is often with an acute onset and is associated with a high mortality. Other less common causes of bacteraemia are Streptococcus agalactiae, Enterococcus faecalis and Pseudomonas aeruginosa. Streptococcus agalactiae, also called group B streptococcus (GBS), is a beta-haemolytic, gram-positive diplococcus. It is an important pathogen in the newborn and can also cause postpartum infections, endocarditis, bacteraemia and sepsis. Enterococcus faecalis is a facultative anaerobic, gram-positive coccus that causes endocarditis, urinary tract, prostate, and epididymal infections. Pseudomonas aeruginosa is an aerobic, gram-negative rod and a common pathogen in hospital acquired infections (5-10).
Table I. The distribution of bacterial species found in blood cultures at Växjö Central Hospital during the time period 100101-101231 (Sundqvist M, MD, Växjö Central Hospital, personal communication)

<table>
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<th>Bacterial species</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase Negative <em>Staphylococcus</em> (CoNS)</td>
<td>27,3</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>20,0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>11,3</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>6,6</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>4,3</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>2,1</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1,8</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>1,8</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>1,6</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em> (group B streptococcus)</td>
<td>1,5</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>1,5</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>1,3</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>1,0</td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>1,0</td>
</tr>
</tbody>
</table>

The mortality rate differs in patients with bacteremia depending on microorganism and the mortality increases markedly in elderly persons. Untreated *S. aureus* bacteremia (SAB), for example, carries a 30 day mortality that exceeds 80 % dropping to 13.8 % in patients with adequately treated SAB. Group B streptococcal bacteremia in adults has a mortality of 31 %, adequately treated *E. faecalis* bacteremia 18 % and *Pseudomonas aeruginosa* bacteremia as high mortality as 61 % (6-10).

**Systems for blood culture**

In patients with suspected blood stream infections, at least 40 ml of venous blood should be inoculated in blood culture vials (two aerobic and two anaerobic). These are then incubated in an incubation device with a temperature of 37°C until reported positive. Vials not reported positive will be reported negative after four to ten days of incubation (Smyth R, Växjö Central Hospital, personal communication). Two systems for blood culture are chiefly on the market: BacT/Alert from bioMérieux and BacTEC from Becton, Dickinson and Company (BD). The main differences between the two blood culture systems are the composition of the growth-medium and also their different ways of neutralizing antibiotics potentially present in the blood sample. BacTEC vials have resin and BacT/Alert have charcoal to capture antibiotics. Studies have shown that there is no significant difference between the systems in detection of bloodstream infections (11) in patients receiving antibiotics at
the time of blood culture. The conclusion of this is that both resin and charcoal works equally well (12).

The two systems have similar principles for detecting growth. The systems detect CO₂ produced when microorganisms metabolize the substrate in the culture medium. The method of detection of increased CO₂ is different in the two systems. Both types have a sensor at the bottom of the blood culture vial. In the BacTEC system the produced CO₂ reacts with a pigment in the sensor. This reaction regulates the amount of light absorbed by a fluorescent material. A photodetector then measures the increased fluorescence, which is proportional to the concentration of CO₂ in the vial. In the BacT/Alert-system the sensor changes colour when CO₂ is produced. A light emitting diode (LED) projects light on the sensor and the increased level of reflected light is registered by a photodetector (13-14).

Methods used for species identification

Today, when a blood culture vial is positive a Gram stain is usually performed followed by a subculture on appropriate solid agar medium and different biochemical tests are performed manually or in automatic systems. Using these conventional microbiological techniques the time from positive signal in the blood culture system to species identification is 6-48 hours (Smyth R, Växjö Central Hospital, personal communication). A variety of different methods have been applied to speed up the species identification from positive blood culture bottles. Examples of such methods are fluorescent in situ hybridization (FISH), Polymerase chain reaction (PCR) followed by sequencing, hybridization, pyrosequencing or single-stranded conformation polymorphism (15). These techniques are in general both expensive and require several hours of hands on time. In a recently published study the technique FISH has been applied on pathogens in blood culture vials and compared with traditional cultivation results. The overall agreement between them was 91,4 %, with better results for gram-negative bacteria than gram-positive (100 % and 89,5 %, respectively). The FISH analysis took 1 hour (16). PCR has in one study been suggested as a method for rapid detection of S. aureus bacteraemia (17). PCR can also be connected to Electrospray ionization (ESI)-MS and provide a species ID within five to six hours (18). Recently, the technique matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used for rapid identification of bacteria and yeasts (15).
**MALDI-TOF MS**

