Rapid diagnosis of bloodstream infections

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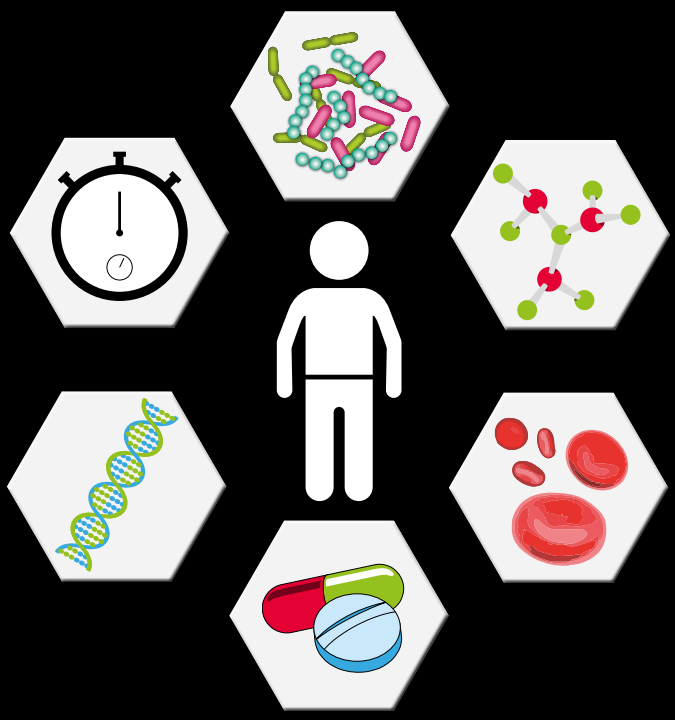
From theory to clinical practice



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Rapid diagnosis of bloodstream infections

From theory to clinical practice

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Contents

Chapter 1

Introduction

1. Preface

Despite major advances in diagnostics and therapy, bloodstream infections still account for more deaths than any other infection[1]. Mortality rates are high, varying from 15 to 35%[1-5].
The reported incidence of bloodstream infection ranges from 80-189/100.000 persons/years in Europe to 240/100.000 persons/year in the USA and the incidence is increasing[6-9]. This is probably secondary to an aging population, more immunocompromised patients (caused by chemotherapy, transplantation, HIV and immunosuppressive medication) as well as an increase of invasive procedures (surgery, intravenous catheters etcetera), and an improved survival rate of patients with severe conditions, such as auto-immune disorders and malignancies[6,7]. Contributing to the increasing numbers are better methods of detection and improved infection registration systems[6,7], although naturally this does not result in an actual increase in the number of patients suffering from bloodstream infections.
Case fatality rates declined over the years, as a result of improved detection and treatment[6,7,10]. However, due to the increasing incidence, the absolute number of deaths that can be attributed to bloodstream infections has risen[6,7,10].
Bloodstream infections can be caused by a multitude of bacteria; most often by *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* species, coagulase-negative *Staphylococcus* species, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*[2,7,8,10,11]. Yeasts and (very rarely) fungi can also cause bloodstream infections. These infections however are beyond the scope of this thesis.

2. Bloodstream infections

2.1 Definitions

Many terms are used to describe the presence of bacteria in the bloodstream, or bacteraemia, and the clinical syndromes this can result in[12].
In general, an infection is defined as the pathologic process of the invasion of a normally sterile tissue or fluid or cavity by pathogenic or potentially pathogenic microorganisms[13]. In case of a bloodstream infection, this concerns an invasion of the bloodstream. This can be secondary to an infection at another body site, or a primary bloodstream infection, when it originates from an intravascular device or an unknown focus[14].
The inflammatory response to a bloodstream infection can result in a systemic inflammatory response syndrome, or SIRS (Table 1). SIRS can have numerous causes, both infectious and non-infectious. Sepsis is SIRS being caused by a bloodstream infection, either suspected or proven (by culture). Severe sepsis is defined by sepsis-induced organ dysfunction or tissue hypoperfusion. If the hypotension persists despite adequate fluid resuscitation, the term 'septic shock' is used[15].

2.2 Criteria for the diagnosis of bloodstream infections

According to CDC-criteria, a bloodstream infection is diagnosed when a recognized pathogen is cultured from one or more blood cultures[16]. However, this criterion does not always suffice, since sensitivity varies and may be as low as 30-40% in some foci of bacteraemia, such as endocarditis[17-19]. Unfortunately, a gold standard for the diagnosis of bloodstream infections is not available. Therefore, signs and symptoms of sepsis, as defined by the SIRS-criteria, are often used, but these are non-specific and can also be caused by non-infectious conditions, such as pancreatitis, trauma and burns[13]. Thus, using sepsis as gold standard may result in an overestimation of the number of bloodstream infections. On the other hand, using SIRS criteria to diagnose bloodstream infections may result in an underestimation of the sensitivity of blood cultures.

Blood cultures also are not 100% specific. The cultured microorganism may be a contamination of the cultured blood due to non-optimal aseptic procedures during venipuncture. It is important to recognise contaminated blood cultures, to prevent unnecessary antibiotic treatment.

The most common blood culture contaminants are coagulase-negative *Staphylococcus* species (CoNS), and, less frequently, *Corynebacterium* species or *Bacillus* species. However, these species do cause bloodstream infections, often associated with prosthetic material[20-23]. Separate CDC-criteria have been developed to diagnose bloodstream infections caused by these species. These criteria are 1) a minimum of 2 positive blood cultures, drawn at separate occasions, and 2) the presence of fever, chills or hypotension[16]. In daily practice, these criteria lack sensitivity since two blood cultures are not always drawn[24,25] and patients do not always present with fever or hypotension[24,26]. Sensitivity of these criteria was found to be as low as 67%, and specificity only 56%[24]. Additional criteria, such as time to blood culture positivity or the ability of a bacterium to produce slime have been proposed[27], as well as alternative algorithms based on clinical findings and number of positive blood cultures[24,26]. Although such criteria may aid in the diagnosis of bloodstream infections, results are still suboptimal. It is not clear whether further identification of CoNS to species level can result in a higher sensitivity and specificity of the criteria.

Table 1. SIRS criteria

Fever of more than 38°C (100.4°F) or less than 36°C (96.8°F)
Heart rate of more than 90 beats per minute
Respiratory rate of more than 20 breaths per minute or arterial carbon dioxide tension (PaCO2) of less than 32 mmHg
Abnormal white blood cell count (>12,000/µl or < 4,000/µl or >10% immature (band) forms)

2.3 Treatment of bloodstream infections

Optimally, adequate antibiotic therapy has to be started in patients with symptoms of bloodstream infections before the microbiological diagnosis has been established (which may take up to 72 hours). In 2002 the Surviving Sepsis Campaign was launched (http://www.survivingsepsis.org). This guideline describes two bundles of elements of care (Table 2). The goal of these elements is early and adequate resuscitation, diagnosis, and antibiotic treatment of the patient. In this bundle, broad-spectrum empirical antibiotic therapy is started as soon as possible. It has been shown that adherence to these bundles reduces mortality and length of hospital stay[28].

The choice of antibiotics depends upon local epidemiology of antibiotic susceptibility and should cover the majority of likely causative microorganisms. However, the use of (combinations of) antibiotics, especially broad-spectrum antibiotics, also has disadvantages[29-32]:

- Selection and potential spread of resistant microorganisms
- An increase of invasive fungus and mould infections
- An increase in usage of expensive antibiotics like tigecycline, daptomycine, etc., potentially leading to more resistance to these, often last resort, antibiotics
- An increased risk of drug toxicity
- More *Clostridium difficile* infections
- An increase in costs

Therefore, a switch to more narrow spectrum antibiotics is necessary as soon as the microbiological information on the causative microorganism and its antibiotic susceptibility spectrum is available. Currently, this requires approximately 48-72 hours. More rapid microbiological methods that reduce the time to identification of the microorganism and the antibiotic susceptibility will decrease the time of use of broad-spectrum antibiotics thereby increasing the occurrence of the above mentioned disadvantages.

Table 2. Surviving sepsis campaign bundles:

To be completed within 3 hours:
Measure lactate level
Obtain blood cultures prior to administration of antibiotics
Administer broad-spectrum antibiotics
Administer 30mL/kg crystalloid for hypotension or lactate >4mmol/l
To be completed within 6 hours:
Apply vasopressors (for hypotension that does nog respond to initial fluid resuscitation) to maintain a mean arterial pressure (MAP) > 65mmHg
In the event of persistent arterial hypotension despite volume resuscitation (septic shock) or initial lactate >4 mmol/l (36 mg/dl):
Measure central venous pressure (CVP)
Measure central venous oxygen saturation
Remeasure lactate if initial lactate was elevated.

2.3.1 Choice of antibiotic therapy and outcome

Antibiotic therapy initiated as soon as possible is only beneficial when the antibiotic used is adequate i.c. the causative microorganism is susceptible for the chosen antibiotic. However, there are no clinical randomised trials that have investigated this issue, since it would be unethical to delay antibiotic therapy in a severely ill patient. Therefore, the only data available come from observational studies.

Kumar *et al*. showed in an often-quoted study that in a cohort of patients with septic shock, every hour delay in initiation of adequate therapy resulted in a 7% increase in mortality[33]. However, data from other studies are less convincing. In a review by McGregor *et al*. in 2007[34], 51 studies on the clinical impact of inadequate antibiotic therapy were evaluated. Only 30 of these studies (59%) showed a significant increase in mortality when inadequate therapy was administered. Definitions of adequate therapy and outcome varied between studies, and time to diagnosis and appropriate therapy were rarely provided, making comparison of the results of the various studies difficult.

Severity of illness is an important prognostic factor, which may influence results. It was measured in only 34 studies (67%), and of these studies, only 23 (68%) adjusted for disease severity in the final analysis. The sample size of most studies was small, only 17 studies showed a power of >80%. Thirteen of these studies showed an effect of inadequate therapy (76%). This suggests that inadequate therapy indeed influences outcome, but that it is difficult to draw a definite conclusion. A similar result was found by Paul *et al.*[35], who assessed 70 studies, of which 26 were pooled into a meta-analysis. Inadequate antibiotic therapy had an OR of 1.60 (95% CI 1.37-186) for mortality, but there was a large degree of heterogeneity between studies.

Antimicrobial resistance of the bacteria causing the infection can be a risk factor for starting inadequate treatment is. Indeed several studies have shown that antimicrobial resistant microorganisms were associated with an increased risk of inadequate treatment and mortality[36,37]. In contrast, in a study by Blot *et al.*[38] no increased risk of inadequate treatment was found in methicillin resistant *S. aureus* (MRSA) bloodstream infections when compared with bloodstream infections with methicillin-susceptible *S. aureus* (MSSA). Nevertheless, methicillin resistance was found to be associated with higher mortality, suggesting that other factors associated with antibiotic resistance also may affect risk of mortality. Resistant strains require different antibiotics that may be less effective in treating bacteraemia. Furthermore, length of hospital stay and previous treatment with antibiotics increase the risk of an infection with a resistant strain[36,37] and may be potential confounding factors. Not all cited studies have corrected for these confounding factors when assessing the correlation between antibiotic resistance and outcome.

In other studies very broad-spectrum antibiotics were used as empiric therapy, thereby reducing the risk of inadequate treatment[39,40]. This approach has the disadvantage of increasing the risk of future antibiotic resistance, making the choice of future adequate empiric therapy much more difficult.

2.3.2 Dosing antibiotic therapy and outcome

Not only the choice of antibiotics is important for adequate therapy, also the dosing and method of administration is important[41]. This depends upon factors involving bacterial characteristics such as MIC and host factors of the patient, for instance:

- The bacterium causing the infection: in case of reduced susceptibility, a higher dose is required
- The primary infection site: the efficacy of an antibiotic depends upon its ability to penetrate the infected tissue or organ
- Underlying conditions of the patient that could influence the distribution of the drug throughout the body or the metabolism of the drug by the body.

When starting treatment for a bloodstream infection, these factors should be taken into account.

2.3.3 Targeting antibiotic therapy

Taken together, it is recommended that empiric antibiotic therapy must be administered as soon as possible and should cover the bacteria causing the infection; this usually is a broad-spectrum antibiotic. This recommendation seems to decrease the risk of an unfavourable outcome of bloodstream infections. The downside of this approach is that the use of broad-spectrum antibiotics may result in selection of antibiotic resistant bacteria and adverse effects. It is thus important to start as soon as possible with targeted antibiotic therapy. To reach this goal, microbiological methods for identification of the microorganism and the susceptibility for antibiotics are the cornerstone. The field of microbiological diagnostics for bloodstream infections is rapidly evolving.

3. Microbiological diagnosis of bloodstream infections

3.1 Blood cultures

For many years, blood cultures have been, and still are, the standard method for the detection of bacteria and fungi in blood. 5-10 ml of blood is injected in a vial containing culture medium, which is then incubated in an automated blood culture device for several days. The vials are regularly automatically checked for bacterial growth, using measurement of CO_2 production or O_2-consumption by bacteria. On average, 15 hours (range 2.6-127 hours) of incubation is required before growth is detected[31].

In the event of bacteraemia, blood may contain >100 colony forming units (cfu) per ml, especially in children and neonates[42], but in the majority of patients it contains approximately 1-30 cfu/ml[43,44], and in as many as 27-62% of patients bacterial load is even lower[45].

Exact data on the sensitivity and specificity of blood cultures are not available, since no gold standard exists for diagnosing bloodstream infections. Theoretically, they have a detection

limit of 1 cfu. But in clinical practice, sensitivity and specificity are suboptimal, since their performance is influenced by many potentially complicating factors:

- The volume of blood in the vial: the sensitivity increases when higher volumes of blood are incubated. In a study by Mermel *et al*. sensitivity increased with 3% for each extra mL of blood incubated[46]. However, in up to 50% of all blood cultures, the volume of blood drawn is too low[47].
- The number of blood cultures drawn: the more blood cultures are drawn, the higher the incubated volume of blood. Also, in bloodstream infections, bacterial load in blood varies during the day[31]. Therefore, drawing multiple blood cultures at multiple time points increases sensitivity. Also, drawing multiple blood cultures allows for the distinction between a true bloodstream infection and blood culture contamination: if a potential pathogen is isolated on more than one occasion, a true infection is more likely.
- The use of antibiotics at the time of blood culture drawing: this results in antibiotics in the blood culture vial, which can inhibit the growth of bacteria, resulting in a false-negative blood culture.
- The type of microorganism in the blood: some bacteria and fungi grow very slowly (HACEK group); others are incapable of growth in blood culture medium (*Coxiella burnettii*).
- Incubation time: increase in incubation time can result in a higher detection rate in blood cultures containing slow growing micro-organisms or very low numbers of micro-organisms.
- Hygienic measures before, during and after blood culture drawing: if these are insufficient, a blood culture can be contaminated with bacteria from the environment, for example the skin, resulting in a false-positive blood culture.

Thus, a positive blood culture is not the end of the diagnostic pathway but the starting point for identification and susceptibility testing.

3.2 Identification

After growth of bacteria in a blood culture bottle, the broth is subcultured on agar. Also, Gram-staining is performed as a first step in species identification (ID). In Dutch hospitals, the result of Gram-staining is usually reported to the attending physician, together with an advice on antibiotic treatment.

When sufficient growth is obtained on agar, the strain can be further identified. For decades, this was done by performing series of biochemical tests on the strain. Some are performed by hand and results are readily available, such as testing for coagulase-production in *Staphylococcus* strains. But for most biochemical reactions, automated systems are available, such as the Phoenix system (BD), Vitek 2 (Biomerieux) and MicroScan WalkAway (Siemens).

Within the last 5 years, in many labs, biochemical tests have been replaced by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry, or MALDI-TOF MS. The strain is

spotted on a slide, matrix solution is added and the slide is placed in the machine. The sample is degraded into peptides, which are vaporised and ionised. The ions are accelerated towards a detector, which measures time-of-flight, which depends upon the mass of the peptide. The various time-of-flights are translated into a mass spectrogram, which is compared to spectrograms in a reference database to identify the strain. The procedure can result in an ID within minutes per strain. For most bacterial species, MALDI-TOF MS performs as well as, or sometimes better, than biochemical ID[48]. The analyser itself is very expensive, approximately $150000[49]. However, this is compensated by the low costs of labour and reagents, which are about $0.50 per sample[50].

3.3 Antibiotic susceptibility testing

Most current methods for antibiotic susceptibility testing follow the same principle: a fixed amount of bacteria (or fungi) are incubated with fixed amounts of an antibiotic, either on solid culture media (disk diffusion, E-test) or in liquid culture media (microbroth dilution). After the incubation period, multiplication of the bacteria, or the lack thereof due to the antibiotic, is measured. The lowest concentration of an antibiotic inhibiting growth of the strain is called the minimal inhibitory concentration, or MIC.

Many automated systems exist for antibiotic susceptibility testing, some of which can also be used for ID of the strain. Most use the principle of microbroth dilution. The machine measures bacterial growth either after a fixed amount of time (Sensititre AWIS 2X (Trek Diagnostic Systems), or continuously, until sufficient growth has occurred (Phoenix (BD), Vitek 2 (Biomerieux), MicroScan WalkAway (Siemens))

To determine whether an antibiotic is effective in the treatment of a strain, breakpoints have been established by various groups, such as CLSI and EUCAST. These breakpoints are based on:

Microbiological data, for example intrinsic resistance mechanisms

- Data on the distribution of the drug throughout the body and the metabolism of the drug (pharmacokinetic properties of the drug).
- Data on the effect of the drug on the body, such as side effects or toxicity (the pharmacodynamic properties of a drug)
- Results of clinical studies on microbiological and/or clinical cure rates of an antibiotic in patients, if available.

If the strain has an MIC that is above the breakpoint, the strain is considered to be resistant to that specific antibiotic. If the MIC of the strain is below that breakpoint, the strain is considered susceptible. In some antibiotic/species combinations, two breakpoints exist. If the MIC of the strain is between the two breakpoints, its susceptibility to the tested antibiotic is reduced, making it unsuitable for the treatment of most infections, although it may still be useful in some situations. This is called intermediate susceptibility.

Sometimes, additional phenotypic tests are required to test for specific resistance mechanisms, such as tests on extended-spectrum beta-lactamase production in Gram-negative rods or screening for methicillin resistance in *Staphylococcus aureus*.
It is clear that culturing of bacteria causing bloodstream infections and subsequent identification and susceptibility testing is laborious and time consuming. New easier to perform and more rapid techniques are urgently needed.

3.4 Laboratory developments to reduce time to results

All of the currently used methods require a positive blood culture (incubation time: 1-5 days) and usually a subsequent overnight subculture of the grown blood culture on agar (incubation time: 18-24 hours). Time to results for the aforementioned techniques varies from 4-24 hours[51], depending upon the method used and the growth rate of the tested strain. All together, time to results, starting from blood culture drawing, is at least 2-3 days. Since inadequate therapy is associated with poorer clinical outcome, and too broad-spectrum antibiotics also have disadvantages, attempts are made to reduce time to results, in order to be able to adjust antibiotic therapy sooner.

3.4.1 Improvement of currently used techniques

Current techniques for ID and AST require a suspension with a fixed amount of bacteria per ml. To obtain this suspension, bacteria from an agar plate are added to saline or culture medium until a standardized turbidity is obtained.
According to manufacturers' guidelines, an overnight culture of bacteria on agar plates is required to prepare this suspension, which takes 18-24 hours. However, bacterial growth on agar is usually visible much earlier, and a sufficient amount of bacteria can usually be harvested from the plate after only 4 hours of incubation.
To further reduce time to results, ideally, the subculture step should be skipped completely. It is however not possible to directly inoculate the currently used devices with the blood culture medium, since these tests require the input of a fixed inoculum of bacteria, whereas the concentration of bacteria varies widely between blood cultures, and measurement of the bacterial concentration is disturbed by the presence of erythrocytes and leukocytes. It is possible, however, to separate the bacteria from other components in the blood culture, by using serum separator tubes, which are designed to remove cells from blood samples. After centrifuging blood culture medium in this tube, human cells have moved to the bottom of the tube, underneath a gel layer. Bacteria remain on top of the gel layer. The liquid components of the blood culture can then be discarded and the bacteria can be harvested from the gel layer to inoculate the saline or culture medium. This requires approximately 15 minutes. For the Vitek 2 device, many studies have shown good results of this method of obtaining bacteria from blood cultures[52-56], however it is less extensively studied for other devices, such as the Phoenix device[57].