The principle of MALDI-TOF MS is quite simple. At first the sample is mixed with a matrix solution on a MALDI target plate, which then results in crystallisation of the sample. The MALDI target plate is after sample preparation loaded into the mass spectrometer and bombarded with pulses of a laser beam. The matrix absorbs energy from the laser and the sample is divided into ions. The electric field accelerates the ions and they will pass a field-free drift region called flight tube with a given length. Smaller ions will travel faster than larger ions. When the ions reach the end of the flight tube they hit a detector. The energy of the impact together with the time of flight that is required to reach the detector is used to calculate the masses of the ions. This information is then used to create a mass spectrum. A mass spectrum (Fig. 1) is a graphical illustration of the different m/z values obtained in a sample (19).

![Diagram of MALDI-TOF MS](image)

**Figure 1.** The principle of MALDI-TOF MS. At first sample and matrix are mixed and bombarded with a laser which leads to a release of ions from the sample. The ions, with different masses and charges travel, down a flight tube and hit the detector. A mass spectrum is then created from the calculation of the ions mass and charge (reproduced with permission of Wiley-Blackwell, from the article “Application and use of various mass spectrometry methods in clinical microbiology”) (20)

To identify bacteria and yeasts with MALDI-TOF MS the mass spectrum is matched with a database containing thousands of identified mass spectra. Every bacterial species has its own mass spectrum. The peaks of the species mass spectrum represent microbial proteins. After the MALDI-TOF analysis the received results are presented in a table by the software (Table II) (21).
Table II. The different levels of certainty of the bacterial species identification using the Biotyper 2.0 software for interpretation of mass spectra obtained in the Microflex system (Bruker Daltonics, Germany)

<table>
<thead>
<tr>
<th>Range of Score Value</th>
<th>Description</th>
<th>Symbols</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.300- 3.000</td>
<td>Highly probable species identification</td>
<td>(+++)</td>
<td>Green</td>
</tr>
<tr>
<td>2.000- 2.299</td>
<td>Secure genus identification, probable species identification</td>
<td>(++)</td>
<td>Green</td>
</tr>
<tr>
<td>1.700- 1.999</td>
<td>Probable genus identification</td>
<td>(+)</td>
<td>Yellow</td>
</tr>
<tr>
<td>0.000- 1.699</td>
<td>No reliable identification</td>
<td>(-)</td>
<td>Red</td>
</tr>
</tbody>
</table>

The type of matrix used can affect the mass spectra gathered from bacterial samples. The selection of the proper matrix is important because different matrices allow analysis of varying types of biomolecules. Examples are 2-(4-hydroxyphenylazo) benzoic acid (HABA), 5-chloro-2-mercaptobenzothiozole (CMBT) and α-cyano-4-hydroxycinnamic acid (HCCA). HCCA has been reported to provide high-quality and reproducible spectra for a diverse set of microorganisms (19).

There is at present two MALDI-TOF systems on the market. The description below is focused on the system from Bruker Daltonics.

**Previous studies**

Most previous studies using MALDI-TOF MS from blood culture vials for the identification of bacterial species have shown promising results. They have used either the BacTEC system or the BacT/Alert system for blood culture, but only in one study a comparison between the two has been made. The same protein extraction protocol for both blood culture systems was used followed by an additional ethanol/formic acid extraction procedure. The protein extraction protocol started with 4 mL of blood culture fluid and contained several centrifugation steps to remove leucocytes and collect the bacteria. The results of the MALDI-TOF analysis were compared with the results of routine species identifications. From non-charcoal-containing (uncommon type) BacT/Alert vials 62 % of the positive vials were correctly identified with the direct method while from BacTEC the corresponding values were 76 %. The results of the study support the routine application of MALDI-TOF MS (22).
Using MALDI-TOF MS direct from BacTEC vials has in other studies shown promising results with a high rate of identification. In two studies 95 % of monomicrobial blood cultures were identified with MALDI-TOF MS. In polymicrobial samples the identification rate was lower. One study showed that most of the polymicrobial samples had a species identification of one of the species (80.9 %) and that 36 % of the isolates were identified. In those studies the results from MALDI-TOF were compared with the routine species identification used in the specific laboratory at the time of the study (23-24).
Two previous studies have concluded that charcoal-containing BacT/Alert vials have a low rate of correct identification and that the method requires further technical development. However, in the proposed protein extraction protocols no extra step to remove the charcoal is suggested or included.(22, 25).