For MALDI-TOF MS, it is also possible to isolate bacteria directly from positive blood cultures, which is slightly more labour and time consuming than the method described above for the Vitek 2 system, requiring several reagents and a centrifuging or filtration step, and approximately 30 minutes to perform. If an ID result is obtained with these extraction methods, it is highly reliable. However in 20-36% of all positive blood cultures, no result is obtained and a subculture on agar is still required[58]. Alternatively, it is possible to incubate the grown blood culture on agar for 4 hours and harvest bacteria from this agar to inoculate the MALDI-TOF MS device, instead of an overnight incubation on agar, thereby still shortening turnaround time with 14-20 hours.

3.4.2 Nucleic acid based techniques for pathogen ID

Introduction

The aforementioned techniques all use phenotypic characteristics of microorganisms for ID. Instead, genotypic ID can be performed using nucleic acid based techniques.

In situ hybridisation

This technique uses fluorescent probes that can adhere directly to specific bacterial DNA-targets. Different probes can be used to discern different bacterial species, allowing for ID of bacteria directly in positive blood cultures. Commercial kits, such as AdvanceDx PNA-FISH (Woburn, MA, USA) target a limited number of bacterial or *Candida* species, within 3 hours, with a hands-on time of 15 minutes. Their sensitivity and specificity are very high, almost 100%[59-63]. But the number of targets is limited and the cost-effectiveness for some targets is hampered by the availability of certain rapid phenotypic tests such as the tube-coagulase test for *S. aureus*[64] or MALDI-TOF MS[50].

Polymerase chain reaction (PCR)

PCR is a technique that is increasingly frequently used in clinical microbiology to detect various pathogens. It uses small DNA-fragments, primers, which adhere to the target DNA. Starting from this primer, using an enzyme called DNA-polymerase, nucleotides are added, resulting in a new DNA-strand complementary to the original target DNA-segment. In new cycles of primer adherence and amplification both the original DNA-segment and the new DNA-strands are copied, resulting in an exponential increase of new DNA-strands. With this technique, large numbers of copies of a selected DNA-segment can thus be obtained.

Several different techniques are available to subsequently measure the presence of these DNA copies.

In conventional PCR, the PCR product is added to an agarose gel under an electric current. DNA is negatively charged, so the DNA-segments move towards the positively charged side of the gel. The speed by which this happens is dependant upon the length of the fragment, so that the length of the fragment can also be determined. Disadvantages of this method are

that it requires an additional step after DNA-amplification to detect the PCR-product, and quantification of the original amount of DNA present in the sample cannot be determined.
An alternative for conventional PCR is real-time PCR, which is now frequently being used. With this technique, DNA-segments are being detected during the amplification process, either by the use of a fluorescent dye that attaches to double stranded DNA, such as SYBR-green, or by the use of fluorescent probes. These probes are short DNA segments that are complementary to the DNA-sequence that is being amplified. If the DNA-sequence is present and the probe adheres, it starts to emit fluorescence, allowing for the amplicons to be detected.
It is possible to perform real-time PCR on several different targets simultaneously in one reaction tube, which is called multiplex PCR. With this technique results can be available within several hours.
However, the number of targets that can be performed simultaneously is currently limited. A broad range of pathogens can cause bloodstream infections. This means that a large number of targets should be included in the PCR. When using real-time PCR for the detection and ID in bloodstream infections, this requires a complex combination of assays.
This problem could be solved by using a technique called micro-array. This is a microchip coated with a large number of targets to which the PCR products can adhere, thereby allowing for testing more targets in one run than with real-time PCR. However, currently the technique is still labour-intensive and expensive. A commercial assay is already available for use on positive blood cultures: Prove-It Sepsis (Mobidiag, Helsinki, Finland), which can detect 50 different pathogens within 3 hours[65].
Another technique that could be useful in the ID of bloodstream infection is PCR followed by DNA-sequencing. First, a DNA-segment present in all bacteria, such as the 16S rRNA gene, is being amplified. Afterwards, in a separate reaction, the sequence of the DNA-amplicons is being determined. This sequence is then compared to sequences in a database to identify the pathogen. An advantage of this technique is that the number of different species that can be identified is not limited by the number of targets present in the test when using a universal PCR. Currently, the technique is relatively time-consuming and expensive due to the sequencing step. However, more rapid and cheaper systems are becoming available.
A new technique is PCR-Electrospray Ionization Mass Spectrometry (PCR-ESI), which measures the mass/charge ratio of PCR-amplicons, which is then compared to a reference database, similarly to MALDI-TOF MS. When using universal PCR, allows for ID of all bacterial species in one assay. Kaleta *et al.* have shown that with this technique, results of ID can be available within 5-6 hours[66,67]. The system is currently being marketed under the name Iridica (Abbott Molecular, Des Plaines, IL, USA) and also includes several antibiotic resistance genes.
In conclusion, PCR-based techniques are very promising in the ID of blood culture pathogens, provided that they are able to identify all most common pathogens, can be performed in any lab, and at a reasonable price.

3.4.3 Antibiotic susceptibility testing (AST)

Introduction

For a new test to be introduced in clinical practise, its results need to be compared with those of a reference method. A system to compare the results of both tests has been described by Jorgenson[51]. If the new method for antibiotic susceptibility testing shows a susceptible result where the reference method shows a strain to be resistant, this is called a very major error. In the case of a resistant result in the new test and a susceptible result in the reference method it is called a major error. Any error involving an intermediate susceptible result is a minor error. Furthermore, a test can be tested for essential agreement: whether the MIC of a strain tested by the new method is comparable to the MIC when tested with the reference method[51].

The FDA has established criteria for the accuracy of a new test for antibiotic susceptibility[68]:

- <1.5% very major errors for individual species/drug-combinations
- <3% major errors
- Essential agreement: > 90%

Nucleic acid amplification based testing

Antibiotic resistance in bacteria can be caused by mutations within genes, or by the introduction of new genes into the genome. Real-time PCR can be used to detect such mutations and genes. In some cases, only a small number of genes are responsible for resistance against an antibiotic. For example, methicillin resistance in *Staphylococcus aureus* is caused by acquisition of the *mecA* or *mecC* gene, and *vanA* or *vanB* genes are responsible for vancomycin resistance in *Enterococcus* species. In these cases, real-time PCR is a relatively simple and reliable method to determine antibiotic resistance. Both in-house developed methods[69] and commercial automated methods, such as GeneXpert (Cepheid, Sunnyvale, CA, USA), the BD MAX system (BD, San Diego, CA, USA), and the previously mentioned Iridica system are already available for rapid detection of these genes in clinical practise. The GeneXpert system can also be used directly on positive blood cultures, thereby significantly reducing time to results, since it requires only several hours starting from a positive blood culture.

However, in other cases, a resistance mechanism can be coded for by a large number of different mutations, in multiple existing genes, sometimes in only one nucleotide. This is the case in extensive spectrum beta-lactamase production in Enterobacteriaceae, in which hundreds of different mutations are implicated. Performing real-time PCR does not suffice in these situations, since a very large number of primers and probes would be required. Instead, a micro-array could be used. Commercial panels are already available, for example the Verigene Gram-positive blood culture (BC-GP) and Gram-negative blood culture (BC-GN) assays (Nanosphere, Inc., Northbrook, IL, USA).

Whole genome sequencing can also be used to detect resistance genes present in the genome of the causative microorganism. However, until recently, the sequencing techniques

available were expensive and time-consuming. Furthermore, it requires extensive data-analysis. Recently, next-generation sequencing techniques have been introduced, as well as better techniques for data-analysis, allowing for faster and cheaper whole genome sequencing and sequence analysis. Zankari *et al.* showed a very high concordance (99.8%) of results of AST using whole genome sequencing with results of phenotypic AST[70].
However, molecular testing for antibiotic resistance has several disadvantages. It is only possible to test for genes or mutations present in the test panel, so those mutations not included in the panel will be missed. The genetic bases of many resistance mechanisms have not yet been fully unravelled, and new mutations and resistance genes will emerge, which would be missed in these tests. Also, a certain genotype does not always result a resistant or susceptible phenotype. As a result, these techniques cannot replace culture-based AST in the near future. But they could be a valuable, potentially fast but expensive addition to culture-based techniques.

MALDI-TOF MS

Several methods have been developed to use MALDI-TOF MS for AST. It can be used to directly detect those proteins that cause antibiotic resistance, such as beta-lactamases[71]. Also, bacteria can be incubated for a short period of time with an antibiotic, after which MALDI-TOF MS can be used to detect metabolites of the antibiotic. This is only feasible if the antibiotic has been degraded by the tested strain[72]. Furthermore, MALDI-TOF MS can be used to discern a specific resistant clone of a bacterium from susceptible clones, which has been shown for vancomycin-resistant enterococci, by comparing the spectrograms of resistant and susceptible clones[73]. These techniques are potentially fast and inexpensive, however, they are still in an early developmental stage. Also, each of these methods can only be used to test for specific resistance mechanisms. Their usefulness for clinical practise thus will have to be proven in the future.

Other techniques

Several new techniques have been described for earlier detection of bacterial growth in the presence of an antibiotic.
Fredborg *et al.*[74] used the oCelloScope, a microscopic camera, to detect bacterial growth in real-time, enabling results of AST within 6,5 hours. Similarly, an automated system called Accelerate ID/AST (Accelerate Diagnostics Inc, Tuscon, AZ, USA) measures the effects of various antibiotics on single cells in real-time by using a computer operated microscope, yielding results of AST within 5 hours. The system is currently available for research purposes only.
Broeren *et al.*[75] used flow cytometry to monitor cell counts in bacterial suspensions with and without antibiotics. Using this technique, antibiotic susceptibility could be determined in 90 minutes for *E. coli* and in 120 minutes for *P. aeruginosa* and *S. aureus*, starting from an overnight culture on agar.

Several studies have tested methods for AST performed in small droplets containing as little as one bacterium[76,77]. These techniques could significantly reduce incubation time, since the technique does not require the large number of replication cycles most other techniques require to establish bacterial growth.
Although promising, these techniques have not yet been tested on a larger scale on clinical materials, including blood cultures.

3.4.4 Diagnosis directly on blood

Introduction

All techniques described above require a positive blood culture, which requires time, 15 hours on average, but sometimes up to 127 hours[31]. Performing ID and AST directly on blood would significantly reduce time to diagnosis. Also, as described earlier, for many reasons bacteria will not always grow in the blood culture medium, reducing sensitivity of blood cultures. Therefore, ideally, a technique should be used that does not rely upon the viability of bacteria in the blood, for example PCR.

Techniques

Several commercial kits are available for ID of bloodstream infections directly on blood. The most tested in clinical practise is Septifast (Roche Molecular Systems, Branchburg, NJ), which is a multiplex real-time PCR that can detect 25 different pathogens, representing approximately 90% of all species causing bloodstream infections. Turnaround time is approximately 6 hours[78]. Sepsitest (Molzym, Bremen, Germany) uses a PCR followed by sequencing and is capable of detecting over 300 pathogens. The sequencing step results in a longer turnaround time of 8-12 hours[79]. VYOO (SIRS-Lab, Jena, Germany) is a multiplex PCR assay that is followed by gel electrophoresis. In addition to being able to detect >40 different pathogens, it also detects several antibiotic resistance genes, such as *mecA*, *vanA* and *vanB*. Turnaround time is approximately 8 hours[80].

Sensitivity and specificity

Since blood of bacteraemic patients contains very few bacteria (sometimes as low as 1 cfu/ml), these methods have to be able to detect very low numbers of bacteria, in a large volume of blood. The detection limit of existing tests varies from 3-40 cfu/ml[65], which is higher than that of a blood culture (1 cfu/ml). Also, species not (yet) included in the test panel will be missed[81-83]. In a meta-analysis of SeptiFast, a sensitivity of 80%[84] was demonstrated. On the other hand, many studies show that PCR based methods for detection of bacteria directly on blood frequently detect bacteria in samples that are negative in blood culture[65,81-83,85-87]. The clinical significance of these bacteria is not always clear, since no gold standard exits for detection of bloodstream infections. In some cases, it was shown that the detected microorganism was very likely to be the cause of a bloodstream infection, for example when it was also isolated from another body site[83,85,86]. Often, the detected microorganisms are

difficult to culture, such as *Candida* or *Aspergillus* species[86,88]. Sometimes the patient had already been treated with antibiotic therapy at the time of blood culture drawing[81,87]. In the meta-analysis by Chang *et al.*, sensitivity of SeptiFast was 76% when using blood cultures as 'gold standard', but it increased to 82% when the clinical diagnosis was used as the reference standard[84].
Specificity in this meta-analysis was 95%, possibly due to contamination[78,86,89]. This risk of contamination probably is higher when a postamplification step such as sequencing or gel electrophoresis is necessary[79]. False-positive results may also be caused by bacterial DNA present in blood, caused by either bacteria from another body site without an actual bloodstream infection[69,90] or circulating DNA without viable bacteria[65].

Disadvantages
AST with these methods is currently very limited. In the future, probably more resistance genes can be added, but genotypic AST has its disadvantages, as described earlier. Blood cultures will thus still be required to perform AST, and because of the approximately 20% false-negative results. Other disadvantages are relatively high hands-on time and high costs (150-200 euros)[65], of nucleic acid based tests compared to blood cultures. The turnaround time is relatively long (6-12 hours), so the reduction in time to diagnosis is still limited. With this turnaround time, in most laboratories, which are closed in the evening and night, results will not be available until the next working day.

4. Clinical effects of rapid diagnosis in bloodstream infections

4.1 Rapid identification

Several studies have been performed to assess the clinical impact of new rapid methods for identification (ID). Two studies on in situ hybridisation on blood cultures containing *Staphylococcus* spp. showed a potential reduction on vancomycin use, length of hospital stay, and in one study also a reduction in mortality[91,92]. In *Enterococcus faecium* bacteraemia, time to ID was reduced with 2.3 days using PNA-FISH, which resulted in a significant reduction of time to adequate therapy and mortality[93].
The introduction of MALDI-TOF potentially allows for ID on positive blood cultures almost as fast as PNA-FISH, but at lower costs. In a Dutch study on the clinical impact of MALDI-TOF MS performed directly on positive blood cultures, time to ID was reduced with 28.8 hours, resulting in an 11.3% increase in the proportion of patients receiving adequate therapy in the first 24 hours after blood culture positivity. However, overall time to adequate therapy was not reduced. Furthermore, MALDI-TOF MS failed to produce an ID in 36.4% of positive blood cultures[58].
Some small studies assessed the impact of PCR directly on blood (SeptiFast) on antibiotic treatment. An antibiotic switch was implemented in 8.0-16.9% of all performed tests[82,85,86]. The study by Lodes *et al.*[85] showed that most switches occurred in *E. faecium*, *Pseudomonas*

aeruginosa, *Stenotrophomonas maltophilia*, *Candida* species and *Aspergillus fumigatus*. They found no effect on mortality. Reduction in time that inadequate or too broad-spectrum antibiotics were used was not assessed. These studies also show that although the test requires 6 approximately to perform, time to results ranged from 5-22 hours[82,86]. A study on VYOO reported a median time to results of 24.2 hours[80]. These delays are caused by laboratory logistics and limited opening hours. The reduction in time to results compared to blood cultures will thus probably limited in most laboratories.

4.2 Rapid antibiotic susceptibility testing

A limitation of the aforementioned methods is that they cannot be used for AST, which is especially important in decision-making in strains and areas with a high rate of antimicrobial resistance.

Cattoir *et al.* assessed the clinical effects of a multiplex PCR that combined discerning *S. aureus* from CoNS and detection of the *mecA* gene, which was performed directly on positive blood cultures. Time to results was significantly reduced, however, no effect was shown on time to appropriate therapy or outcome[94].

Since genetic susceptibility testing is not feasible for most other species, and other rapid methods for AST are not yet suitable for use in clinical practise, the few available studies on the clinical effects rapid ID and AST have focussed on improving existing techniques. The majority of these studies compare inoculation of an automated device directly from a positive blood culture with using a subculture on agar. In 1994, Doern *et al.* showed a reduction in mortality, morbidity and costs[95]. However, two later studies[96,97] did not find a reduction in mortality, in spite of a reduction in antibiotic use. One study failed to show any effect of rapid diagnosis in infections[98]. These four studies involved all culture types, and no subanalysis was performed on blood culture results. The only study that focussed solely on bloodstream infections[99] was published in 1989 and showed that more antibiotic switches occurred after rapid diagnosis, however, the effects on clinical outcome were very limited. Results thus vary greatly between these studies, as well as setting, methods and patient population. Furthermore, the methods for rapid AST used in these studies are now routinely used in most labs. As a result, it is difficult to extrapolate their results to the present day clinical practise in patients with positive blood cultures.

4.3 Other methods to reduce time to results

Several studies have been performed to assess the impact of improvement of lab flow in order to provide results on ID and AST earlier. Kerremans *et al.*[100] showed that immediate incubation of blood culture bottles resulted in a 10-hour reduction in time to blood cultures signaling positive, which led to significantly earlier antibiotic switches. However, no impact on mortality or morbidity was observed. In a study by Galar *et al.*[101] ID and AST were initiated earlier on the working day, enabling same day results (before 10 pm) for the majority of

cultures. For blood cultures, this resulted on a significantly earlier report on ID and AST (9.2 hours versus 23.5 hours), but no impact on mortality or length of stay was observed. Eveillard *et al.*[102] hypothesized that introduction of a night shift had resulted in earlier appropriate therapy in 27,9% of patients.

The timing of reporting results may also influence antibiotic switching. Several studies show that implementation of results on ID and AST is suboptimal[103,104], thereby limiting their clinical impact. Munson *et al.*[105] assessed the effects of laboratory reporting on antimicrobial management. They found that most therapy interventions occurred after blood culture drawing and after reporting of Gram-stain results, and significantly less interventions occurred after AST-results became available, a finding that was confirmed in a Dutch study[106]. This may explain the lack of effect of earlier reporting of ID and AST in many of the aforementioned studies. The impact of AST-results on antimicrobial treatment could thus potentially improve if data are available sooner.

Also, the method of reporting can influence adequate therapy. It is shown that more patients receive adequate antimicrobial therapy if a clinical microbiologist or infectious diseases specialist was involved in the choice of antibiotic[106,107]. If this has occurred before AST results become available, impact of AST results will probably be smaller, since more patients already receive adequate therapy. This may have been a confounding factor in the PNA-FISH studies, since reporting the ID result was accompanied by an advice of the antimicrobial therapy team, which likely has influenced the adequacy of therapy positively and thus may provide an alternative explanation of the favourable results in these studies.

In the future, time to results may be negatively influenced by the trend of lab mergers, since transportation time of blood culture bottles will increase, as is already shown in Germany[108].

5. Outline of this thesis

ID and AST of micro-organisms causing bloodstream infections is currently very time-consuming, thereby delaying targeted treatment of patients with bloodstream infections. New, faster methods are being developed, but most of these involve methods for ID only, whereas for targeting of antibiotic therapy, AST is required. Faster methods for AST either have a limited scope (for example *mecA* testing in *S. aureus* infections) or are far from use in clinical practise.

The goal of these faster techniques is to enable earlier targeting of antibiotic therapy, thereby improving clinical outcome of patients with bloodstream infections. Studies on the clinical effects of rapid ID and AST on the outcome of bloodstream infections are sparse, and often outdated since they used 'fast' methods that are now considered standard. As a result, it is unknown what the effects are of methods more rapid than those currently available.