It seems as if gram-negative bacteria, independently of the type of vial, have a higher rate of identification than gram-positive bacteria. One study showed that 89% of the gram-negative bacteria were correctly of 89 % and only 61% of the gram-positive bacteria. Another study showed an identification frequency of 91 % (gram-negative) and 89 % (gram-positive) (21-22). These differences are partly due to the incapacity of MALDI-TOF to separate Streptococcus pneumoniae from Streptococcus oralis and Streptococcus mitis (23).

In the present study blood culture vials from bioMérieux (BacT/Alert) and Becton, Dickinson and Company (BD) (BacTEC) were inoculated with five reference strains and incubated at 35-37°C for approximately 18 hours. The Sepsityper™ kit from Bruker Daltonics was used for extraction of bacterial proteins according to the manufacturer’s instructions. The suspension was analysed for species ID using the Microflex™ with the software Biotyper 2.0 from Bruker Daltonics, Germany. At the same time samples from the blood culture vials were plated on chocolate agar. This step was performed to evaluate if a reliable species ID could be obtained after 1, 2, 3, 4 and 5 hours incubation when later analysed using MALDI-TOF MS.

The aims
The aim of the present study was to evaluate the time to species ID with identification from commonly used blood culture vials using a commercial kit for sample preparation in relation to a plating method followed by species ID using
MALDI-TOF. An additional aim was to evaluate the hands on time for both methods.

MATERIALS AND METHODS

Study design
This study was an experimental study with focus on performance of two methods for rapid species identification from blood culture vials and focused on the possibility of an accurate species ID and short hands on time for the laboratory technician.

Reference strains
The reference strains used in the present study were *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Streptococcus agalactiae* ATCC 12386. The reference strains were, before inoculation in blood-culture vials, grown on blood agar plates overnight in a 35-37°C aerobic environment.

Culture media
The blood culture vials used in this study were BacTEC Plus aerobic/F (BD), BacTEC Plus anaerobic/F (BD), BacT/Alert FA (bioMérieux) and BacT/Alert FN (bioMérieux).

The agar plates used in the study were blood agar plates and chocolate agar plates. The blood agar plate contained defibrinated horse blood and blood agar base (Merck). The chocolate agar plate contained GC agar base (Acumedia) with defibrinated blood and IsoVitox (Acumedia).
**Inoculation of blood culture vials**

The four types of blood culture vials were inoculated in triplicate with *S. aureus* ATCC29213, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212 and *S. agalactiae* ATCC 12386. *P. aeruginosa* ATCC 27853 was inoculated in triplicate in the two types of aerobic blood culture vials.

Colonies from each reference strain was suspended into 1.5 mL 0.85 % NaCl to McFarland 0.5 (range 0.4-0.6) (dilution A) by using Densichek (Vitek) from bioMérieux, Durham, North Carolina, USA. From this suspension 1 µL was mixed with 1.5 mL 0.85 % NaCl (dilution B). From dilution B 1 µL was inoculated on a blood agar plate for control of concentration and contamination. The remaining volume was inoculated in a blood culture vial. The blood culture vial were incubated at 35-37°C for approximately 18 hours.

**Viable count**

To evaluate the amount of bacteria in the BC vials after the incubation time (18 h in 35-37°C) a viable count was performed in triplicate. From the blood culture vials 5 mL suspension was drawn after 18 h incubation. From this suspension 100 µL was mixed with 900 µL 0.85 % NaCl. This step was followed by six 1 in 10 dilutions using 100 µL suspension to 900 µL 0.85 % NaCl. The final dilution was 1x10⁷. From each of the four last dilutions 20 µL was inoculated on blood agar plates and air-dried until the fluid had evaporated. The agar plates were then incubated in an 35-37°C aerobic environment overnight. Colonies from each suspension were counted and the concentration of bacteria in the blood culture vial was calculated.

**Sample preparation**

For sample preparation the Sepsityper™ kit (Bruker Daltonics, Germany) was used according to the manufacturer’s instructions, including the extra steps suggested for the BacT/Alert vials.
Sample preparation from BacTEC vials
After 18 hours incubation of the BacTEC blood culture vial 1 mL was transferred to a reaction tube. To the suspension 200 µL Lysis buffer (Bruker Daltonics) was added. Thereafter it was vortexed on Labdancer S40 (VWR) for approximately 10 seconds. The solution was centrifuged 1 minute at 13,000 rpm in a miniSpin microcentrifuge (Eppendorf, Germany). The supernatant was removed by pipetting and discarded. The remaining pellet was thoroughly suspended in 1 mL Washing Buffer (Bruker Daltonics) by pipetting up and down. The suspension was centrifuged at 13,000 rpm for 1 minute and the supernatant was removed by pipetting. The pellet was then treated according to the ethanol/formic acid extraction protocol.

Sample preparation from BacT/Alert vials
After 18 hours incubation of the BacT/Alert vial 1 mL was transferred to a reaction tube and 200 µL Lysis buffer (Bruker Daltonics) was added. The suspension was vortexed on Labdancer S40 for approximately 10 seconds. From the suspension 800 µL was transferred to a SigmaPrep™ Spin Column (Prod. No. SC1000, Sigma-Aldrich) and centrifuged in a miniSpin microcentrifuge for 1 minute at 2,000 rpm. The filter was discarded and the filtrate was centrifuged at 13,000 rpm for 2 minutes. The supernatant was removed by pipetting and discarded. The remaining pellet was resuspended in 1 mL Washing Buffer (Bruker Daltonics) by pipetting up and down and centrifuged 1 minute at 13,000 rpm. The supernatant was removed by pipetting and the pellet was treated according to the ethanol/formic acid extraction protocol.

Ethanol/formic acid extraction and preparation of target plate
The pellet, from the Sepsityper™ extraction, was suspended in 300 µL deionized water by pipetting up and down. Thereafter 900 µL 99.5 % ethanol was added. The mixture was centrifuged at 13,000 rpm for 2 minutes and the supernatant was decanted. The pellet was centrifuged for another 2 minutes at 13,000 rpm and the residual ethanol was carefully removed. The pellet was let to dry for roughly 2 minutes at room temperature. For *P. aeruginosa* 20 µL 70 % formic acid was added to the pellet and the sample mixed thoroughly by pipetting up and down. Thereafter 20 µL of 100 % acetonitrile was added to the tube and mixed carefully. For all other reference strains the volume of formic acid and acetonitrile added was 10 µL. The suspension was centrifuged at 13 000 rpm for 2 minutes. Of the supernatant 1 µL was loaded in duplicate onto a polished steel TF MTP 384 target plate (Bruker
Daltonics, Germany) and air-dried before overlaid with 0.9 µL of matrix solution containing HCCA (α-cyano-4 hydroxycinnamic acid, C_{10}H_{7}NO_{3}) (Bruker Daltonics) in organic solvent. The organic solvent contained acetonitrile (Sigma Aldrich GmbH), deionized water and trifluoroacetic acid (Sigma Aldrich GmbH). The matrix was then air-dried and the steel plate immediately covered to avoid light exposure. The target plate was then sent by standard transportation (bus) to the department of Clinical Microbiology, Karlskrona, Sweden, for analysis in the Microflex LT system (Bruker Daltonics). The total transport time was 20 hours.

**Preparation of target plates with culture from chocolate agar plates**

After 18 hours incubation of the blood culture vial 100 µL of the blood culture fluid was spread on chocolate agar plates containing GC agar and incubated in CO₂ (5 %) environment at 35-37°C for 1, 2, 3, 4 and 5 hours. After the specified time the plates were taken out and the growth was harvested by scraping on the surface of the plate with a toothpick. Thereafter the material from each plate was smeared onto two sample targets on a polished steel TF MTP 384 target plate and then air-dried. Then 0.9 µL of matrix was loaded onto each sample target and left to air-dry. The steel plate was then sent by standard transportation (bus) to the department of Clinical Microbiology, Karlskrona, Sweden, for analysis in the Microflex LT system (Bruker Daltonics). Total transportation time was 20 hours.