The first aim of this thesis was to develop new faster methods for ID and AST that would readily applicable in current clinical practise. The second aim of the thesis was to explore the clinical effects of these more rapid methods in a clinical trial.

According to manufacturers' guidelines, current methods for ID and AST require an overnight culture on agar before inoculation. For some automated systems, such as Vitek 2, the use of serum separator tubes has been extensively tested for harvesting bacteria directly from positive blood cultures, thereby significantly reducing time to results. However, for the BD Phoenix system, very few studies are available and only for specific groups of bacteria. Therefore, in **chapter 2** the accuracy of AST using the Phoenix system with bacteria harvested directly from blood cultures was investigated.

Time to ID using currently available automated systems depends upon the metabolism and growth rate of the tested bacteria. PCR does not require bacterial growth and can therefore significantly reduce time to results. **Chapter 3** describes a multiplex real-time PCR method combining one primer set with multiple probes to identify the most common bacterial species in bloodstream infections. This method can be applied directly on blood cultures and requires only 2 hours to obtain results.

To reduce time to AST, a rapid method was developed that combines culture and PCR, described in **chapter 4**. Bacteria harvested directly from positive blood cultures were incubated with antibiotics in order to determine whether growth was inhibited by the antibiotic, similar to current techniques. Real-time PCR was used to determine bacterial load. **Chapter 5** describes the results of a randomised clinical trial on the effects of rapid ID and AST on antibiotic treatment and clinical outcome in patients with bloodstream infections, using the techniques described in chapter 3 and 4.

MALDI-TOF MS has rapidly replaced other methods for ID in clinical practise. It has been shown to be a reliable, fast and cheap method for ID of coagulase-negative *Staphylococcus* species[109]. These were previously not routinely identified to species level, since the available methods were either not reliable, or too expensive and time-consuming. Distinguishing blood culture contamination from true infection is difficult, and little is known about different CoNS species and their ability to cause infection. In the study in **chapter 6** the value of CoNS speciation by MALDI-TOF MS in the diagnosis of CoNS bloodstream infections was tested.

In **chapter 7**, the findings of the previous chapters are summarised and discussed.

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Chapter 2

Evaluation of direct inoculation of the BD PHOENIX system from positive BACTEC blood cultures for both Gram-positive cocci and Gram-negative rods

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Abstract

Background

Rapid identification (ID) and antibiotic susceptibility testing (AST) of the causative micro-organism of bloodstream infections result in earlier targeting of antibiotic therapy. In order to obtain results of ID and AST up to 24 hours earlier, we evaluated the accuracy of direct inoculation of the Phoenix system from positive blood cultures (BACTEC) by using Serum Separator Tubes to harvest bacteria from positive blood cultures.

Methods

Direct inoculation of the Phoenix system from positive blood cultures (BACTEC) was performed using Serum Separator Tubes to harvest bacteria from positive blood cultures.
Results were compared to those of standard Phoenix procedure. Discrepancies between the two methods were resolved by using the API system, E-test or microbroth dilution.

Results

ID with the direct method was correct for 95.2% of all tested Enterobacteriaceae (N = 42) and 71.4% of *Pseudomonas aeruginosa* strains (N = 7). AST with the direct method showed a categorical agreement for Gram-negative rods (GNR) of 99.0%, with 0.7% minor errors, 0.3% very major errors and no major errors. All antibiotics showed an agreement of >95%.
The direct method for AST of *Staphylococcus* (N = 81) and *Enterococcus* (N = 3) species showed a categorical agreement of 95.4%, with a minor error rate of 1.1%, a major error rate of 3.1% and a very major error rate of 0.4%. All antibiotics showed an agreement of >90%, except for trimethoprim-sulfamethoxazole and erythromycin.

Conclusions

Inoculation of Phoenix panels directly from positive blood cultures can be used to report reliable results of AST of GNR a day earlier, as well as ID-results of Enterobacteriaceae. For *Staphylococcus* and *Enterococcus* species, results of AST can also be reported a day earlier for all antibiotics, except for erythromycin and trimethoprim-sulfamethoxazole.

Introduction

Bloodstream infections are a common condition, affecting approximately 2% of all hospitalised patients and up to 70% of all patients in the Intensive Care Unit, and the incidence is rising[1-4]. Mortality is high, ranging from 14 to 57%[5]. In this group of patients, rapid identification (ID) and antibiotic susceptibility testing (AST) of the causative microorganism are essential since they result in earlier targeting of antibiotic therapy[6-9].
Early administration of adequate antibiotic therapy has been shown to reduce mortality[10-12].
The introduction of automated blood culture systems and automated systems for ID and AST have reduced the time to diagnosis in bloodstream infections. However, for these systems, blood cultures have to be subcultured on agar before ID and AST can be performed, which can take up to 24 hours.
An alternative for subculture on agar is harvesting the bacteria needed for inoculation of these systems directly from positive blood cultures by using Serum Separator Tubes, thereby reducing the time needed to obtain results of ID and AST by a day. Although this method has been successfully tested for many automated systems[13-17], direct inoculation was reported only twice for the BD Phoenix Automated Microbiology System (BD), once for Gram-negative rods (GNR)[18] and once for Gram-positive cocci (GPC)[19]. Both studies compared their results of the direct method with results of the Vitek system.
No studies are available comparing results of direct inoculation with the routinely used method of inoculating the Phoenix system, which is the standard procedure for ID and AST in many microbial diagnostic laboratories. Here, we evaluated the accuracy of direct inoculation of the Phoenix system with positive blood culture isolates, compared to the routinely used procedure.

Methods

Sample collection

Between January and April 2009, blood cultures grown in the previous 24 hours in the Bactec automated blood culture device (Bactec™ 9240, BD Diagnostic Systems, Sparks, MD, USA) and containing *Staphylococcus* species, *Enterococcus* species or obligate aerobic and facultative anaerobic GNR were evaluated. Polymicrobial cultures as well as cultures containing anaerobes or fungi were excluded from the analysis. *Streptococcus* species are not routinely processed in the Phoenix system in our lab and were therefore also excluded from the analysis. One positive blood culture per patient per episode of bloodstream infection was included in the study. The study was performed in the Department of Medical Microbiology of the Maastricht University Medical Center (MUMC), a 750-bed referral hospital.
All samples were used according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies.

Blood cultures

Blood drawn from patients admitted in the MUMC and suspected for bloodstream infection was incubated in blood culture bottles (Plus+Aerobic (product no. 442192; BD) and Plus+Anaerobic (product no. 442193; BD)) and monitored for microbial growth in the Bactec^{TM} 9240 instrument (BD). When growth was detected by the instrument, Gram-staining was performed.

Direct inoculation

For the direct method, 5 ml of grown blood culture was aspirated from the blood culture bottle and the aspirate was injected in a Serum Separator Tube (SST) (BD Diagnostic Systems, Sparks, MD, USA). This tube was centrifuged at 2000 × *g* for 10 minutes, after which the supernatant was discarded. Bacteria were harvested from the gel layer using a sterile cotton swab and suspended in a Phoenix system ID broth tube (product no. 246000; BD) until a 0.5 McFarland standard suspension was obtained. To obtain optimal results, for Gram-negative isolates, 25 µl of this suspension were transferred into a tube of Phoenix system AST broth (product no. 246002; BD) in which one drop of AST indicator (product no. 246004; BD) was previously added, according to manufacturers' guidelines. Since the inoculum of GPC in ID broth was shown to be almost 10 times lower than is standard in a 0.5 McFarland suspension, 250 µl of inoculated ID broth was added to AST broth for GPC, instead of the 25 µl in the manufacturers' guidelines. For GNR, the Phoenix system panel NMIC/ID-75 (product no. 448087; BD) was used. For GPC, the PMIC-58 panel (product no. 448052; BD) was used.
To calculate the original number of cfu/ml in the ID broth and to serve as purity control, dilutions of ID broth were also subcultured, using the Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain).

Routinely used inoculation

For the routinely used method, a small volume of positive blood culture was inoculated on Columbia sheep blood agar plates and incubated at 35°C with 5% CO_2. A standard inoculum in ID broth was prepared from the bacteria grown on the agar medium and inoculated into Phoenix panels, following the manufacturer's recommendations.

Identification of GPC

Since the Phoenix system is not used for ID of GPC in routine diagnostics, ID by direct inoculation was not tested in this group. To discern Staphylococcus species from other GPC, a catalase test was performed. For the identification of *Staphylococcus* species, catalase-positive strains were tested for coagulase and DNAse production. If both tests were negative, the strain was identified as a coagulase-negative *Staphylococcus* species (CoNS).

To discern Enterococcus species from other catalase- negative GPC, bile esculin and tellur diagnostic tablets (Rosco Diagnostica, Taastrup, Denmark) were used, according to manufacturer's guidelines. If both tests were positive, the strain was identified as *Enterococcus faecalis*, whereas in case of a positive bile esculin test but a negative tellur test, an API 20 Strep test (Biomérieux SA, Marcy l'Etoile, France) was performed to further identify the strain.
Results of identification were adjusted in the Phoenix results retrospectively for both the standard and direct method, after which the software automatically adjusted MIC cutoff values to those of the identified species.

Discrepancy analysis

To resolve differences in ID of GNR, the API system was used (API 20E for Enterobacteriaceae and API 20NE for non-fermenters (Biomérieux)). In case of discrepancies in AST between results of the direct method and the routinely used method for ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, clindamycin, levofloxacin, moxifloxacin, linezolid, penicillin, piperacillin, piperacillin-tazobactam, and tobramycin an E-test (Biomérieux) was performed according to manufacturer's guidelines, and used as gold standard[20,21]. Discrepancies for amoxicillin, amoxicillin-clavulanate, erythromycin, gentamicin, oxacillin, rifampin, tetracycline and trimethoprim-sulfamethoxazole were resolved using microbroth dilution, as described in the CLSI- guidelines[22].

Data analysis

Results of AST with the direct method (susceptible, intermediate or resistant) were analysed for categorical agreement with the results of the standard method. Also, minor errors (any false result involving an intermediate result), major errors (false-resistant results) and very major errors (false-susceptible results) were calculated.

Statistical analysis

Bacterial load in ID broth for GPC and GNR was compared using an independent samples t-test.

Results

Inoculum of bacteria in ID broth after use of serum separator tubes (SSTs)

In total, 134 blood cultures were included, from 116 patients. The inoculum of GPC in ID broth was on average 3.6×10^7 cfu/ml, whereas that of GNR was 1.8×10^8 cfu/ml, which was a significant difference (95% CI between -1.7×10^8 and -1.2×10^8; $P < 0.001$).

ID of GNR with the direct Phoenix method

ID with direct inoculation was correct for 95.2% of all tested Enterobacteriaceae. One *Escherichia coli* strain was incorrectly identified as *Salmonella choleraesuis* with the direct method. One *Serratia marcescens* strain could not be identified with the direct method. Identification for *Pseudomonas* spp. was correct in 71.4%. Both errors in this group involved strains of *Pseudomonas aeruginosa* that were incorrectly identified as *Pseudomonas fluorescens* (Table 1). No errors in ID were observed for the routine method.

Table 1. Results of identification of GNR with the direct method

	Total no. of strains	No. of unidentified strains	No. of misidentified strains	ID of misidentified strains
Enterobacteriaceae				
E. coli	26		1	*Salmonella choleraesuis*
K. pneumoniae spp. *pneumoniae*	8			
S. marcescens	4	1		
K. oxytoca	1			
P. mirabilis	1			
E. cloacae	1			
M. morganii	1			
Non-fermenters				
P. aeruginosa	7		2	*Pseudomonas fluorescens*

Antibiotic susceptibility testing (AST) of GNR

Results of AST were available for 49 strains, one *P. aeruginosa* strain failed to grow sufficiently in the Phoenix system so no results were available for the direct method. Categorical agreement of the direct method with results of the standard method for GNR was 97.6%. After discrepancy analysis of the results of AST, this percentage rose to 99.0%, with 5 minor errors (0.7%), no major errors, and 2 very major errors (0.3%) (Table 2). Both very major errors occurred with trimethoprim-sulfamethoxazole in *P. aeruginosa* strains. Categorical agreement of the standard method after discrepancy analysis was 98.4% (Table 2). One very major error occurred with trimethoprim-sulfamethoxazole. No antibiotic showed a categorical agreement of <95% (Table 3).

Table 2. Agreements and errors for AST of GPC and GNR for the direct and routinely used Phoenix method

	Direct vs routinely used method	Direct method after discrepancy analysis	Routine method after discrepancy analysis
GPC (n = 84)			
Categorical agreement	93.1%	95.4%	97.3%
Minor errors	1.7%	1.1%	0.7%
Major errors	4.2%	3.1%	0.8%
Very major errors	0.9%	0.4%	1.6%
GNR (n = 49)			
Categorical agreement	97.6%	99.0%	98.4%
Minor errors	1.9%	0.7%	1.4%
Major errors	0.1%	0.0%	0.1%
Very major errors	0.4%	0.3%	0.1%

AST of GPC

AST using the direct method was performed for 84 GPC (22 *Staphylococcus aureus*, 59 CoNS, 2 *Enterococcus faecalis* and 1 *Enterococcus faecium*). Categorical agreement for the tested GPC was 93.1% compared with results of the standard method. After discrepancy analysis this was 95.4%, with a minor error rate of 1.1%, a major error rate of 3.1% and a very major error rate of 0.4% (Table 2). Except for erythromycin and trimethoprim-sulfamethoxazole, all antibiotics showed a categorical agreement of the direct method of >90% (Table 4). Again, all very major errors (n = 4) occurred with trimethoprim-sulfamethoxazole, all in CoNS strains. The major errors were divided as follows: 10 for *S. aureus*, 23 for CoNS and 1 for *Enterococcus* spp..

Categorical agreement for the standard method after discrepancy analysis was 97.3% (Table 2). One very major error occurred for amoxicillin-clavulanate, 1 for ampicillin, 1 for erythromycin, 4 for gentamicin, 1 for moxifloxacin, 2 for oxacillin, 1 for tetracycline and 3 for trimethoprim-sulfamethoxazole (Table 4).

Table 3. Agreement and errors of the direct method for AST for GNR, after discrepancy analysis

Antimicrobial agent	No. of tested strains	% categorical agreement	No. of minor errors (%)	No. of major errors (%)	No. of very major errors (%)
Amikacin	49	100	0	0	0
Amoxicillin/clavulanate	49	98.0	1 (2.0)	0	0
Ampicillin	49	98.0	1 (2.0)	0	0
Ceftazidime	49	100	0	0	0
Ceftriaxone	49	98.0	1 (2.0)	0	0
Cefuroxime	49	98.0	1 (2.0)	0	0
Ciprofloxacin	49	100	0	0	0
Colistin	49	100	0	0	0
Gentamicin	49	100	0	0	0
Levofloxacin	49	100	0	0	0
Meropenem	49	100	0	0	0
Piperacillin	49	98.0	1 (2.0)	0	0
Piperacillin/tazobactam	49	100	0	0	0
Tobramycin	49	100	0	0	0
Trimethoprim/ sulfamethoxazole	49	96.0	0	0	2 (4.0)
Total	735	99.0	5 (0.7)	0 (0)	2 (0.3)

Table 4. Agreement and errors of the direct method of AST for GPC after discrepancy analysis

Antimicrobial agent	No. of tested strains	% categorical agreement	No. of minor errors (%)	No. of major errors (%)	No. of very major errors (%)
Amoxicillin/clavulanate	84	91.7	0.0	7 (8.3)	0
Ampicillin	84	94	0	5 (6.0)	0
Clindamycin	84	96.4	2 (2.4)	1 (1.2)	0
Erythromycin	84	86.9	8 (9.5)	2 (3.6)	0
Gentamicin	84	100	0	0	0
Linezolid	84	91.6	1 (1.2)	6 (7.2)	0
Moxifloxacin	84	100	0	0	0
Oxacillin	84	96.4	0	3 (3.6)	0
Penicillin	84	98.8	0	1 (1.2)	0
Rifampin	84	98.8	0	1 (1.2)	0
Tetracycline	84	97.6	1 (1.2)	1 (1.2)	0
Trimethoprim/ Sulfamethoxazole	84	89.2	0	5 (6.0)	4 (4.8)
Vancomycin	84	98.8	0	1 (1.2)	0
Total	1090	95.4	12 (1.1)	34 (3.1)	4 (0.4)

Discussion

This study shows SSTs can be used to inoculate Phoenix ID broth to a 0.5 McFarland standard, as was also shown by Funke *et al.* for GNR[18]. However, a 0.5 McFarland standard for GPC obtained by using SSTs was shown to consistently contain a lower inoculum than 1.5×10^8 CFU/ml. This may be due to the presence of blood culture components other than bacteria, since they could also contribute to the turbidity in the ID broth.

This study shows very good results for ID of Enterobacteriaceae. Only two errors occurred with ID in this group. One strain was not identified and one strain of *E. coli* was misidentified as *S. choleraesuis*. Results of ID for *Pseudomonas* species were less reliable. Both errors in this group were *P. aeruginosa* strains that were identified as *P. fluorescens*, a rare cause of bloodstream infections. These misidentifications did not lead to errors in interpretation of AST, but rare or unlikely results of ID should be dealt with carefully and be con- firmed using additional tests. Other studies also showed that ID of non-fermenting GNR was less reliable than that of Enterobacteriaceae[18,23]. This may be due to the lower growth rate of non-fermenters, which could result in weaker fluorescent biochemical reactions in the Phoenix ID panel. Errors in ID with the direct method could also be caused by traces of blood culture components in the ID broth. This however seems less likely, since with Enterobacteriaceae, errors in ID were rare.

Since the Phoenix system was not used for ID of GPC, ID by direct inoculation was not tested in this group. But since ID is required for interpretation of AST, in clinical practice, rapid AST will have to be combined with a rapid method of ID, such as PCR-based methods on whole blood, like LightCycler® SeptiFast Test MGRADE (Roche), VYOO Sepsis Test (SIRS-Lab), SepsiTest™ (Molzym), or MALDITOF-MS on positive blood cultures[24].

Some studies on direct methods for AST showed poor results for GPC[15,16] or focus on GNR only due to unfavorable results for GPC[17]. However, in this study, direct AST for *Staphylococcus* species and *Enterococcus* species showed good agreement with conventional methods, comparable to results of the standard method, but with fewer very major errors. Lupetti *et al.*[19], who tested the direct Phoenix method for GPC and compared their results with those of the Vitek 2, found an even higher agreement. They incubated a portion of the positive blood culture with saponin in order to harvest more bacteria from a positive blood culture through the release of intracellular bacteria.

Other studies that presented results of direct methods for AST of GPC showed variable results[13-16,25,26], which makes comparison difficult. But our results were comparable to those of the routine Phoenix method. Moreover, categorical agreement for most tested antibiotics in this study, including oxacillin and vancomycin, were well over 90% and the percentage of major and very major errors is low, meeting the standards proposed by Jorgensen *et al.*[27]. Only erythromycin and trimethoprim-sulfamethoxazole showed lower agreements. The majority of errors for erythromycin were minor errors, but also some major errors occurred. Trimethoprim-sulfamethoxazole was the only antibiotic for both GPC and GNR showing very major errors. Lupetti *et al.* showed a lower agreement of 94% for erythromycin as well, but

observed no very major errors for trimethoprim-sulfamethoxazole. Some other studies on direct methods for AST showed some very major errors for trimethoprim-sulfamethoxazole[15,16,18], but only Kerremans *et al.*[13] found very high percentages of very major errors for this antibiotic in GPC, but not in GNR. Therefore, we conclude that the direct Phoenix method using SSTs can be used to reliably report results of AST for GPC, except for trimethoprim-sulfamethoxazole and erythromycin.