**MALDI-TOF MS**

Measurements were performed in a Microflex LT MALDI-TOF MS (Bruker Daltonics). Spectra were recorded in the linear positive mode at a laser frequency of 60.0 Hz within a mass range from 2,000 – 20,137 Da. For each sample 240 laser shots were required. Automated spectrum processing and species identification were performed using the MALDI-Biotyper 2.0 software. The software compared the acquired sample spectra to reference spectra in the provided database containing 3995 species (latest update: April 2011). The species identity together with the ID score was recorded.
**Quality control**

Before use of the Densicheck, when inoculating the blood culture vials, it was calibrated with Densichek™ Calibration Standard, BioMérieux (lot 835366601) as described by the manufacturer.

For control of bacteria in dilution B, 1 µL was inoculated on a blood agar plate and incubated overnight at 35-37°C in an aerobic environment. After the incubation the plate was examined for bacterial growth.

Every new matrix solution was controlled using the reference strain. This was achieved by smearing material, from a colony on the blood agar plate with the actual reference strain, onto two sample targets on the polished steel TF MTP 384 target plate that were air-dried. Thereafter 0.9 µL of the new matrix was loaded onto the sample targets and air-dried. The used steel plate was then sent by standard transportation (bus) to the department of Clinical Microbiology, Karlskrona, Sweden, for analysis in the Microflex LT system (Bruker Daltonics). The results of the MALDI-TOF analysis were recorded and compared with previous results obtained using the reference strain.

**Data/results processing**

The MALDI-TOF identifications were classified based on the score values as proposed by the manufacturer (table II) and transferred to an Excel spreadsheet together with the results from the viable count. The viable count results were recorded after calculation. The calculation was based on the number of colonies present on blood agar plates multiplied by the dilution, using the following formula:

\[
\text{No. of colonies} \times 50 \times \text{dilution} = \ast \text{CFU/ml}
\]

**Ethical aspect**

Reference strains were used in the study and therefore no ethical considerations are applicable.
RESULTS

Analysis of calibration and reference samples

The Densichek™ Calibration Standard was used to calibrate the densicheck instrument during inoculation of the blood culture vials. It was at all times within the given range. All “dilution B” suspensions, used when inoculating the blood culture vials, grew the expected number of colonies of the reference strain analysed.

Viable count of blood culture vials

Viable count was performed as a control of growth in the blood culture vials. The detailed results are shown in table III. All blood culture vials had a concentration in the range 10^7-10^9 colony forming units (CFU)/ml after 18 hours incubation. *E. coli* showed the highest mean concentration with 5 x 10^9 CFU/ml and *S. aureus* the lowest with 1 x 10^7 CFU/ml. Bacterial yield was similar from both BacT/Alert vials and BacTEC vials.

Table III. The total viable count (TVC) of *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212 and *S. agalactiae* ATCC 12386 in BacT/Alert vials and BacTEC vials, both aerobic and anaerobic (Colony forming units (CFU)/ml after 18h incubation at 37°C).

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>BacTEC Plus anaerobic/F (CFU/ml)</th>
<th>BacTEC Plus aerobic/F (CFU/ml)</th>
<th>BacT/Alert FN (CFU/ml) anaerobic</th>
<th>BacT/Alert FA (CFU/ml) aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> ATCC 27853</td>
<td>*</td>
<td>2 x 10^7</td>
<td>*</td>
<td>2 x 10^8</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>1 x 10^7</td>
<td>1 x 10^7</td>
<td>4 x 10^7</td>
<td>2 x 10^7</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>3 x 10^9</td>
<td>5 x 10^9</td>
<td>4 x 10^9</td>
<td>3 x 10^9</td>
</tr>
<tr>
<td><em>E. faecalis</em> ATCC 29212</td>
<td>9 x 10^8</td>
<td>2 x 10^9</td>
<td>4 x 10^6</td>
<td>1 x 10^9</td>
</tr>
<tr>
<td><em>S. agalactiae</em> ATCC 12386</td>
<td>4 x 10^8</td>
<td>3 x 10^8</td>
<td>2 x 10^9</td>
<td>1 x 10^9</td>
</tr>
</tbody>
</table>

* not analysed
**Species identification with MALDI-TOF**

Sample preparation, with the Sepsityper™ kit followed by MALDI-TOF analysis showed high ID-scores when applied to samples from the BacTEC vials, both aerobic and anaerobic, while none of the BacT/Alert cultures were identified using the Sepsityper™.

The time to result using the plating method was about the same for both blood culture systems. The details for each species are presented below. Species ID >2.0 from 2 of 3 cultures were judged as a species identification.