The direct method of AST for GNR showed very good agreement with conventional methods for both Enterobacteriaceae and *Pseudomonas* species, comparable to the routinely used method, with essential agreements and categorical agreements of over 95% for all antibiotics tested (Table 3). Both very major errors occurred with trimethoprim-sulfamethoxazole in *P. aeruginosa* strains that were correctly identified. For these strains, it would never be considered an adequate treatment, due to intrinsic resistance. These errors thus would not have clinical consequences. Funke *et al.* [18] also described a categorical agreement of 99.0%, which is comparable with or higher than results from studies on other direct methods of AST[13-17,26]. Therefore, we conclude that also for GNR, results of the direct Phoenix method for AST can be used to guide antibiotic therapy in bloodstream infections.

The strains tested in this study are a representative sample of the strains most frequently encountered in clinical practice. A limitation of the study is the low number of tested *Enterococcus* and *Pseudomonas* strains (3 and 7, respectively), however, both groups show very good agreement, with only few errors.

Inoculating ID and AST broth by using SSTs can be performed as soon as blood culture bottles are taken out of the BACTEC system and takes approximately 30 minutes, whereas a subculture takes up to 24 hours.

Therefore, by using the direct method, results of ID and AST can be available up to 23.5 hours earlier than with the routinely used method.

From these results we conclude that AST by inoculating Phoenix panels with bacteria harvested directly from positive blood culture bottles is as reliable as using bacteria from a subculture on agar, with the exception of results for erythromycin and trimethoprim-sulfamethoxazole in *Staphylococcus* and *Enterococcus* spp., which should not be reported due to their low agreement. Results of ID of Enterobacteriaceae were shown to be very reliable. ID of *Staphylococcus* and *Enterococcus* spp. was not performed with the direct method. Caution is warranted about interpretation of results of *Enterococcus* and *Pseudomonas* spp., of which only a limited number of strains was tested.

Thus, by only a small change in daily laboratory routine, results of ID and AST of positive blood culture isolates can be obtained up to a day earlier than with the standard method, thereby leading to earlier targeting of antibiotic therapy in patients with bloodstream infections.

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Chapter 3

Molecular probes for diagnosis of clinically relevant bacterial infections in blood cultures

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Abstract

Background

Broad-range real-time PCR and sequencing of the 16S rRNA gene region is a widely known method for the detection and identification of bacteria in clinical samples. However, because of the need for sequencing, such identification of bacteria is time-consuming. The aim of our study was to develop a more rapid 16S real-time PCR-based identification assay using species- or genus-specific probes.

Methods

The Gram-negative bacteria were divided into *Pseudomonas* species, *Pseudomonas aeruginosa*, *Escherichia coli*, and other Gram-negative species. Within the Gram-positive species, probes were designed for *Staphylococcus* species, *Staphylococcus aureus*, *Enterococcus* species, *Streptococcus* species, and *Streptococcus pneumoniae*. The assay also included a universal probe within the 16S rRNA gene region for the detection of all bacterial DNA.

Results

The assay was evaluated with a collection of 248 blood cultures. In this study, the universal probe and the probes targeting *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Streptococcus* spp., *S. pneumoniae*, *Enterococcus* spp., and *Staphylococcus* spp. all had a sensitivity and specificity of 100%. The probe specific for *S. aureus* showed eight discrepancies, resulting in a sensitivity of 100% and a specificity of 93%.

Conclusions

These data showed high agreement between conventional testing and our novel real-time PCR assay. Furthermore, this assay significantly reduced the time needed for identification. In conclusion, using pathogen-specific probes offers a faster alternative for pathogen detection and could improve the diagnosis of bloodstream infections.

Introduction

Bloodstream infections are a major cause of death in the world and need a thorough and adequate therapeutic strategy. Inadequate antibiotic therapy is associated with higher mortality rates, the appearance of antibiotic resistance, and longer hospitalization lengths[1]. Conventional identification and susceptibility testing have several limitations, such as lack of rapidity and sensitivity. The current gold standard, i.e., blood culture, usually requires 6 to 12 hours of incubation before growth is detected and a further 24 to 48 hours for the definitive identification of the infectious agent and its susceptibility to antibiotics[2,3]. Routine diagnostics already use molecular techniques for the direct detection of viral and bacterial pathogens. However, most in-house assays are targeted against one specific bacterium and/or virus and do not offer broad-range pathogen detection. Recently, several PCR assays have been developed targeting a panel of the most relevant bacterial and fungal blood- stream pathogens, which can be performed directly with blood, such as SeptiFast (Roche Diagnostics GmbH, Mannheim, Germany), SepsiTest (Molzym GmbH & Co. KG, Bremen, Germany), and VYOO (SIRS-Lab GmbH, Jena, Germany), or using positive blood cultures, such as the microarray-based system Prove-it Sepsis (Mobidiag, Helsinki, Finland).

As discussed in our previous work, direct detection in whole blood is hampered by several factors, such as the presence of PCR inhibitors and background DNA, low bacterial load, insufficient sensitivity, and difficulty of establishing an assay capable of detecting a wide range of pathogens[4]. In contrast, molecular testing of growth-positive blood cultures does not require highly sensitive assays because of the presence of a high bacterial load. Furthermore, until now, the use of culturing remains essential to determine the microorganism's anti-microbial profile. Therefore, the role of blood cultures remains important for the detection and identification of causative bacterial agents. Molecular testing of blood cultures, possibly in combination with conventional testing, could enable more rapid identification and, consequently, more rapid diagnosis and start of correct therapy. Molecular approaches such as broad-range real-time PCR and sequencing of the 16S rRNA gene region are widely known methods for the detection and identification of bacteria in clinical samples[5-8]. However, because of the need for sequencing, the identification of bacteria is time-consuming.

The aim of our study was to develop a more rapid 16S real-time PCR-based identification assay using species- or genus-specific probes. The assay is particularly intended for the identification of positive blood cultures, for which Gram staining results are known. In this proof-of-concept study, priority was given to the genera or species most frequently found in blood cultures and/or those that could direct the choice of a suitable antibiotic therapy. Therefore, we selected a panel of eight species- or genus-specific probes. The Gram-negative bacteria were divided into *Pseudomonas* species, *Pseudomonas aeruginosa*, *Escherichia coli*, and other Gram-negative species. Within the Gram-positive species, probes were designed for *Staphylococcus* species, *Staphylococcus aureus*, *Enterococcus* species, *Streptococcus* species, and *Streptococcus pneumoniae*.

Hence, a first indication about the causative microorganism is given after 2 hours, while confirmation and precise identification can be achieved with sequencing. Consequently, multiple species can be detected in samples with polymicrobial infections. The present paper reports a retrospective study performed on blood cultures obtained from patients with suspected bloodstream infections. Results of this new multiprobe assay were compared with conventional blood culture findings.

Methods

Clinical samples

A total of 248 blood cultures were collected at the Maastricht University Medical Center (MUMC; Maastricht, Netherlands). All samples were analyzed with standard conventional testing. Blood specimens drawn from patients suspected of having bloodstream infections were incubated in blood culture bottles (Plus+Aerobic [product no. 442192] and Plus+Anaerobic [product no. 442193]; BD Diagnostic Systems, Sparks, MD, USA) and monitored for microbial growth in the Bactec 9240 automated blood culture device (BD). When growth was detected, Gram staining was performed. A small aliquot of each blood culture (1 ml) was requested for the novel molecular assay. Two separate assays were developed for Gram-negative and Gram-positive bacteria. Hence, further analysis was based on the results of Gram staining.

Conventional bacterial identification

Regarding the Gram-positive cocci (GPCs), to discern *Staphylococcus* spp. from other GPCs, a catalase test was performed by adding 1 colony to a drop of 3% H_2O_2. For the identification of *Staphylococcus* spp., catalase-positive strains were tested for coagulase and DNase production. If both tests were negative, the strain was identified as coagulase-negative *Staphylococcus* (CoNS). To discern *Enterococcus* spp. from other catalase-negative GPCs, bile esculin, Tellur diagnostic tablets (Rosco Diagnostica, Taastrup, Denmark), and an API 20 Strep test (bioMerieux SA, Marcy l'Etoile, France) were used, according to the manufacturer's guidelines. Optochin susceptibility (OXOID) was used to differentiate *S. pneumoniae* from the other viridans group streptococci, which were further identified by API 20 Strep. In the case of beta-hemolytic streptococci, latex agglutination was performed using the Prolex streptococcal grouping latex kit (product code PL.030; Bio Trading).

Bacterial strains

Reference strains were used to validate the specificity of the assay, including *S. aureus* ATCC 25923 and ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 and ATCC 14990,

S. pneumoniae ATCC 49619, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* BM 4147, and *E. coli* ATCC 35218.

Multiprobe assay

An aliquot (0.1 ml) of blood culture was 1:100 diluted in 0.9% NaCl. Dilutions were centrifuged for 5 min at 12,000 rpm, and the bacterial pellet was resuspended in 100 µl nuclease-free water. The primers and the universal bacterial TaqMan probe have been described previously[9]. The probes for *P. aeruginosa*, *Pseudomonas* spp., *E. coli*, *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp., *Streptococcus* spp., and *S. pneumoniae* were designed by using the BLAST tool and ClustalW software. Multiple sequence alignments were made and are partly shown in Figure 1. The designed probe sequences are given in Table 1.

All primers and probes were tested for specificity and cross- reactions both manually and with use of the NCBI-BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). Primer and probe matrices were performed to determine optimal concentrations. Each test contained a 5 µl purified sample and a 20 µl reaction mixture. The reaction mixture contained 12.5 µl of TaqMan environmental master mix 2.0 (Applied Biosystems, Foster City, CA, USA), 0.9 µM forward primer, 0.6 µM reverse primer, and 0.2 µM each probe. There were three separate reactions. The first reaction included the universal probe and the *P. aeruginosa* probe. The second reaction included the probes targeting *E. coli* and *Pseudomonas* spp. The third reaction included the *Staphylococcus* probe, the *S. aureus* probe, and the *Enterococcus* probe, the fourth and final reaction included the *Streptococcus* probe and the *S. pneumoniae* probe. Reactions were performed using the ABI Prism 7000 real-time PCR system (Applied Biosystems), and optimal thermal cycling conditions used were as follows: 2 min at 50°C, initial denaturation at 95°C for 15 min, 42 cycles of denaturation for 15 s at 95°C, and annealing at 60°C for 1 min. The cycle threshold (Ct) value, the cycle number at which amplicon fluorescence exceeded the preset detection threshold, was recorded for all samples. The cutoff value to consider a PCR result as positive was set to a Ct value of 35.

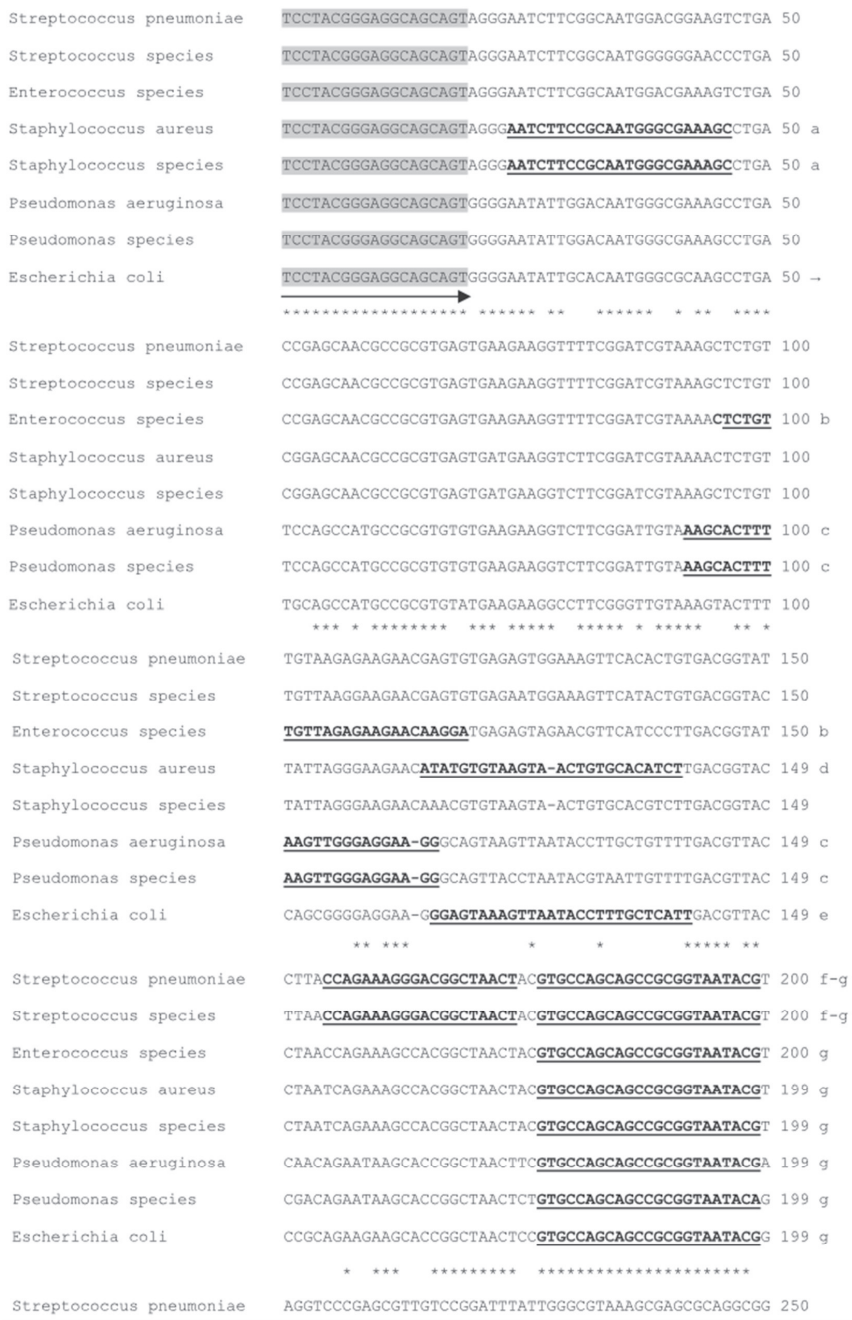

Figure 1. Multiple sequence alignments of the amplified part of the bacterial 16S rRNA gene. Sequences of the bacteria included in the multiprobe assay are shown. The arrows represent the forward and reverse primer targeting the 16S rRNA gene. The sequences of the probes are in boldface and underlined. a, *Staphylococcus* probe; b, *Enterococcus* probe; c, *Pseudomonas* probe; d, *S. aureus* probe; e, *E. coli* probe; f, *Streptococcus* probe; g, universal probe; h, *S. pneumoniae* probe; i, *P. aeruginosa* probe.

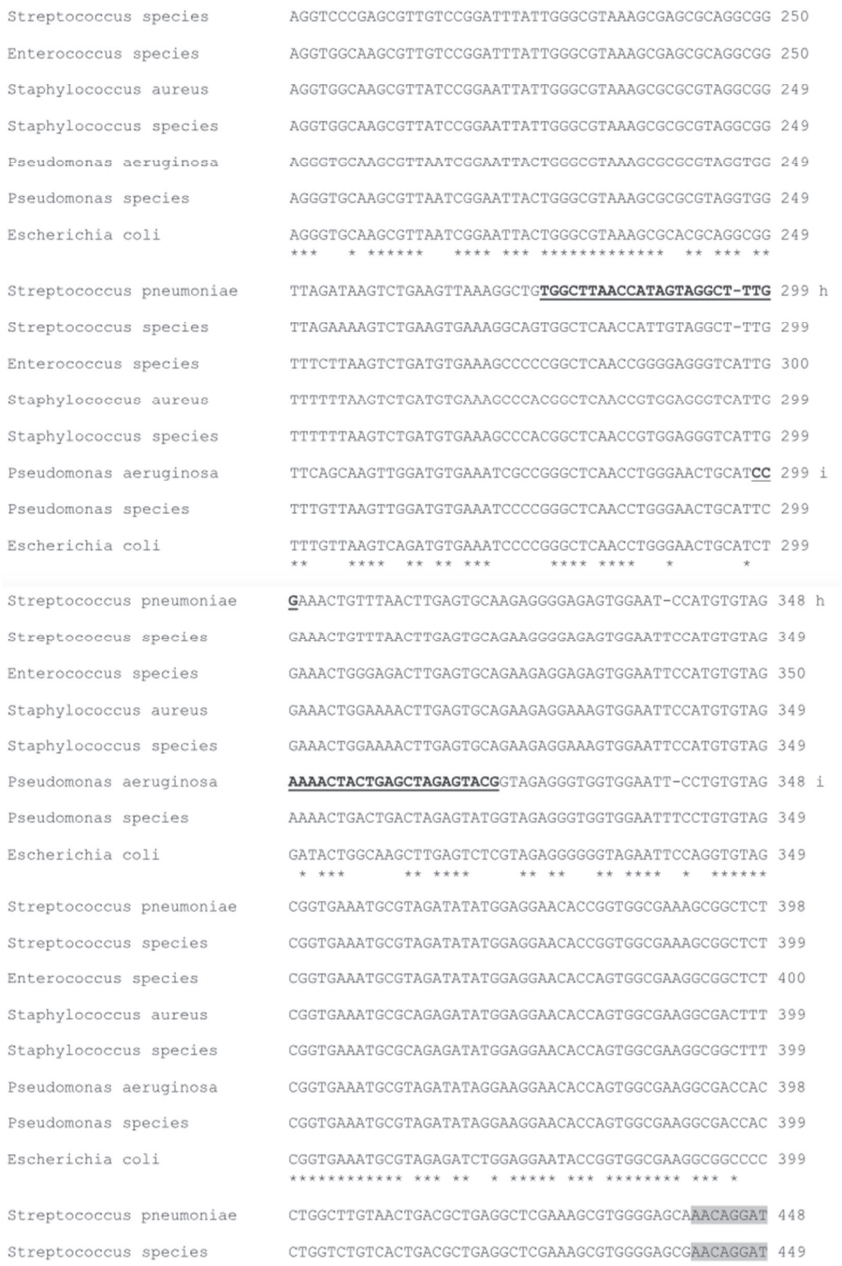

Figure 1. *Continued*.

Table 1. Probes designed for use in multiplex PCR

Probe	Sequence (5'–3')[a]
Pseudomonas species	NED-CCTTCCTCCCAACTTAAAGTGCTT-MGB
P. aeruginosa	JOE-CCAAAACTACTGAGCTAGAGTACG-BHQ1
E. coli	JOE-GGAGTAAAGTTAATACCTTTGCTCATT-BHQ1
Staphylococcus species	NED-AATCTTCCGCAATGGGCGAAAGC-MGB
S. aureus	FAM-AGATGTGCACAGTTACTTACACATAT-BHQ1
Enterococcus species	JOE-TCCTTGTTCTTCTCTAACAACAGAG-BHQ1
Streptococcus species	NED-CCAGAAAGGGACSGCTAACT-MGB
S. pneumoniae	JOE-CCAAAGCCTACTATGGTTAAGCCA-BHQ1

[a] NED, fluorescent label (Applied Biosystems); MGB, minor groove binder; JOE, 6-carboxy-4,5-dichloro-2,7-dimethyoxyfluorescein; BHQ1, black hole quencher 1; FAM, 6-carboxyfluorescein.

DNA sequencing

Samples with discrepant results were further analyzed with sequencing. The DNA was purified using the MSB PCRapace PCR cleanup kit (Invitek, Berlin, Germany) and dissolved in 30 µl NASBA H_2O. Cyclic sequencing was performed using BigDye 3.0 (Applied Biosystems, Foster City, CA). The resulting sequence product was purified and separated with the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Results

Design of the species- or genus-specific probes

The specificity of the probes was evaluated using BLAST. *In silico* analysis revealed that nearly only cross-reactivity was found with not clinically relevant microorganisms such as *Geobacillus* spp. and *Lactobacillus plantarum. In silico* cross-reactivity with clinically relevant microorganisms was tested *in vitro* with reference strains. For example, the *in silico E. coli* probe cross-reacted with *Acinetobacter* spp. and *Enterobacter* spp.. However, *in vitro* tests showed no positive signals for these microorganisms. Furthermore, cross-reactions were found with *Escherichia albertii* and *Shigella* spp.. Based on the literature, these microorganisms occur only rarely in blood cultures, and therefore, the risk of false identification was minimal. In a few cases such as with the *Streptococcus* and the *Staphylococcus* probes, cross-reactivity with *Bacillus cereus* was found. The species conferring cross-reactivity in PCR were then sequenced to check for mismatches between the probe-target hybrids. Sequencing results showed that this occurred because of at least two mismatches at the binding site of both probes (data not shown). However, in all these cases, the cross-reacting fluorescent signals generated by the mismatched probes were weak

compared to that of the positive template control and uncharacteristically appeared over 5 cycles later than signals from 100% matched probes (data not shown).