**Pseudomonas aeruginosa**

Sepsityper™ resulted in a species identification (ID score >2.0) from all three of the aerobic BacTEC vials. The BacT/Alert vials resulted in no species ID. The incubation time needed for a species ID using the plating method was three hours from BacT/Alert and five hours from BacTEC (table IV).

<table>
<thead>
<tr>
<th>Blood culture vial</th>
<th>ID score</th>
<th>Sepsityper™</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>2.000-3.000</td>
<td>3/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>BacT/Alert FA</td>
<td>2.000-3.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>

**Staphylococcus aureus**

Sepsityper™ resulted in a species identification (ID score >2.0) from all six of the BacTEC vials while only one out of six BacT/Alert vials resulted in a reliable species ID. Surprisingly *S. aureus* was hard to identify from growth on chocolate agar plates (Table V).

<table>
<thead>
<tr>
<th>Blood culture vial</th>
<th>ID score</th>
<th>Sepsityper™</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacTEC Plus anaerobic/F</td>
<td>2.000-3.000</td>
<td>3/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>2.000-3.000</td>
<td>3/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BacT/Alert FN</td>
<td>2.000-3.000</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>-</td>
</tr>
<tr>
<td>BacT/Alert FA</td>
<td>2.000-3.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>-</td>
</tr>
</tbody>
</table>
Escherichia coli
Sepsityper™ resulted in a species identification (ID score >2.0) from three of the anaerobic BacTEC vials and two of the aerobic vials. The BacT/Alert vials resulted in no species ID. The incubation time needed for a species ID using the plating method was two hours incubation from BacTEC vials. The same incubation time was needed for the aerobic BacT/Alert vials while the anaerobic needed three hours (Table VI).

Table VI. Number of BC vial (maximum three) that showed results with ID score >2.000 for ATCC 25922 E. coli using the Sepsityper™ and plating on chocolate agar in CO₂ both followed by species ID using Microflex™ and Biotyper 2.0 (Bruker Daltonics).

<table>
<thead>
<tr>
<th>ID score</th>
<th>Sepsityper™</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacTEC Plus anaerobic/F</td>
<td>2.000-3.000</td>
<td>3/3</td>
<td>-</td>
<td>2/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>2.000-3.000</td>
<td>2/3</td>
<td>-</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>BacT/Alert FN</td>
<td>2.000-3.000</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>BacT/Alert FA</td>
<td>2.000-3.000</td>
<td>-</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>

Enterococcus faecalis
Sepsityper™ resulted in a species identification (ID score >2.0) from three of the anaerobic BacTEC vials and from two of the aerobic vials. The BacT/Alert vials resulted in no species ID. The incubation time needed for a species ID using the plating method was three hours from both culture systems. Interestingly ID scores dropped after four and five hour’s incubation resulting in fewer ID (Table VII).

Table VII. Number of BC vials (maximum three) that showed results with ID score >2.000 for ATCC 29212 E. faecalis using the Sepsityper™ and plating on chocolate agar in CO₂ both followed by species ID using Microflex™ and Biotyper 2.0 (Bruker Daltonics).

<table>
<thead>
<tr>
<th>ID score</th>
<th>Sepsityper™</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacTEC Plus anaerobic/F</td>
<td>2.000-3.000</td>
<td>3/3</td>
<td>1/3</td>
<td>2/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>2.000-3.000</td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BacT/Alert FN</td>
<td>2.000-3.000</td>
<td>-</td>
<td>1/3</td>
<td>2/3</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td>BacT/Alert FA</td>
<td>2.000-3.000</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>2/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>
*Streptococcus agalactiae*

Sepsityper™ resulted in a species identification (ID score >2.0) from three of the anaerobic BacTEC vials and from two of the aerobic. Analysis of the aerobic BacT/Alert vials resulted in species identification for only one vial. The BacT/Alert anaerobic vials resulted in no species ID. The incubation time needed for a species ID using the plating method was three hours from the aerobic BacTEC vials. Altogether, *S. agalactiae* was hard to identify from chocolate agar plates (Table VIII).