Evaluation of the multiprobe assay by testing clinical blood culture samples

From a total of 248 blood cultures, the presence or absence of bacterial DNA was determined in 232 growth-positive and 16 growth-negative samples using the universal probe, yielding a sensitivity and specificity of 100% (Table 2). Our multiprobe assay was performed after Gram staining of the positive blood cultures. Considering the growth-positive blood cultures, results from the multiprobe assay were in accordance with conventional identification in 222 cases (96%). The specific probes targeting *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Streptococcus* spp., *S. pneumoniae*, and *Staphylococcus* spp. all had a sensitivity and specificity of 100%. The majority of Gram-positive blood cultures, 85 of 145 (59%), contained a coagulase-negative *Staphylococcus* species. In 31 blood cultures, the causative agent was identified as *S. aureus*. Regarding the Gram-positive staphylococcal blood cultures, the multiprobe assay was in conflict with culture results in nine cases. The nine discrepancies were further analyzed by coagulase testing, specific *S. aureus* real-time PCR, and sequencing (Table 3; Figure 2). One out of nine cases, clinical isolate (CI) 7, tested coagulase positive and was positive for two targets specific for *S. aureus*, *i.e.*, *femA* and *sa442*. Sequencing of the PCR product also confirmed the results of the multiprobe assay. The sequences of the eight other clinical isolates showed three mismatches with the reference *S. aureus* sequence, as shown in Figure 2A. Hence, the remaining eight cases were confirmed as discordant. Consequently, sensitivity and specificity of 100% and 93%, respectively, and positive and negative predictive values of 79% and 100%, respectively, were achieved when sequencing was considered the gold standard. The probe specific for *Enterococcus* spp. showed one conflicting result. One blood culture was determined to contain *Aerococcus viridans*, while our multiprobe assay identified the infectious agent as an *Enterococcus* species (Table 3). Further analysis revealed that the partial sequence of CI 10 was completely similar to the sequence of a reference *Enterococcus* strain, as shown in Figure 2B. Comparison of the reference *A. viridans* and *Enterococcus* strains showed five mismatches. Consequently, the probe specific for *Enterococcus* spp. yielded a sensitivity and specificity of 100% when sequencing was considered the gold standard.

Table 2. Bacterial isolates in 232 positive blood cultures and results from universal and specific real-time PCR

	Result of blood culture (N (%))[a]								
Pathogen	UNI	PSEU	PSEUAE	ECOLI	STAPH	STAU	ENTE	STREPT	STREPN
Gram-negative pathogens									
Acinetobacter lwoffii/haemolyticus	1 (100)	0	0	0	NA	NA	NA	NA	NA
Bacteroides fragilis	2 (100)	0	0	0	NA	NA	NA	NA	NA
Citrobacter koseri	1 (100)	0	0	0	NA	NA	NA	NA	NA
Enterobacter cloacae	3 (100)	0	0	0	NA	NA	NA	NA	NA
Escherichia coli	43 (100)	0	0	43	NA	NA	NA	NA	NA
Gram-negative rod	2 (100)	0	0	0	NA	NA	NA	NA	NA
Klebsiella oxytoca	5 (100)	0	0	0	NA	NA	NA	NA	NA
Klebsiella pneumonia	7 (100)	0	0	0	NA	NA	NA	NA	NA
Moraxella catarrhalis	1 (100)	0	0	0	NA	NA	NA	NA	NA
Morganella morganii	1 (100)	0	0	0	NA	NA	NA	NA	NA
Neisseria meningitides	1 (100)	0	0	0	NA	NA	NA	NA	NA
Prevotella buccae	1 (100)	0	0	0	NA	NA	NA	NA	NA
Proteus mirabilis	2 (100)	0	0	0	NA	NA	NA	NA	NA
Pseudomonas aeruginosa	10 (100)	10	10	0	NA	NA	NA	NA	NA
Pseudomonas oryzihabitans	1 (100)	1	0	0	NA	NA	NA	NA	NA
Serratia marcescens	4 (100)	0	0	0	NA	NA	NA	NA	NA

Table 2. *Continued*

	Result of blood culture (N (%))[a]								
Pathogen	UNI	PSEU	PSEUAE	ECOLI	STAPH	STAU	ENTE	STREPT	STREPN
Gram-positive pathogens									
Aerococcus viridians	1 (100)	NA	NA	NA	0	0	1	0	0
Coagulase-negative *Staphylococcus* spp.	85 (100)	NA	NA	NA	85	9	0	0	0
Corynebacterium spp.	2 (100)	NA	NA	NA	0	0	0	0	0
Enterococcus avium	1 (100)	NA	NA	NA	0	0	1	0	0
Enterococcus faecalis	3 (100)	NA	NA	NA	0	0	3	0	0
Enterococcus faecium	4 (100)	NA	NA	NA	0	0	4	0	0
Lactobacillus spp.	1 (100)	NA	NA	NA	0	0	0	0	0
Propionibacterium acnes	1 (100)	NA	NA	NA	0	0	0	0	0
Staphylococcus aureus	31 (100)	NA	NA	NA	31	31	0	0	0
Streptococcus agalactiae	1 (100)	NA	NA	NA	0	0	0	1	0
Streptococcus group A and/or D	2 (100)	NA	NA	NA	0	0	0	2	0
Streptococcus milleri	3 (100)	NA	NA	NA	0	0	0	3	0
Streptococcus oralis	1 (100)	NA	NA	NA	0	0	0	1	0
Streptococcus pneumoniae	6 (100)	NA	NA	NA	0	0	0	6	6
Streptococcus pyogenes	1 (100)	NA	NA	NA	0	0	0	1	0
Streptococcus sanguis	1 (100)	NA	NA	NA	0	0	0	1	0
Mixed									
Alcaligenes faecalis + *Propionibacterium* spp.	1 (100)	0	0	0	0	0	0	0	0
Klebsiella oxytoca + *Serratia marcescens*	1 (100)	0	0	0	NA	NA	NA	NA	NA
Streptococcus salivarius + *Streptococcus viridans*	1 (100)	NA	NA	NA	0	0	0	1	0
Totals									
Concordant		11	10	43	116	31	8	16	6
Discordant		0	0	0	0	9	1	0	0

[a] UNI, universal probe; PSEU, *Pseudomonas* spp.; PSEUAE, *P. aeruginosa*; ECOLI, *E. coli*; STAPH, *Staphylococcus* spp.; STAU, *S. aureus*; ENTE, *Enterococcus* spp.; STREPT, *Streptococcus* spp.; STREPN, *S. pneumoniae*; NA, not applicable.

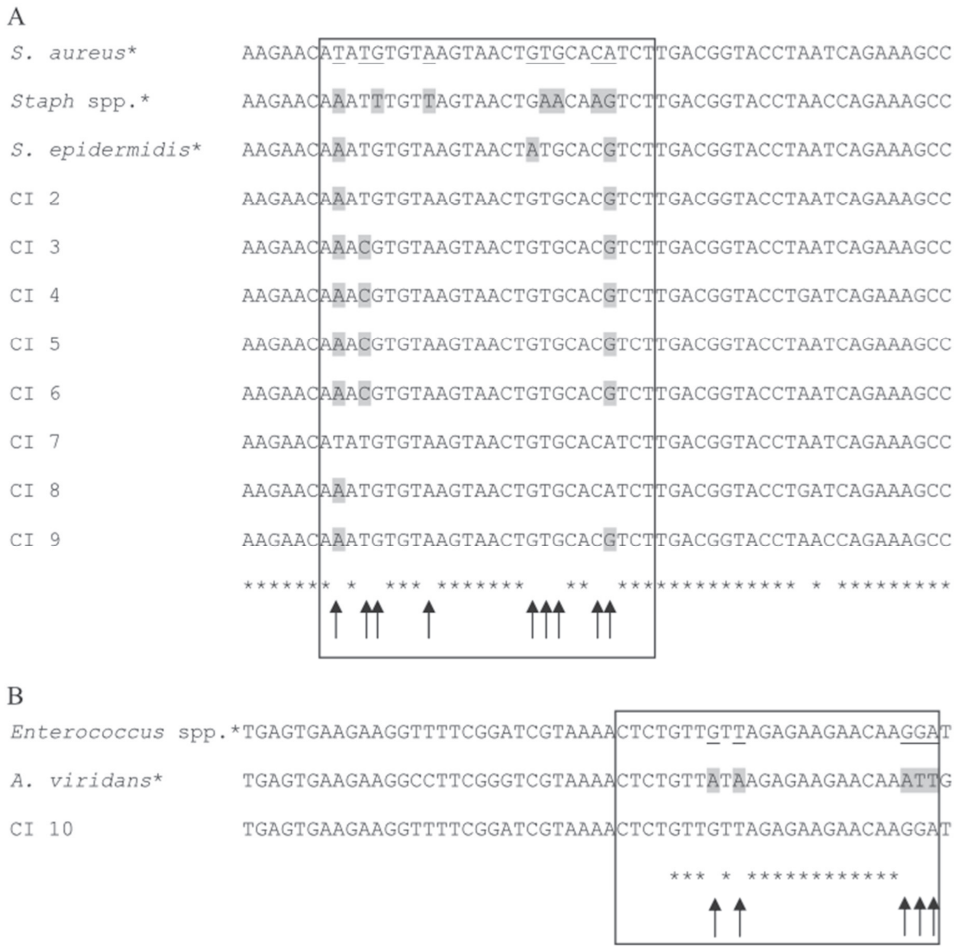

Figure 2. Partial sequence alignments of the 16S rRNA gene, derived from the clinical isolates with discordant results. (A) Region where the probe specific for *S. aureus* binds. The sequences of the eight discrepancies are shown in the rectangle. The nucleotides underlined in the rectangle represent the nucleotides which distinguish *S. aureus* from *Staphylococcus* (*Staph*) spp. The sequence alignment of clinical isolate (CI) 7 is the exact reverse complement of the *S. aureus* probe sequence, which implicates that this isolate, which was identified as CoNS, is in fact *S. aureus*. CI 1 was not shown because the sequence could not be determined. GenBank accession numbers of reference strains are as follows: *S. aureus*, FN433596; *Staphylococcus* spp., GQ222246; and *S. epidermidis*, GQ911565. (B) Region where the probe specific for *Enterococcus* spp. binds. The nucleotides underlined in the rectangle represent the nucleotides which distinguish *Enterococcus* spp. from *A. viridans*. Conventional testing identified CI 10 as *Aerococcus viridans*, whereas our assay and sequencing classified it as an *Enterococcus* sp. GenBank accession numbers of reference strains are as follows: *Enterococcus* spp., AB489105; *A. viridans*, M58797.

* reference sequences.

Table 3. Clinical isolates with discordant blood culture and PCR results

	Result of blood culture[a]			
CI	**Specific probe(s)**	***femA/sa442***	**Coagulase activity**	**Sequence**
1	STAPH, STAU	N/N	N	Unidentified
2	STAPH, STAU	N/N	N	*Staphylococcus hominis*
3	STAPH, STAU	N/N	N	*S. hominis*
4	STAPH, STAU	N/N	N	*Staphylococcus pasteuri*
5	STAPH, STAU	N/N	N	*S. hominis*
6	STAPH, STAU	N/N	N	*Staphylococcus schleiferi*
7	STAPH, STAU	P/P	P	*S. aureus*
8	STAPH, STAU	N/N	N	*S. pasteuri*
9	STAPH, STAU	N/N	N	*S. epidermidis*
10	ENTE	NA	NA	*Enterococcus* spp.

[a] Clinical isolates (CI) 1 to 9 are CoNS species, while CI 10 is *A. viridans*. STAPH, *Staphylococcus* spp.; STAU, *S. aureus;* ENTE, *Enterococcus* spp; N, negative; P, positive; NA, not applicable.

Discussion

New strategies for the detection and identification of bacterial pathogens in blood are continuously under investigation worldwide. Current diagnostic tools are hampered by several factors, such as a lack of sensitivity, a long time to results, the presence of PCR inhibitory compounds in certain sample materials, and the occurrence of fastidious and nonculturable microorganisms.

Rapid universal 16S rRNA gene-based PCR is often combined with sequence analysis, and a considerable number of commercial assays have been generated and tested recently, i.e., SeptiFast (Roche Diagnostics GmbH, Mannheim, Germany), Prove-it Sepsis (Mobidiag, Helsinki, Finland), and SepsiTest (Molzym GmbH & Co. KG, Bremen, Germany). The latter includes a universal 16S rRNA gene-based PCR assay combined with sequence analysis, while the two other commercial assays use a panel of probes for the detection of a range of bacterial and mycotic pathogens. Virtually all studies conducted with the SeptiFast and SepsiTest assays found similar results, i.e., an overall agreement of 77 to 83%[8,10-15]. Both assays are validated for whole-blood samples and can be completed in 4 to 6 h. Tissari *et al.* performed an observational study comparing conventional culture with Prove-it Sepsis. The assay, based on a microarray platform and performed with blood cultures, had a clinical sensitivity of 94.7% and a specificity of 98.8%[16].

In our study, the 16S rRNA gene was used for the design of eight species- or genus-specific probes. The sequence similarities found in *in silico* analysis (BLAST) were derived mainly from not clinically relevant microorganisms such as *Geobacillus* spp., *Paenibacillus* spp., *L. plantarum*, and *Lactobacillus fermentum*. Remaining similarities were, if available, tested *in vitro* with reference strains. A limited number of microorganisms showed cross-reactivity. For example, streptococcal and staphylococcal probes weakly cross-reacted with *Bacillus cereus*.

In these cases, sequencing determined the correct identification. Additionally, as the assay is always preceded by Gram staining, in most cases, morphology data obtained by Gram staining was in contradiction with these false-positive signals and targeted the samples for confirmatory sequencing.

Our multiprobe assay was designed for use with blood culture material and can be completed within 2 hours. Use of whole-blood samples could significantly improve the turnaround time of our assay. However, since culturing still remains essential to determine the microorganism's antimicrobial profile, we chose to use blood cultures instead of whole blood. In this study, we found an overall agreement of 97% between conventional testing and our multiprobe assay. One of the strengths of our approach is that the assay can be extended by adding more probes for other bacterial pathogens to the identification panel. The grouping of Gram-negative and Gram-positive bacteria was based on the identifications needed for antimicrobial susceptibility testing. The assay was designed to use a universal probe for detection instead of Sybr green, because it is known that Sybr green can generate false-positive signals because of the presence of background human DNA or the formation of primer-dimers.

Analysis of the conflicting results showed that, in some cases, our assay was in accordance with sequencing results. The reference sequence derived from a *Staphylococcus* sp. and a *S. epidermidis* strain showed seven and three mismatches, respectively, with the reference sequence derived from an *S. aureus* strain. The sequences derived from the discrepant clinical isolates only showed three or fewer mismatches with the *S. aureus* sequence. This indicated that within the group of staphylococci, more homologous sequences can cause false positives because of the less efficient binding capacity of the *S. aureus*-specific probe.

Overall, these results showed strong agreement between conventional testing and our novel, real-time PCR assay. Furthermore, this assay significantly reduced the time needed for identification in comparison to that of routine diagnostics. In conclusion, using pathogen-specific probes offers a faster alternative for pathogen detection and could improve the diagnosis of bloodstream infections. The assay will be implemented in a clinical trial investigating the impact of earlier pathogen identification in combination with susceptibility testing with the choice of therapy.

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Chapter 4

Antibiotic susceptibility testing of grown blood cultures by combining culture and real-time polymerase chain reaction is rapid and effective

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Abstract

Background

Early administration of appropriate antibiotic therapy in bacteraemia patients dramatically reduces mortality. A new method for RApid Molecular Antibiotic Susceptibility Testing (RAMAST) that can be applied directly to positive blood cultures was developed and evaluated.

Methods

Growth curves and antibiotic susceptibility of blood culture isolates (*Staphylococcus aureus*, enterococci and (facultative) aerobic Gram-negative rods) were determined by incubating diluted blood cultures with and without antibiotics, followed by a quantitative universal 16S PCR to detect the presence or absence of growth.

Results

Testing 114 positive blood cultures, RAMAST showed an agreement with microbroth dilution of 96.7% for Gram-negative rods, with a minor error (false-susceptibility with a intermediate resistant strain) rate of 1.9%, a major error (false resistance) rate of 0.8% and a very major error (false susceptibility) rate of 0.6%. Agreement for *S. aureus* was 97.9%, with a very major error rate of 2.1%. *Enterococcus* species showed 95.0% agreement, with a major error rate of 5.0%. These agreements are comparable with those of the Phoenix system. Starting from a positive blood culture, the test was completed within 9 hours.

Conclusions

This new rapid method for antibiotic susceptibility testing can potentially provide accurate results for most relevant bacteria commonly isolated from positive blood cultures in less time than routine methods.

Introduction

Invasion of bacteria in the bloodstream (bacteraemia) can result in sepsis. Sepsis occurs in about two percent of all hospitalized patients and in as much as 37.4% of all ICU patients and the incidence is rising[1-4]. Mortality is high, varying from 14 to 57%[5]. Although this percentage has declined during the past decades, due to the rising incidence of sepsis[1], the total number of deaths through sepsis is still rising, making it the tenth leading cause of death in the United States[6].

Early administration of appropriate antibiotics reduces mortality of sepsis dramatically[7-9]. Usually, empiric therapy, consisting of one or more broad-spectrum antibiotics, is started as soon as bacteraemia is suspected. Broad-spectrum antibiotics are active against most bacteria causing bacteraemia and are often used in combination. However, their use favors the selection of antibiotic resistant bacteria[10]. Moreover, the more antibiotics a patient uses, the greater the risk of drug toxicity. Additionally, the empiric antibiotic therapy may not cover the causative micro- organism, especially with the rising incidence of multi-drug resistant bacteria[10]. This is the case in up to 40% of all bacteraemia patients[9,11,12].

Rapid analysis of the antibiotic susceptibility pattern of the causative microorganism in bacteraemia leads to earlier targeting of antibiotic therapy and may be lifesaving[13-15]. A major drawback of the conventional blood culture systems is that once blood cultures are positive, additional one or two overnight incubations are required for identification of the causative micro- organism, antibiotic susceptibility testing and targeting of antibiotic therapy.

A new method combining culture and real-time PCR for antibiotic susceptibility testing within 7 hours was reported by Rolain *et al.*[16]. In that study, reference strains were cultured for several hours in the presence of an antibiotic, after which growth was measured with real-time PCR. The absence or presence of growth indicated susceptibility or resistance to the tested antibiotic.

The aim of this study was to modify the method of Rolain *et al.* in order to develop a new rapid method for RApid Molecular Antibiotic Susceptibility Testing (RAMAST) that can be applied directly on positive blood cultures. The study was conducted in 2 phases: (I) establishment of growth curves of bacteria harvested from positive blood cultures using real-time PCR, to determine the required minimal incubation time for adequate antibiotic susceptibility testing, and (II) antibiotic susceptibility testing of 114 blood culture isolates with RAMAST, to investigate the accuracy of RAMAST in clinical isolates.

Materials and Methods

Ethics statement

All data in this study were analyzed anonymously. Therefore, no consent from the patients was required and the ethics committee did not have to be approached. This is in agreement with the code for proper use of human tissue as formulated by the Dutch Federation of

Medical Scientific Societies and the policy of the Medical Ethics Committee of the Maastricht University Medical Center.