**Table VIII.** Number of BC vials (maximum three) that showed results with ID score >2.000 for ATCC 12386 *S. agalactiae* using the Sepsityper™ and plating on chocolate agar in CO₂ both followed by species ID using Microflex™ and Biotyper 2.0 (Bruker Daltonics)

<table>
<thead>
<tr>
<th></th>
<th>ID score</th>
<th>Sepsityper™</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacTEC Plus anaerobic/F</td>
<td>2.000-3.000</td>
<td>3/3</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>1/3</td>
<td>-</td>
</tr>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>2.000-3.000</td>
<td>2/3</td>
<td>-</td>
<td>-</td>
<td>2/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>BacT/Alert FN</td>
<td>2.000-3.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>BacT/Alert FA</td>
<td>2.000-3.000</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Hands on time**

The sample preparation time before MALDI-TOF using the Sepsityper™ kit followed by the Ethanol/ Formic Acid Extraction, with twelve samples, were for both BacT/Alert and BacTEC approximately 60 minutes. The corresponding total time for plating of samples from twelve vials on chocolate agar plates were 10 minutes. The incubation time was different for each species as described above. The time it took to apply the samples on the target plate was the same for the two methods.
DISCUSSION

The time to correctly instituted therapy has a crucial impact on the outcome of patients with septic shock. A rapid species identification and Antibiotic Susceptibility Testing (AST) is therefore important. When a positive signal is reported from a blood culture system a sample is withdrawn, a Gram stain prepared and analysed, a preliminary report is given and a rapid disk diffusion method can be used for a preliminary AST after 6 hours. The disk diffusion method is in line or even faster than automated susceptibility testing (Vitek®2 and Phoenix™) (26). However the species ID after 6 hours is usually preliminary and not depending on the method. Species ID using MALDI-TOF has been shown to be rapid and safe (22-25). The present study evaluated species ID using MALDI-TOF directly from blood culture vials following extraction with a new commercial kit (Sepsityper™) in relation to subcultivation on chocolate agar.

The Sepsityper™ kit manufacturer primarily recommends the Sepsityper™ kit for blood culture vials without charcoal. The present study supports this recommendation as a high identification rate with the Sepsityper™ from BacTEC vials was obtained. All the species tested were identified with an ID score >2.0. Interestingly no species from BacT/Alert vials was identified using the Sepsityper™ despite using the manufacturer’s protocol suggested for this kind of blood culture vials. The problem seen is likely to be caused by the charcoal included in the BacT/Alert vials (25). In our hands, when following the manufacturer’s instructions for the BacT/Alert vials not all of the charcoal was caught in the SigmaPrep™ Spin Column. Some charcoal went through the filter to the pellet and contaminated the other steps in the analysis. In the protocol suggested by the manufacturer, 800 µL suspension of blood culture broth and lysis buffer is suggested to be filtered using the SigmaPrep™ Spin Columns. The product information from Sigma however states that 400 µL should be used for optimum performance, with a maximum capacity of 800 µL (27). This could be one reason for the low performance of the Sepsityper™ with samples from BacT/Alert vials as charcoal particles will affect the result of the MALDI-TOF (25). The charcoal removal from BacT/Alert samples before protein extraction and ID with MALDI-TOF thus seems to require further technical development. When contacting the manufacturer concerning the lack of results after the sample preparation from BacT/Alert, they proposed two ways to improve the results. The particle size of the charcoal can vary from bottle to bottle and if the liquid stands for 15 minutes the bigger particles form a sediment. Thereafter some of the supernatant is harvested and the protocol is continued. If this doesn’t work either a short subcultivation (3 hours) of some supernatant in combination with Sepsityper™ is one way to go. These steps have not been tested in the present study but at least the latter will give slower species ID because you get one extra step.
BioMérieux recently launched a new vial for blood cultures with a new broth and, most important, without charcoal. The future will tell whether the Sepsityper™ will perform better with the new system.

The plating method gave similar results from BacT/Alert and BacTEC vials, but was in general slower to species ID than using the Sepsityper™. For *E. coli* the species result seems to be available only marginally later with the plating method than with the Sepsityper™ and the difference in hands on time was 50 min for 12 samples. For *E. faecalis* the hands on time and incubation time using the plating method was 3 hours and 10 minutes. Importantly the hands on time for the technician using the Sepsityper™ was almost an hour for 12 samples while for the other method only 10 min was needed. This “spare time” can be used for preparing rapid susceptibility tests (24), or other analyses. The plating method is also cheap compared to the Sepsityper™ kit. Every laboratory has to evaluate which method works best in their local situation.