Sample collection

The study was performed in the Department of Medical Microbiology of the Maastricht University Medical Center, a 750- bed referral hospital. Blood cultures were incubated in the Bactec automated blood culture device (Bactec™ 9240, BD Diagnostic Systems, Sparks, MD, USA). Bacterial growth in the bottles was detected through continuous monitoring of the CO_2 level by the Bactec device. Between October 2009 and July 2010, all blood cultures that were signaled positive in the previous 24 hours and contained *Staphylococcus aureus*, *Enterococcus* species or (facultative) aerobe Gram-negative rods (GNRs) were included. Blood cultures with anaerobes, *Streptococcus* species, coagulase-negative staphylococci (CoNS) or with growth of more than one species were excluded from the study.

Establishment of growth curves of bacteria harvested from positive blood cultures

Positive blood cultures (0.5 ml) were diluted 10^{-5} in double concentrated Mueller Hinton II broth (212322, BBL™ Mueller Hinton II Broth (Cation-adjusted), BD Diagnostic Systems). 50 µl of this diluted blood culture was added to each well of a microtiter plate containing 50 µl of antimicrobial solution (Table 1) or sterile demineralised water. The concentrations of the antibiotics were based on the breakpoints for susceptibility according to the CLSI-guideline for microbroth dilution[17]. This plate was incubated for 0, 2, 4, 6 and 8 hours at 37°C. After incubation, the content of the wells was transferred to sterile tubes. These were centrifuged at 16000 x *g* for 5 minutes, after which the supernatant was removed. The remaining pellet of bacteria was suspended in demineralised water and stored at 4°C until PCR was performed.
The real-time PCR assay used in this study was described previously[18]. In short, the PCR-reaction mix included two universal 16S rRNA gene primers (5'-TGGAGAGTTTGAT-CCTGGCTCAG-3' and 5'-TACCGCGGCTGCTGGCAC-3'), iQ SYBR Green Supermix (Bio-Rad Laboratories BV, Veenendaal, the Netherlands), water and bacterial isolate. The MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories BV) was used for amplification and melt curve analysis. The threshold was calculated automatically.
The threshold cycle value (Ct-value) was plotted against time to establish a growth curve, in which a descending line indicates growth.

Assessment of antibiotic susceptibility using RAMAST

After Gram-staining, 5 ml of grown blood culture was aspirated from the blood culture bottle and the aspirate was injected in a Serum Separator Tube (SST) (BD). This tube was centrifuged at 2000 x *g* for 10 minutes, after which the supernatant was discarded. Bacteria were harvested from the gel layer using a sterile cotton swab and suspended in 0.9% saline until a

0.5 McFarland standard suspension was obtained. This suspension was diluted in double-concentrated Mueller-Hinton II broth to an inoculum of $5x10^5$ cfu/mL. This dilution was incubated in a microtiter plate with and without antibiotics (Table 1) for 6 hours at 37°C.

Table 1. Tested antibiotics[a] and concentrations[b].

Gram-positive panel	Gram-negative panel
AMX 0.25 mg/l[S]	AMX 8 mg/l[Eb]
AMX 8 mg/l[Ec]	AMC 8/4 mg/l[Eb]
OXA 2 mg/l[S]	PIP 16 mg/l[N]
VAN 2 mg/l[S]	PIP 64 mg/l[P]
VAN 4 mg/l[Ec]	TZP 16/4 mg/l[Eb,N]
GEN 4 mg/l[S]	CIP 1 mg/l[Eb,N,P]
STX 2/38 mg/l[S]	CAZ 1 mg/l[Eb,N]
	CAZ 8 mg/l[Eb,N,P]
	GEN 4 mg/l[Eb,N,P]
	STX 2/38 mg/l[Eb,N,P]
Mixture of antibiotics[A] containing: VAN 4 mg/l, PIP 64 mg/l, CIP 1 mg/l, GEN 4 mg/l	
Growth control (water)[A]	

[a] AMX, amoxicillin; OXA, oxacillin; VAN, vancomycin; GEN, gentamicin; STX, sulfamethoxazole/trimethoprim; AMC, amoxicillin/clavulanate; PIP, piperacillin; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; CAZ, ceftazidime;
[b] These concentrations were based upon the CLSI breakpoints for susceptibility.
[S] For *Staphylococcus aureus*.
[Ec] For *Enterococcus* species.
[Eb] For Enterobacteriaceae.
[N] For non-fermenters.
[P] For *Pseudomonas aeruginosa*.
[A] For all strains.

After incubation, the content of each well was processed for PCR as described above, as well as a sample of diluted bacterial suspension that was not incubated at 37°C but had instead been stored at 4°C. To prevent inhibition of the PCR-reaction due to high loads of bacteria, all samples were diluted 10 times in demineralized water.

To determine antibiotic susceptibility, cut-off Ct-values were calculated. These cut-off Ct-values were chosen from initial growth curve experiments to obtain optimal agreement for each drug-organism combination (data not shown). They represent the mean of the Ct-value of the positive and negative growth control. The positive growth control was the sample that was incubated without antibiotics. For GNRs, the sample incubated with the antibiotic mixture (see table 1) was used as negative growth control. For *S. aureus* and *Enterococcus* spp. the unincubated sample was used as negative growth control.

To calculate the cut-off Ct-values, the following formula were used:

Cut-off Ct-value 1 = Ct-value positive growth control + 0.5 x (Ct- value negative growth control – Ct-value positive growth control)

And:

Cut-off Ct-value 2 = Ct-value positive growth control + 0.25 x (Ct- value negative growth control – Ct-value positive growth control)

For *S. aureus* and enterococci, the two cut-off values were used as follows:
– Cut-off value 1: for vancomycin and gentamicin
– Cut-off value 2: for amoxicillin, oxacillin and trimethoprim/ sulfamethoxazole

For GNRs, the two cut-off values were used for the following antibiotics:
– Cut-off value 1: for amoxicillin, amoxicillin/clavulanate, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole
– Cut-off value 2: for piperacillin, piperacillin/tazobactam and ceftazidim

A Ct-value higher than the cut-off Ct-value indicated susceptibility for the antibiotic. A Ct-value lower than the cut-off value indicated antibiotic resistance.
If a positive blood culture grew less than 1 log (a difference of less than 3,32 Ct between the positive and negative growth control) within 6 hours of incubation, it was excluded from analysis, since the difference between growth and inhibition was too small to reliably determine susceptibility or resistance.

Rapid identification of blood culture isolates

For identification, a portion of the bacterial suspension in 0.9% saline that was used for RAMAST was centrifuged 16000 x *g* for 5 minutes. The supernatant was carefully removed, after which the bacterial pellet was re-suspended in sterile demineralized water. Along with RAMAST, after Gram-staining, rapid identification of the strains was performed using a multiplex 16S DNA based PCR-assay described by Hansen *et al.*[19]. By using genus- and species-specific probes, Gram-negative bacteria were divided into *Pseudomonas aeruginosa* and other Gram-negative species. Within the Gram-positive species, probes were used to identify *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp. and *Streptococcus* spp..

Routine methods for identification and antibiotic susceptibility testing

Routine identification of GNRs was performed by the BD Phoenix Automated Microbiology System (BD Diagnostic Systems), simultaneously with antibiotic susceptibility testing. For the

identification of *Staphylococcus* species, catalase-positive strains were tested for coagulase and DNAse production. If both tests were positive, the strain was identified as *S. aureus*. *Enterococcus* species were identified using bile esculin and tellur diagnostic tablets (Product no. 40411 and 45041 resp.; Rosco Diagnostica, Taastrup, Denmark) were used, according to manufacturer's guidelines. If both tests were positive, the strain was identified as Enterococcus faecalis, whereas in case of a positive bile esculin test but a negative tellur test, an API 20 Strep test (Biomerieux SA, Marcy l'Etoile, France) was performed to further identify the strain.
For routine antibiotic susceptibility testing, the Phoenix system was used, except or *Pseudomonas aeruginosa*, for which disk diffusion according to CLSI-guidelines was performed[17].

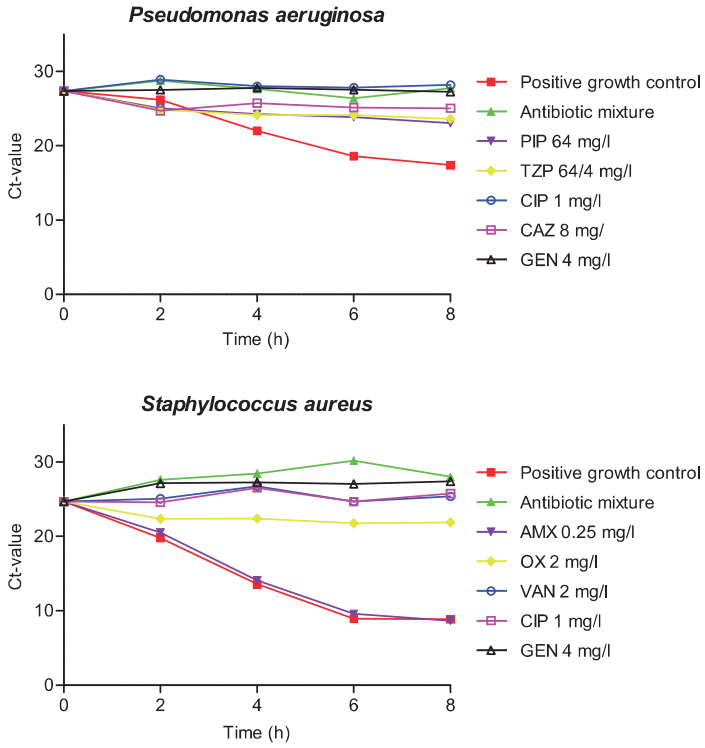
Gold standard for antibiotic susceptibility testing

Results of antibiotic susceptibility testing with the new method were analyzed anonymously for categorical agreement with the results of microbroth dilution according to CLSI-guidelines, which was used as the gold standard[17]. Errors were defined as minor, major or very major. A major error represented a false resistant result, and a very major error was defined as a false susceptible result, as described previously[20]. When RAMAST showed a susceptible result where the gold standard showed an intermediate resistant result, this was a minor error.

Results

Phase I: Growth curves of bacteria from positive blood cultures

First, real-time PCR was used to determine the kinetics of growth and the optimal incubation time for Gram-positive and Gram-negative bacteria, with and without antibiotics. Two representative examples of growth curves of Gram-positive cocci and Gram-negative rods established by RApid Molecular Antibiotic Susceptibility Testing (RAMAST) are shown in Figure 1. The growth curves showed that blood culture isolates require 6 hours of incubation before sufficient growth has occurred for reliable antibiotic susceptibility testing with RAMAST.



AMX, amoxicillin; OXA, oxacillin; VAN, vancomycin; CIP, ciprofloxacin; GEN, gentamicin; AMC, amoxicillin/clavulanic acid; PIP, piperacillin; TZP, piperacillin/tazobactam; CAZ, ceftazidim.

Figure 1. Growth kinetics of *P. aeruginosa* and *S. aureus* from blood cultures with and without antibiotics.

Phase II: Susceptibility testing on positive blood cultures with RAMAST

A total of 114 blood cultures met the inclusion criteria and were tested for antibiotic susceptibility with the new method for RAMAST: 85 GNRs, 19 *S. aureus* and 10 *Enterococcus* spp. (Table 2). Two blood cultures had to be excluded due to insufficient growth within the 6 hours incubation period. These were one *Stenotrophomonas maltophilia* strain and one non-fermenting strain that could not be further identified. In total, 836 antibiotic-isolate combinations were tested.

Table 2. Tested isolates.

Species	N
Gram-positive cocci	**29**
Staphylococcus aureus	19
Enterococcus faecium	7
Enterococcus faecalis	3
Gram-negative rods	**85**
Escherichia coli	49
Pseudomonas aeruginosa	11
Enterobacter cloacae	7
Klebsiella pneumoniae species *pneumoniae*	7
Klebsiella oxytoca	2
Proteus mirabilis	2
Serratia marcescens	1
Proteus vulgaris	1
Klebsiella species	1
Salmonella typhimurium	1
Morganella morganii	1
Aeromonas hydrophila	1

Categorical agreement of RAMAST for *S. aureus* was 97.9%. Both errors in this group occurred with amoxicillin. All other tested antibiotics showed an agreement of 100% Table 3). In the group of *Enterococcus* spp., one major error was found, resulting in an agreement of 95%.
Gram-negative rods showed an agreement of 96.7%, with a minor error rate of 1.9%, a major error rate of 0.8% and a very major error rate of 0.6%. The majority of errors in this group (N = 11) occurred with piperacillin and all but one of these errors occurred in *Escherichia coli* strains, one error occurred with an *Enterobacter cloacae* strain. All other antibiotics showed an agreement of >93% (Table 3).
In this study, the routine methods (Phoenix system and disk diffusion) were shown to have an agreement with the gold standard of 96.8% for *S. aureus*, 95.0% for *Enterococcus* spp. and 97.4% for GNRs.

Table 3. Agreement and errors in the tested population, per antibiotic.

Antibiotic	% Susceptible	% Agreement	No. minor errors	No. major errors	No. very major errors
Staphylococcus aureus (N = 19)					
Amoxicillin	36.8%	89.5%	0	0	2
Gentamicin	100%	100%	0	0	0
Oxacillin	100%	100%	0	0	0
Trimethoprim/sulfamethoxazole	100%	100%	0	0	0
Vancomycin	100%	100%	0	0	0
Enterococcus spp (N = 10)					
Amoxicillin	30%	95.0%	0	1	0
Vancomycin	100%	100%	0	0	0
Gram-negative rods (N = 85)					
Amoxicillin	40.0%	97.3%	0	2	0
Amoxicillin/clavulanate	70.7%	93.3%	4	0	1
Ceftazidime	91.9%	98.8%	0	1	1
Ciprofloxacin	89.4%	98.8%	1	0	0
Gentamicin	94.1%	100%	0	0	0
Piperacillin	87.1%	87.1%	9	0	2
Piperacillin/tazobactam	97.3%	100%	0	0	0
Trimethoprim/sulfamethoxazole	71.8%	96.5%	0	3	0

Discussion

Here we describe a new method, RAMAST, for antibiotic susceptibility testing directly on positive blood cultures combining culture and real-time PCR. An overall agreement of >95% was shown for *S. aureus*, *Enterococcus* spp. and GNRs.

For *S. aureus*, all antibiotics showed a 100% agreement, except for two errors for amoxicillin. This is comparable with the results of the Phoenix system, which also showed errors for amoxicillin in *S. aureus* in this study.

In GNRs, the majority of antibiotics showed an agreement of >93%. Only piperacillin showed a high percentage of errors, which was also found in other methods for antibiotic susceptibility testing[21-23]. All but one of these errors occurred in amoxicillin-resistant *E. coli* strains, for which piperacillin would never be considered an appropriate treatment, so in clinical practice, these errors would not result in an inappropriate treatment. Nevertheless, although piperacillin would thus not be appropriate treatment of amoxicillin resistant *E. coli*, RAMAST results for piperacillin should not be reported in clinical practice.

The overall agreement of RAMAST with results of microbroth dilution was comparable to the agreement we found for the results of routinely used methods compared to microbroth dilution, which in this study was 96.8% for *S. aureus*, 95.0% for *Enterococcus* spp. and 97.4% for GNRs. The results of RAMAST also meet the criteria for selecting an antibiotic susceptibility system proposed by Jorgensen *et al*.[20].

RAMAST is based on a method published by Rolain *et al.*[16], which combined culture and PCR for antibiotic susceptibility testing for a selection of ATCC reference strains. However, the method described by Rolain *et al.* was not useful for rapid antibiotic susceptibility testing on positive blood cultures. It used bacteria from agar, thus requiring an overnight subculture of the blood culture. Instead we used SSTs to harvest bacteria directly from positive blood cultures, which was described previously in other systems for antibiotic susceptibility testing[24-29]. We found that these bacteria require 6 hours of incubation time for reliable antibiotic susceptibility testing. Rolain *et al.* used bacteria from a fresh culture of reference strains on agar, which required only 2-4 hours of incubation time. Bacteria from positive blood cultures may be in a stationary state because nutrients in the blood culture broth are depleted due to the high bacterial load[30]. This might explain why bacteria harvested directly from positive blood cultures require more incubation time for sufficient growth. In contrast to Rolain *et al.*, the PCR-assay that was used for RAMAST did not require an extensive DNA-isolation[18]. Instead, washing the incubated diluted blood culture once was sufficient, saving time and money. This method was also used for the identification PCR-assay and may also prove useful for other PCR-assays on positive blood cultures. Another advantage of this universal 16S rRNA PCR-assay is that, in contrast to the genus- or species specific PCR-assays used by Rolain *et al.*, it can be used for all bacteria, which further simplified the method. Time required for antibiotic susceptibility testing using RAMAST could therefore be reduced to only 9 hours, starting from a positive blood culture.

Turnaround time of many systems for antibiotic susceptibility testing, like broth microdilution[24,25], or automated systems like the BD Phoenix system[26] or Vitek 2[27,28] can also be reduced with one day by the use of SSTs. Many of these direct methods however showed disappointing results for Gram-positive cocci (GPCs)[28,31] or they were not tested at all[26,32]. Our method can be applied to the majority of clinically relevant GPCs and (facultative) aerobic GNRs. It allows for accurate antibiotic susceptibility testing within 9 hours, which for most strains is more rapid than with these direct methods. This makes the test especially useful for laboratories with extended opening hours or 24-hour laboratories.

Many studies have shown that starting early with appropriate empirical therapy leads to a better prognosis for the patient[7-9]. More rapid identification and antibiotic susceptibility testing on positive blood cultures can reduce the time that inadequate antibiotic therapy is administered[13-15,33,34]. In this study RAMAST was combined with the rapid identification method described by Hansen *et al.*[19], using a multiplex PCR-assay that required only 3 hours to perform. Alternatively, other rapid methods for identification can be used in combination with RAMAST; for example matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry[35] or Light-Cycler SeptiFast Test MGRADE (Roche).

In conclusion, our study shows that RAMAST can potentially provide accurate results for antibiotic susceptibility testing for the majority of clinically relevant blood culture isolates. Since the procedure can be applied directly on positive blood cultures and can be completed within 9 hours, results are available in less time than other available methods for antibiotic susceptibility testing.

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Chapter 5

Impact of same-day antibiotic susceptibility testing on time to appropriate antibiotic treatment of patients with bacteraemia: a randomised controlled trial

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Abstract

Background

Inadequate therapy in bloodstream infections is suggested to be associated with higher mortality. We evaluated the reduction in inappropriate antibiotic therapy using rapid identification and antibiotic susceptibility testing (FAST) compared to standard of care (SOC) testing in patients with bloodstream infections in a randomised controlled clinical trial.

Methods

The FAST method used PCR for identification and to detect growth in the presence or absence of antibiotics after only 6 hours. For SOC testing, the BD Phoenix system was used. Patients with blood cultures growing *Staphylococcus*, *Streptococcus* or *Enterococcus* species or Gram-negative rods were randomised for FAST or SOC tests.

Results

129 patients were randomised for FAST and 121 for the SOC group. At the time SOC results became available, 78 patients in the FAST group could have been switched to more appropriate therapy. Although FAST results were highly accurate (agreement with SOC was 94%), they were only implemented in a minority (16) of patients. However, significantly fewer patients in the FAST group used inappropriate therapy at the time of SOC results (P=0.025). Time to results in the FAST group was reduced with 15.6 hours (P<0.001). In the patients switched after FAST, this was done after a mean of 42.3 hours compared to 61.4 hours in those switched after SOC tests (P<0.001).

Conclusions

In bacteraemic patients, FAST resulted in significantly more patients using appropriate antibiotic therapy at the time SOC results were available and 15.6 hours earlier than SOC tests. However, implementation of FAST results was not optimal and no benefit on clinical outcome was shown.

Introduction

Inadequate empirical therapy for bacteraemia is suggested to be associated with an increase in mortality rate[1-4]. Empiric antibiotic therapy is not appropriate in up to 40% of bacteraemia patients[2,3,5-8] and methods to decrease this percentage are necessary.
Standard testing of blood cultures requires at least 12-24 hours of incubation before the blood culture signals positive and an additional 24-48 hours for identification (ID) and antibiotic susceptibility testing (AST) of the bacteria. Reduction in total testing time may result in earlier targeting of antibiotic therapy, ultimately resulting in decreased mortality. Additionally, early narrowing of antibiotic therapy will minimise the use of broad-spectrum antibiotics and selection of antibiotic resistant bacteria[9].
However, use of more rapid ID and AST in various bacterial infections yielded varying results with regard to mortality, morbidity and antibiotic use[8,10-13]. This may be caused by inclusion of different study populations, different countries with different antimicrobial resistance levels and by a difference in methods used for rapid ID and AST. No clear conclusion can yet be drawn from these studies for patients with bacteraemia.
Recently, we developed a new protocol for same-day AST, called FAST[14]. This method uses highly sensitive PCR to detect bacterial growth in the presence of antibiotics after only 6 hours. Results are thus based on culture-based antibiotic susceptibility profiles and were shown to be as reliable as most commonly used automated AST systems. Here, we present a randomised clinical trial assessing the effects of FAST for patients with bloodstream infections. Primary endpoint was reduction in the number of patients receiving inappropriate antibiotics at the time results of standard-of-care results became available.

Materials and methods

Setting

The study was conducted in the Maastricht University Medical Center, a 750-bed referral hospital. The department of medical microbiology has an integrated laboratory and an active consultation service of medical microbiologists and infectious disease specialists that is operational 24h a day. The study was approved by the hospitals' Medical Ethics Committee and registered in the Nederlands Trialregister (NTR2043).

Patient population

Patients were eligible for inclusion if the blood culture grew Gram-positive cocci (GPCs) or (facultative) aerobic Gram-negative rods (GNRs). Exclusion criteria were a positive blood culture in the previous 3 days, age <18 years, and blood cultures that contained anaerobic micro-organisms or more than one micro-organism. Patients were included after written informed consent.

To avoid confounding, patients with a community acquired bacteraemia or nosocomial bacteraemia, as defined by CDC-criteria[15] were randomised separately in blocks of 4 patients for either FAST or for the standard of care (SOC) according to a computer-generated list, which was not accessible to the researchers. Randomisation was performed by drawing a sealed envelope. Per patient, only one blood culture was included.
Due to the shorter turnaround time of the FAST ID and AST, medical microbiologists could not be blinded for randomisation of the patient. Patients were unaware of the group they were randomised for.

Standard of care (SOC)

Blood cultures bottles were incubated and automatically monitored for microbial growth in the Bactec™ 9240 instrument (BD Diagnostic Systems, Sparks, MD, USA). Results of Gram-staining after growth of the blood culture were reported to the patients' attending physician by telephone as soon as the results were available.
After sub-culturing, the BD Phoenix Automated Microbiology System (BD) was inoculated for ID and AST of GNRs, as well as for AST of *Staphylococcus* and *Enterococcus* species.
For ID of *Staphylococcus* species strains were tested for coagulase and DNAse production. ID of *Enterococcus* species was done with bile esculin and tellur diagnostic tablets (Product no. 40411 and 45041 resp.; Rosco Diagnostica, Taastrup, Denmark) and an API 20 Strep test (Biomérieux SA, Marcy l'Etoile, France). *Streptococcus pneumoniae* was identified using optochin susceptibility (OXOID, Hampshire, United Kingdom). Results of ID and/or AST were reported to the medical microbiologist as soon as results were available. The medical microbiologist then advised by telephone the optimal antibiotic therapy to the attending physician based on ID or AST, whichever result was first available, in the majority of patients within 1 hour of obtaining such results.

Fast antibiotic susceptibility testing (FAST) for ID and AST

FAST ID was performed with a multiplex PCR-assay directly on the positive blood culture as described previously[16].
Streptococcus species, *Streptococcus pneumoniae*, *Enterococcus* species, *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), *Pseudomonas aeruginosa*, *Pseudomonas* species and *Escherichia coli* were identified combining the PCR results with Gram-staining.
Simultaneously with the ID multiprobe-assay, a PCR was performed to test for the presence of the *mecA* gene in *Staphylococcus* spp.[17].
For AST, a semi-molecular method called FAST, previously described by our group[14] was used for *S. aureus*, *Enterococcus* species and all GNRs, but not for streptococci, since they require different culture conditions.
All results were available the same day the blood culture signalled positive[14,16,17]. Results were then reported to the medical microbiologist who subsequently advised the optimal antibiotic

therapy to the attending physician by telephone. For patients in the FAST testing group, the SOC testing method for ID and AST was performed simultaneously.

Data collection and definitions

Primary outcome was reduction in the number of patients receiving inappropriate antibiotics at the time results of standard-of-care results became available. Inappropriate therapy was defined as inadequate or unnecessarily broad-spectrum antibiotic therapy. Inadequate antibiotic therapy was defined as not covering the cultured microorganism based on the in vitro susceptibility data or no antibiotic therapy at all when antibiotic therapy was required. Patients receiving multiple antibiotics when only one antibiotic was required, or a broad-spectrum penicillin or cephalosporin which, based on in vitro susceptibility data, could be replaced by an antibiotic with a more narrow spectrum were characterised as receiving unnecessarily broad-spectrum antibiotic therapy. Antibiotic switch was defined as use of a different antibiotic. Contamination was defined according to CDC criteria[15]. Definitions were used as judged by 2 authors (JB and AV).
Secondary outcome parameters were: the implementation of the PCR-results by medical microbiologists and attending physicians, reduction in time in hours that inappropriate antibiotics were used, mortality and duration of hospital stay.
The following data were collected from the electronic hospital information system: age, sex, duration and department of hospital stay, mortality data, microbiological culture results, underlying diseases, use of immunosuppressive drugs, as well as start and stop dates of all antibacterial agents. For classification of the severity of disease at the time of blood culture drawing, the Pitt bacteraemia score was calculated[18].

Statistics

Before start of the study, the percentage of patients with bacteraemia receiving inadequate or too broad-spectrum empirical antibiotic therapy was estimated to be 10%. We hypothesized that the novel PCR-based method would provide appropriate antibiotic therapy faster than the conventional method in 5% of the patients with bacteraemia. Power analysis showed that 340 patients would have to be included (power of 80% and α=0.1).
However, interim analysis showed that approximately 35% of all patients received inadequate or too broad-spectrum therapy before results of routine ID and AST became available. In the FAST group, this percentage was reduced to 20%. With these data, power calculation showed that 218 patients were required (power of 80% and α=0.1).
Baseline characteristics, focus of infection and causative microorganisms were analysed using chi-square tests and t-tests. Time to diagnosis and appropriate therapy were compared using t-tests. To compare the number of antibiotic switches and mortality, chi-square tests were used. Duration of hospital stay was compared using the Mann-Whitney U test. Differences between groups with a two-sided P-value <0.05 were considered to be statistically significant.

Results

Patient characteristics

Between October 2009 and May 2011, 250 patients were randomised for the FAST group (n=129) or the SOC group (n=121) (Figure 1). Baseline characteristics were not different between the two groups (Table 1), except for solid malignancies, which were significantly more prevalent in the FAST group (P=0.018).

About half of the infections (51.2%) was hospital-acquired. Urinary tract infections (18.0%) and infection of intravenous catheters (18.4%) were the most common source of bacteraemia. In 37 patients (14.8%), no focus for bacteraemia could be identified.

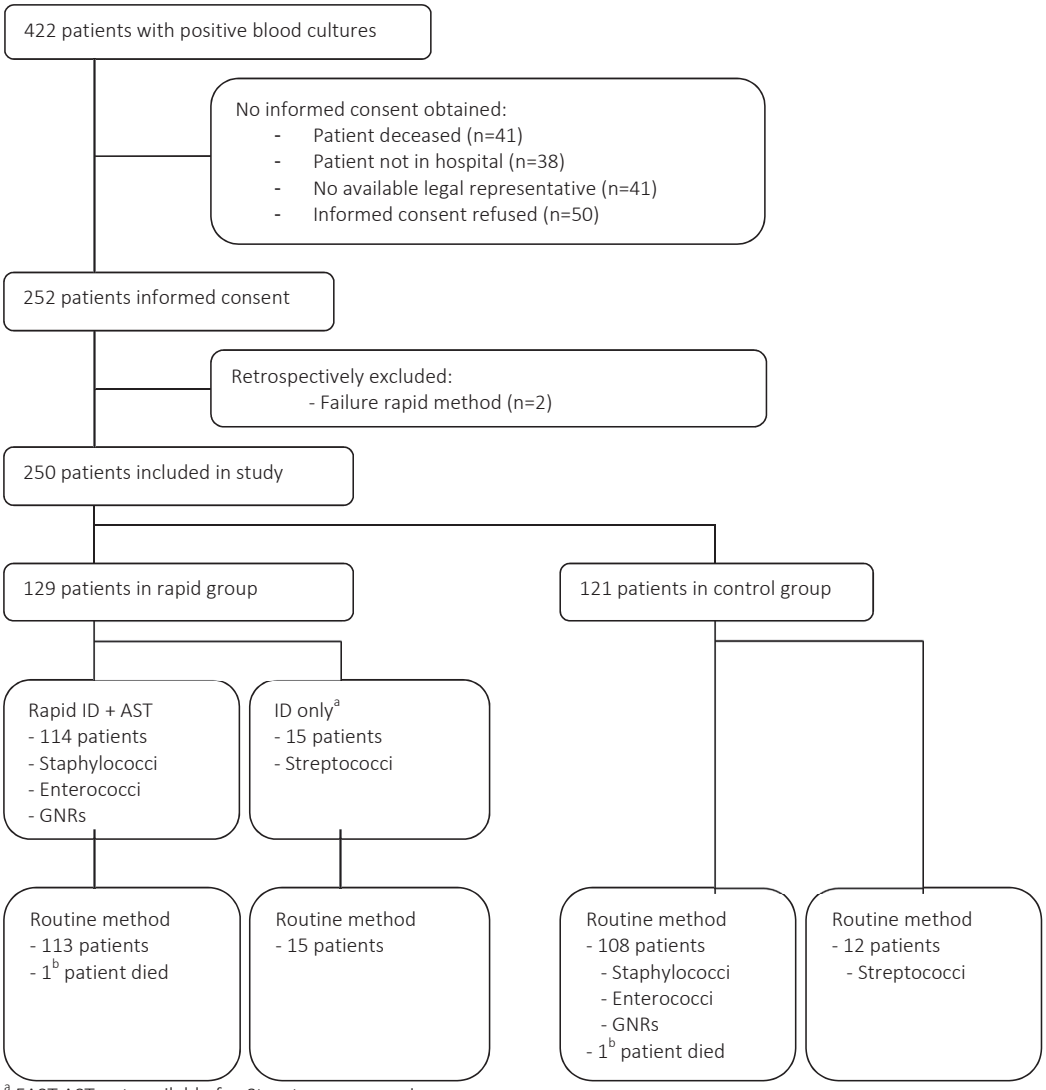

Figure 1. Flow chart of included patients

Table 1. Baseline characteristics of patients in the rapid and control group

	FAST (n=129)	SOC (n=121)
Age in years (SD)	63.3 (15.1)	63.8 (15.6)
Male sex (%)	76 (58.9%)	78 (64.5%)
Ward (%)		
Surgery	17 (13.1)	16 (13.2)
Medicine	61 (47.3)	64 (52.9)
ICU	13 (10.1)	13 (10.7)
Cardiology and thoracic surgery	13 (10.1)	9 (7.4)
Urology	4 (3.1)	5 (4.1)
Pulmonology	13 (10.1)	8 (6.6)
Others[a]	8 (6.2)	6 (5.0)
Infection (%)		
Community-acquired	65 (50.4)	57 (47.1)
Nosocomial	64 (49.6)	64 (52.9)
Pitt bactaeremia score ≥4[b] (%)	11 (8.7)[c]	14 (11.7)[c]
Source of bacteraemia		
Intravenous catheter	27	19
Respiratory tract	9	6
Urinary tract	24	21
Intra-abdominal	6	11
Biliary tract	7	7
Cardiac valves	2	3
Skin or mucous surface	5	7
Prosthetic devices	5	3
Other	3	1
Unknown	19	18
Contamination	22	25
Isolated microorganisms		
Coagulase-negative staphylococci	34	37
Staphylococcus aureus[d]	19	14
Enterococcus species[e]	9	5
Streptococcus pneumoniae	9	2
Other Streptococcus species	6	10
Enterobacteriaceae[f]	50	46
Pseudomonas aeruginosa	2	3
Other Gram-negative rods	0	4

[a] Neurology (n=5), obstetrics (n=4), orthopaedics (n=4), otorhinolaryngology (n=1)
[b] A score of ≥4 is associated with an increased risk of mortality [18]
[c] No data available for 4 patients (1 in FAST group, 3 in SOC group)
[d] No MRSA
[e] No VRE
[f] Of which 4 ESBL-positive strains: 3 Escherichia coli and 1 Klebsiella pneumoniae

Performance of FAST for ID and AST

Overall, FAST ID had a >99% agreement with the SOC ID. The FAST method for ID was inconclusive in 2 patients, 1 *Streptococcus* spp. and 1 CoNS, and since no FAST result could be reported, these patients were not included in the analyses (Figure 1).
Results of the FAST AST in 114 cultures (Figure 1) had an overall agreement of 94% with SOC AST; 100% (53/53) in staphylococci, 78% (7/9) in enterococci and 90% (47/52) in Gram-negative rods.
Starting from blood culture drawing, mean time to definite ID and AST was 50.7 hours (SD 21.0) in the FAST group and 66.3 hours (SD 20.1) in the SOC group (Table 2), with a difference between the means of 15,6 hours in favour of the FAST testing group (P<0.001). Mean time-to-results was significantly longer for contaminated blood cultures (66.7h) than for other blood cultures (55.9h) (P=0.01)

Table 2. Outcome parameters in FAST versus SOC group, in patients in which both ID and AST were performed

	FAST (N=114)	SOC (N=109)	P
Mean time to Gram stain (SD)[a]	41.5 (21.0)	42.5 (20.3)	0.712
Mean time to results, in hours (SD)[a]	50.7h (21.0)	66.3h (20.1)	<0.001
Mean time to appropriate therapy, in hours (SD)[a]	28.2h (32.5)	26.9h (30.1)	0.962
Patients switched after FAST AST (N=12)	42.3h (13.3)		<0.001
Patients switched after SOC AST (N=34)		61.4h (14.7)	
Length of hospital stay, in days (range)	11d (0-75)	11d (1-133)	0.820
Patients switched after FAST AST (N=12)	13d (7-43)		0.486
Patients switched after SOC AST (N=34)		11d (1-133)	
Mortality	12.3%	7.3%	0.216
Patients switched after FAST AST (N=12)	8.3%		0.959
Patients switched after SOC AST (N=34)		8.8%	

[a] Starting from blood culture drawing
[b] Empiric therapy was appropriate in 64 patients of the FAST group and 57 of the SOC group resulting in a low mean time to appropriate therapy

Implementation of FAST results

Overall, empiric antibiotic therapy was inadequate in 26.0% of all patients. Nosocomial bacteraemia was associated with a higher risk of inadequate therapy than community-acquired bacteraemia (32% versus 20.8%, P=0.061). Examples of inadequate therapy are: bacteraemia with Enterobacteriaceae resistant to broad-spectrum beta-lactam antibiotics (mostly amoxicillin-clavulanic acid, but also piperacillin-tazobactam) which were treated with those antibiotics; bacteraemia with Enterobacteriaceae or *Staphylococcus* species which were not treated until results of the bloodculture became available; and bacteraemia with beta-

lactam resistant *Enterococcus* species which were initially treated with beta-lactam antibiotics.

Antibiotic therapy was inappropriate in 95/129 patients in the FAST group and 114/121 in the SOC group (Table 3A). Examples of inappropriate therapy are: bacteraemia with Enterobacteriaceae for which a too broad-spectrum beta-lactam was used (most commonly amoxicillin-clavulanic acid) that could have been narrowed down to amoxicillin; bacteraemia with *S. aureus* strains that were oxacillin susceptible, but were treated with a broad-spectrum antibiotic, such as amoxicillin-clavulanic acid; bacteraemia with a penicillin susceptible *Streptococcus* species which was treated with a broad-spectrum antibiotic, most commonly amoxicillin-clavulanic acid; bacteraemia with Enterobacteriaceae that were treated with two or more antibiotic drugs while only one antibiotic drug would have sufficed.

Table 3A. Possible switch to appropriate antibiotic treatment using FAST and SOC ID and AST test results

	FAST (N=129)	SOC (N=121)
After Gram stain	17	18
After FAST ID		
CoNS not requiring antibiotic therapy[a]	20	0
Streptococcus pneumoniae	9	0
After FAST AST	49	0
No switch possible with FAST[b]	7[c]	NA
After SOC ID		
CoNS not requiring antibiotic therapy[a]	0	26
After SOC AST		
Streptococcus species	4	12
Other strains	0	58
No switch required		
CoNS not requiring antibiotic therapy[a]	6	1
Streptococcus species	2	0
Other strains	15	6

FAST, fast antibiotic susceptibility testing; SOC, standard of care; ID, identification; AST, antibiotic susceptibility testing; NA, not applicable

[a] Either blood culture contaminantion or central line infection with subsequent catheter removal

[b] Test results of FAST were not concordant with SOC test results

[c] 2 enterococci and 5 Gram-negative rods

After the Gram-stain, antibiotic therapy was switched to appropriate antibiotic therapy in 17 patients in the FAST group (13.2%) and 18 patients in the SOC group (14.9%) (P=0.699). After FAST ID, inappropriate therapy was adjusted in 4 patients: antibiotic therapy was narrowed down in 3 of 9 patients with *S. pneumoniae* bacteraemia and antibiotics were stopped in 1 patient with CoNS deemed to be contamination (Table 3B).

Table 3B. Implementation of switch to appropriate antibiotic treatment using FAST and SOC ID and AST test results

	FAST (N=129)	SOC (N=121)	P
After Gram stain	17	18	0.699
After FAST ID			
CoNS not requiring antibiotic therapy[a]	1	0	0.268
Streptococcus species	3	0	0.100
After FAST AST	12	0	<0.001
After SOC ID/AST	32	46	0.024
CoNS not requiring antibiotic therapy[a]	2	4	0.469
Streptococcus species	7	8	0.299
Other strains	23	34	0.025
No switch implemented	64	57	0.692
CoNS not requiring antibiotic therapy[a]	23	23	0.360
Streptococcus species	5	4	1.000
Other strains	36	30	0.651

FAST, fast antibiotic susceptibility testing; SOC, standard of Care; ID, identification; AST, antibiotic susceptibility testing

[a] Either blood culture contamination or central line infection in which catheter was already removed

Implementation of the FAST AST resulted in a switch to appropriate antibiotic therapy in an additional 12 patients: 6 with *S. aureus* and 6 with Enterobacteriaceae bacteraemia (Table 3B). This resulted in significantly less patients in the FAST group that used inappropriate antibiotic therapy at the time SOC AST became available: 23 (26%) in the FAST group versus 34 (43%) in the SOC group (P=0.025) (Table 3B).

Overall, the mean time that patients received inappropriate antibiotic therapy, starting from blood culture drawing, was 28.2 hours (SD 32.5) in the FAST group and 26.9 hours (SD 26.9) in the SOC group (P=0.962). The mean time to switch to appropriate antibiotic therapy was statistically significantly lower in the 12 patients in whom the FAST AST results were implemented compared to the 57 patients (23 in the FAST group and 34 in the control group) in whom SOC AST tests were used: 42.3 hours (SD 13.3) compared to 65.9 hours (SD 19.7), respectively (P<0.001).