Some results from the plating method were unexpected. *Enterococcus faecalis* ID score dropped after four and five hour incubation resulting in fewer ID with the plating method. This might be due to the limited experience of the smear procedure of the investigator as the inoculum is important for optimal ID-score. When smearing the samples on the target plate, more material stuck on the first target than the second. After the MALDI analysis a better ID score was observed from the second target. This underlines the importance of preparation of samples in duplicates.

Surprisingly no species ID was obtained from the plating method for *S. aureus* and *S. agalactiae* despite 5 hours incubation. Harvesting colonies from chocolate agar for MALDI-TOF is a bit tricky as it is easy to “contaminate” the bacteria with agar. Because of this *S. aureus* was reanalysed from one of each vials tested. The protocol was followed but in addition 100 µl of the blood culture broth was inoculated on blood agar plates that were incubated in CO₂. The first harvest was performed after 2 hours. This experiment showed that already after three hours of incubation a species ID was obtained for all types of vials when inoculated on blood agar plates. Chocolate agar plates resulted in species ID after 5 hours from the BacTEC vials but not from the BacT/Alert vials. The details of these results are presented in Appendix I. Blood agar might thus be a better medium than chocolate agar at least for *S. aureus*. *Staphylococcus aureus* was the species that resulted in the lowest viable count from the blood culture vials (10⁷ CFU/mL). This might have contributed to the longer time to ID as too few bacteria on the target spot for the MALDI-TOF analysis will result in less reliable mass spectra and thus lower chance of a species ID. Further
studies have to be performed to determine the optimal inoculum and medium for the plating method.

Overall the performance of the tested techniques was best when performed from BacTEC vials. The difference was obvious for the Sepsityper™. However with exception of *P. aeruginosa* and *E. faecalis*, the BacTEC vials also resulted in a faster species identification using the plating method. Although a tendency towards more rapid species ID from the BacTEC vials was observed in this study, larger studies using clinical samples has to be performed before any firm conclusions regarding this can be drawn.

**Conclusion**

The present study shows that the sample preparation from blood culture vials with the Sepsityper™ kit in combination with BacTEC vials gives rapid species ID with a total preparation time of 60 min for 12 samples. Species ID from growth on chocolate agar led to a longer time to species ID but the hands on time was only 10 minutes for 12 samples. The plating method is thus slower but has some advantages as it is not dependent on the type of BC vial, is cheap and gives room in the working schedule for other analyses to be performed.

**Acknowledgments**

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APPENDIX I. ID score for ATCC 29213 S. aureus from BacT/Alert vials and BacTEC vials, both aerobic and anaerobic, using inoculation on both chocolate agar and blood agar in CO₂ followed by species ID using Microflex™ and Biotyper 2.0 (Bruker Daltonics).

<table>
<thead>
<tr>
<th>Growth medium and type of vial</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chocolate agar</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BacT/Alert FN aerobic</td>
<td>-/-</td>
<td>-/-</td>
<td>1,329/2,007</td>
<td>1,348/1,315</td>
</tr>
<tr>
<td>BacT/Alert FA aerobic</td>
<td>1,906/1,697</td>
<td>1,195/1,485</td>
<td>1,473/1,747</td>
<td>~1,661</td>
</tr>
<tr>
<td>BacTEC Plus anaerobic/F</td>
<td>-/-</td>
<td>1,302/1,485</td>
<td>1,429/2,190</td>
<td>2,109/2,240</td>
</tr>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>1,393/1,809</td>
<td>1,395/1,241</td>
<td>~1,829</td>
<td>2,264/2,311</td>
</tr>
<tr>
<td><strong>Blood agar</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BacT/Alert FN anaerobic</td>
<td>-/-</td>
<td>1,218/2,173</td>
<td>2,101/1,503</td>
<td>1,420/2,149</td>
</tr>
<tr>
<td>BacT/Alert FA aerobic</td>
<td>1,411/1,758</td>
<td>2,182/2,167</td>
<td>2,108/1,894</td>
<td>2,085/2,265</td>
</tr>
<tr>
<td>BacTEC Plus anaerobic/F</td>
<td>-/-</td>
<td>2,124/-</td>
<td>2,163/1,807</td>
<td>1,944/2,050</td>
</tr>
<tr>
<td>BacTEC Plus aerobic/F</td>
<td>-/-</td>
<td>2,155/2,104</td>
<td>2,107/2,066</td>
<td>1,958/2,094</td>
</tr>
</tbody>
</table>

- no peak found