Clinical endpoints

No difference in 30-day in-hospital mortality could be demonstrated: 12.3% in the FAST group and 7.3% in the SOC group (P=0.216) (Table 2). Mortality in patients receiving inadequate empirical therapy for more than 24 hours was 15.0% and for patients receiving adequate empirical therapy 8.0% (P=0.119). Median length of hospital stay (LOS) after inclusion in the study was 13 days (range 7-43) in the FAST group and 11 days (range 1-133) in the SOC group (Table 2, P=0.802). However, patients receiving inadequate empirical antibiotic therapy had a longer LOS than patients receiving adequate empirical therapy (median 9 days (range 0-133) versus 13 days (range 1-75), P=0.03).

Discussion

This study illustrates that a rapid test for identification and susceptibility testing can significantly reduce the time to switch to appropriate antibiotic therapy in patients with bacteraemia. By employing FAST, the time to results of ID and AST was at least 15.6 hours reduced as compared to SOC and same day susceptibility results were available in all patients. Although the results obtained by FAST were highly comparable to SOC with an overall agreement of 94% with SOC, the results of FAST were only used to change therapy in 12 patients. As a result, no difference in secondary clinical endpoints (mortality and length of stay) could be shown. In 1994, a randomised trial comparing a rapid test with SOC by Doern *et al.*[10] showed a reduction in mortality, morbidity and costs in patients with bacterial infections, even though results of rapid ID and AST were available at least 24 hours later than in our study. Five other studies reported on the effect of introduction of rapid ID and AST methods with varying results[8,11-13]. The only study focussing on patients with bloodstream infections[13] showed that more antibiotic switches occurred after the rapid test (direct inoculation in the Vitek system) compared to standard inoculation. This led to a decrease in cost of 158 dollar per patient. However, no effect on reduction in mortality was shown.

There are several factors that may limit antibiotic switching after AST results become available. First and most importantly, it is not always possible to narrow down antibiotic therapy when in vitro susceptibility data indicate a potential switch, for example when a second infection is suspected, or when a source of infection is suspected with multiple microorganisms involved, for example in the case of an intra-abdominal infection or an abscess. Second, in the Netherlands, antibiotic resistance is relatively low[19], thus fewer patients receive inadequate empirical antibiotic therapy than in areas with a high prevalence of antibiotic resistance. Third, the duration of incubation time that is required before a blood culture becomes positive, which was on average 42 hours in this study, is a major drawback for quick adjustment of antibiotic therapy. Implementation of antibiotic targeting has been shown to be directly associated with the time required to obtain results; switching is less likely when time to results is longer[12,20]. Although results of our FAST ID and AST were available on the day the blood culture signalled positive, blood cultures still require at least one day of incubation. Thus, performing ID and AST directly on blood samples without culturing blood would be the ultimate rapid test with the greatest potential effect on clinical outcome. Nonetheless, the previous factors are impacting all methods providing AST results and our study showed that the implementation rate of FAST tests was significantly lower than that of SOC tests, although FAST test results were shown to be reliable with respect to agreement of the tests and number of potential switches. This can likely be explained by the reluctance of the medical microbiologist or attending physicians to change therapy after 2-3 days in patients with improving status on the basis of a newly introduced test[21,22]. A similar result was reported by Oosterheert *et al.*[23], showing that PCR results were rarely implemented for antibiotic switches in respiratory infections, in spite of being more sensitive than the routine method. Since the impact of FAST was hampered by limited usage of the

results we assessed the potential maximum impact of FAST. We investigated which patients could have been switched to appropriate therapy before results of the SOC ID and AST were available, on top of the 12 patients in the trial that were indeed switched after FAST results. In this hypothetical scenario 95/129 patients in the FAST group could have been rapidly switched to appropriate therapy before SOC results would become available as compared to 18/121 of the SOC group (P<0.001) (Table 3A). These findings emphasize the need for implementation research, identifying critical bottlenecks in introduction of new diagnostic technologies to allow maximum impact of the new methods on clinical practise.

In conclusion, we demonstrated that FAST ID and AST in bloodstream infections reduce time to targeting of antibiotic therapy with a mean of at least 15.6 hours compared to SOC tests for a limited set of bacterial species and antibiotics. In addition we showed that introduction of FAST resulted in significantly more patients using appropriate antibiotic therapy at the time SOC results were available. However, implementation of the FAST test results was limited and no clinical benefit could be shown. Studies towards new and rapid ID and AST tests need to focus on implementation by medical microbiologists or infectious disease specialists to obtain insight in the effect of such tests on clinical outcome.

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Chapter 6

The clinical relevance of speciation of coagulase-negative staphylococci from positive blood cultures using MALDI-TOF MS

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Submitted

Abstract

Background

Discrimination between blood culture contamination or bloodstream infection by coagulase-negative staphylococci (CoNS) has not yet been related to CoNS species. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) offers a cheap and reliable method for identification to species level of CoNS. In this study, we investigated the clinical relevance of different CoNS species isolated from positive blood cultures.

Methods

In two Dutch hospitals, CoNS blood culture isolates were retrospectively identified to species level using MALDI-TOF MS. Infection or contamination was defined by CDC-criteria and by clinical diagnosis by clinical microbiology/infectious diseases specialists.
CoNS bloodstream infections were more often diagnosed by clinicians than by using CDC-criteria.

Results

Of 265 strains collected from 245 patients, *Staphylococcus epidermidis* was the most common species (N=182, 68.7%) and the majority of these strains represented contamination (83.5% by CDC-criteria and 59.3% by clinical microbiologists/infectious diseases specialists). *Staphylococcus haemolyticus* strains were most likely to be considered to cause a bloodstream infection (40.0% by CDC-criteria and 70.0% by clinical microbiologists/infectious diseases specialists). In contrast, some species, such as *Staphylococcus warneri*, were always found to be contaminants.

Discussion

This study shows that discrimination between infection and contamination remains difficult. The various CoNS species seem to vary in pathogenicity and speciation of CoNS by MALDI-TOF MS can thus be helpful in the diagnosis of CoNS bloodstream infection.

Introduction

Coagulase-negative staphylococci (CoNS) have long been considered to be blood culture contaminants in the majority of patients, but nowadays CoNS is considered to be a true pathogen in special patient groups or conditions[1,2]. For instance, *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* cause bloodstream infections and endocarditis, and *S. saprophyticus* causes urinary tract infections[3]. However, most cases of CoNS bacteraemia are associated with prosthetic device and intravenous catheter infections[4].

Whether a CoNS from a grown blood culture represents true bacteremia or contamination is based on the number of grown blood culture vials and on clinical data, for which strict CDC-criteria are available (www.cdc.gov/nhsn/pdfs/pscmanual/17pscnosinfdef_current.pdf). However, in clinical practice, treatment of CoNS bacteremia is also frequently started or maintained in patients not meeting these criteria[5]. Until now, it has been unclear whether identification of CoNS species may be helpful in the discrimination between contamination and infection.

Phenotypic methods (Vitek 2, API system) for identification of CoNS to species level are unreliable[6,7]. PCR followed by sequencing is more reliable but time- consuming and expensive. Furthermore, the gene most commonly used for identification of bacteria, the 16S rRNA-gene, does not have sufficient specificity to differentiate between different species of CoNS[7]. Therefore, most laboratories do not further determine the species of CoNS, except in selected cases.

The recent introduction of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) systems, offers a cheap, rapid and reliable method for identification of staphylococci[7-9].

The value of CoNS species identification in discriminating infection from contamination is unclear. Therefore, we retrospectively assessed the clinical relevance of various CoNS species isolated from positive blood cultures.

Methods

Ethics statement

All data and strains in this study were collected retrospectively and anonymised before analysis. No consent from the patients nor the ethics committee was required. This is in agreement with the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies.

Sample collection

Strains were collected in two hospitals: the Erasmus Medical Centre in Rotterdam, The Netherlands, a 1200-bed university hospital, and the Maastricht University Medical Centre, Maastricht, The Netherlands, a 750-bed university hospital.
In Rotterdam, blood culture CoNS strains were collected retrospectively between January and June 2011. In Maastricht, blood culture samples were collected between October 2009 and May 2011. Only the first positive blood culture set of a patient was included. If this blood culture set yielded 2 strains of CoNS, both strains were included in the study. Exclusion criteria were: insufficient clinical data, age <18 years, and blood cultures containing additional non-CoNS strains.

Blood cultures

In both centres, blood cultures bottles were cultured and automatically monitored for microbial growth in the Bactec™ 9240 instrument (BD Diagnostic Systems, Sparks, MD, USA). If growth was detected, blood culture broth was subcultured on Columbia sheep blood agar.
In Maastricht, from this subculture, *Staphylococcus* species were tested for clumping factor and DNAse production. If both tests were negative, the strain was identified as coagulase-negative *Staphylococcus*. In case of discrepancy between both tests, a coagulase test was performed as a gold standard. In Rotterdam, Slidex Staph Plus kit 73116 (Biomérieux SA, Marcy l'Etoile, France) was used to differentiate between coagulase-negative and –positive strains. Slidex positive strains were confirmed as *S. aureus* by the AccuProbe hybridization assay (Gen-Probe, San Diego, CA). All strains were stored at -80^{o}C.

MALDI-TOF MS

Strains stored at -80^{o}C were subcultured twice on Columbia sheep blood agar, after which MALDI-TOF MS analysis was performed in duplicate, as described elsewhere[7]. Scores <1.7 were considered to be unreliable. Scores between 1.7 and 2 were considered to be reliable to the genus level, however, if database match number 1 and 2 resulted in the same strain name, it was considered correct to the species level. Scores ≥2 were considered to be reliable to the species level. Samples that could not be identified directly by MALDI-TOF MS were retested after pretreatment with formic acid and acetonitrile[7].

Data collection

The following clinical data were collected from the patients' electronic medical record: number of blood cultures drawn within 24 hours of drawing of the positive blood culture, number of positive blood cultures, positive cultures from other body sites, presence or absence of fever (>38^{o}C) within 24 hours of blood culture drawing, presence or absence of

hypotension (systolic blood pressure <90 mmHg), other potential foci of fever, and results of consultations of clinical microbiologists and infectious diseases specialists regarding the positive blood culture. The clinical microbiologists and infectious diseases specialists in this study used both laboratory results and clinical data to determine whether or not a blood culture with CoNS represented true bloodstream infection, but no predefined set of criteria was used.

CDC-criteria for laboratory-confirmed bloodstream infection

A patient with a positive blood culture with CoNS was considered to have a laboratory confirmed bloodstream infection if the following criteria were met[10]:
- Fever (>38°C) or chills or hypotension
And:
- Results were not related to an infection at another site
And:
- CoNS cultured from two or more blood cultures drawn on separate occasions

Statistical analysis

For comparison of binary data, Pearson chi-square tests were used, unless the expected count in one of the cells of the contingency table was <5, in which case Fisher's exact tests were used. Differences between groups with a P-value <0.05 were considered to be statistically significant.

Results

Identification of CoNS strains

In total, 304 strains were collected, 172 in a 6 months period in Rotterdam and 132 in a 15 months period in Maastricht. These strains were cultured from 279 blood cultures. Four patients were excluded because their strains failed to grow after storage at -80^{o}C. Of 29 patients, insufficient clinical data were available. One blood culture was excluded because it grew >1 genus (CoNS and *Escherichia coli*). From 18 cultures, more than one species of CoNS was cultured, based on colony morphology: 17 grew 2 species and 1 grew 4 species. Thus, 265 strains (152 from Rotterdam and 113 from Maastricht) from 245 patients were included in the study (Figure 1).

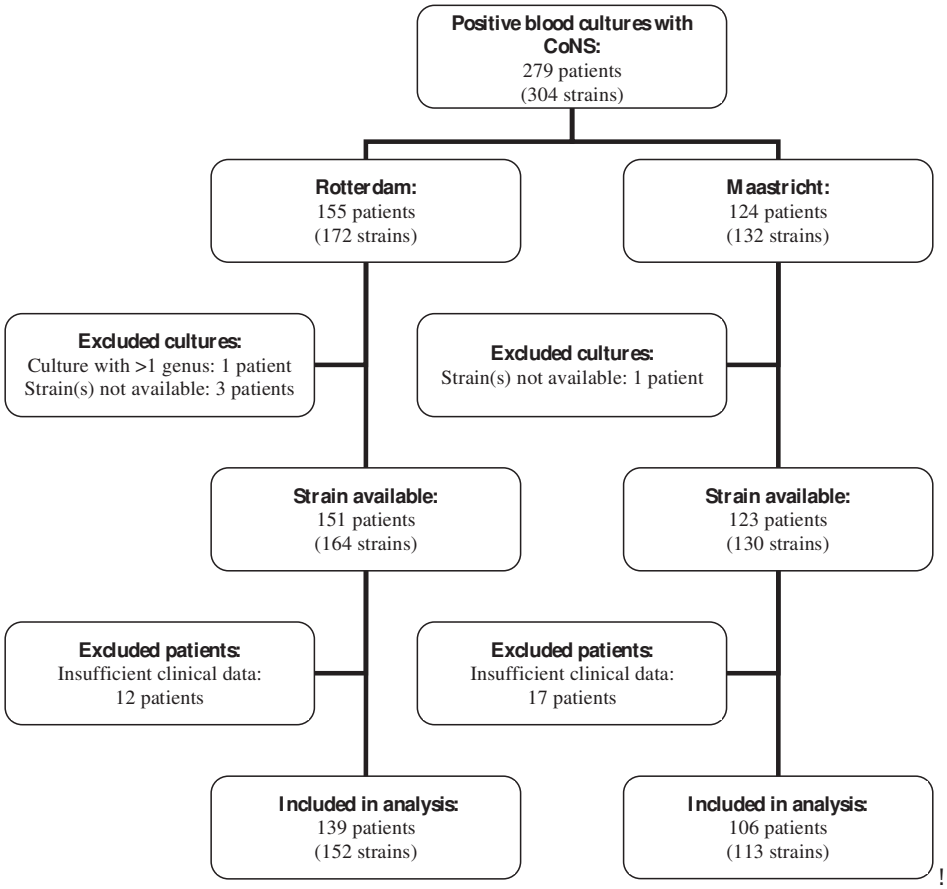

Figure 1. Included patients and strains

The most commonly isolated species was *Staphylococcus epidermidis*, followed by *Staphylococcus hominis*, *Staphylococcus capitis* and *Staphylococcus haemolyticus*. No *S. lugdunensis* or *S. schleiferi* grew from any of the blood cultures (Table 1).
Six strains were identified by MALDI-TOF MS as not being *Staphylococcus* species: two were *Rothia mucilaginosa*, four were *Micrococcus luteus*. One strain could not reliably be identified to species level in four attempts.

Table 1. Results of MALDI-TOF MS

	Total number of strains	Number of strains in Rotterdam*	Number of strains in Maastricht*
S.epidermidis	182 (68,7%)	112 (73,7%)	70 (61,9%)
S.hominis	38 (14,3%)	16 (10,5%)	22 (19,5%)
S.capitis	17 (6,4%)	9 (5,9%)	8 (7,1%)
S.haemolyticus	10 (3,8%)	5 (3,3%)	5 (4,4%)
S.warneri	4 (1,5%)	2 (1,3%)	2 (1,8%)
S.cohnii	2 (0,8%)	1 (0,7%	1 (0,9%)
S.pettenkoferi	2 (0,8%)	1 (0,7%)	1 (0,9%)
S.caprae	1 (0,4%)	1 (0,7%)	0 (0,0%)
S.intermedius	1 (0,4%)	1 (0,7%	0 (0,0%)
S.saprophyticus	1 (0,4%)	1 (0,7%)	0 (0,0%)
Micrococcus luteus	4 (1,5%)	1 (0,7%	3 (2,7%)
Rothia mucilaginosa	2 (0,8%)	1 (0,7%	1 (0,9%)
No ID	1 (0,4%)	1 (0,7%	0 (0,0%)

* Species distribution did not show significant differences between the two centres

Discrepancies between CDC criteria and clinical diagnosis of infection

According to the CDC-criteria, 17.0% of all positive blood cultures with CoNS represented bloodstream infections. A significantly higher percentage (37.0%) of infections was diagnosed by clinical microbiologists and infectious diseases specialists (P<0.001) (Table 2).
Since no gold standard for CoNS infection is available we compared diagnosis by CDC criteria and clinicians in depth. In total, 55 isolates were defined as bloodstream infection by clinicians, but as contaminants by CDC-criteria (Table 2). Of 100 patients with only one set of blood cultures drawn, 22 (22%) were diagnosed as infection by clinical microbiologists and infectious diseases specialists and by definition not by CDC criteria ('CoNS cultured from two or more blood cultures drawn on separate occasions'). In contrast, of 67 patients with 1 positive blood culture bottle out of ≥2 blood culture sets drawn, only 4 (6%) patients were regarded by clinicians to have a CoNS infection. Furthermore, 82 patients did not meet the CDC criteria for hypotension or fever and 19 of these patients were considered by clinicians to suffer from CoNS bloodstream infection.
Because of these discrepancies, we compared the relation between CoNS species and infection using both CDC criteria and clinical diagnosis.

Table 2. Number of true bloodstream infections, according to CDC-criteria and clinical microbiologist/infectious diseases specialist

	Total	Rotterdam*	Maastricht*
Infection according to CDC-criteria	45 (17.0%)	24 (15.8%)	21 (18.6%)
Infection according to clinical microbiologist/infectious diseases specialist	98 (37.0%)	59 (38.8%)	39 (34.5%)
Infection according to clinical microbiologist/infectious diseases specialist but not according to CDC-criteria	55 (20.8%)	35 (23.0%)	20 (17.7%)
Infection according to CDC-criteria but not according to clinical microbiologist/infectious diseases specialist	2 (0.8%)	0 (0.0%)	2 (1.8%)

* No significant differences were observed between the two centres

CoNS species and infection

S. epidermidis was considered to cause 66,7% of all bloodstream infections by CDC-criteria, and 75,5% of infections according to clinical microbiologists or infectious diseases specialists. *S. saprophyticus* and *R. mucilaginosa* were always associated with infections (Table 3A and B). Although *S. haemolyticus* strains represented only 3.8% of all isolates, 70% of these strains represented infection by the clinicians' criteria (80% in Erasmus MC and 60% in MUMC), which was significantly more than other species (P=0.042). According to CDC-criteria, 40% of these strains represented infection, which did not reach statistical significance (P=0.071) (Table 3A and B). *S. hominis* was significantly less likely to cause an infection than the other species by clinicians' criteria (P=0.011), but not by CDC-criteria (P=0.824).

The vast majority (83,5%) of isolated *S. epidermidis* strains represented blood culture contamination as defined by CDC criteria. Of all species isolated more than once, *S. pettenkoferi, S. cohnii, S. warneri* and *M. luteus* were always considered contamination by CDC-standards and only 1/12 (*S. warneri*) as infection by clinicians (Table 3A and B).

Table 3A. Species distribution between contaminants and true bloodstream infection according to CDC-criteria.

CDC-criteria: contamination or infection, per species	No. of strains	No. of infections	% of species representing infection	% of total number of infections
S.epidermidis	182	30	16,5%	66,7%
S.hominis	38	6	15,8%	13,3%
S.capitis	17	2	11,8%	4,4%
S.haemolyticus	10	4	40,0%	8,9%
S.warneri	4	0	0,0%	0,0%
S.cohnii	2	0	0,0%	0,0%
S.pettenkoferi	2	0	0,0%	0,0%
S.caprae	1	0	0,0%	0,0%
S.intermedius	1	0	0,0%	0,0%
S.saprophyticus	1	1	100,0%	2,2%
Micrococcus luteus	4	0	0,0%	0,0%
Rothia mucilaginosa	2	2	100,0%	4,4%
No ID	1	0	0,0%	0,0%
Total	265	45	17,0%	

Table 3B. Species distribution between contaminants and true bloodstream infection according to clinical microbiologist/infectious diseases specialist.

Clinical microbiologist/infectious diseases specialist: contamination or infection, per species	No. of strains	No. of infections	% of species representing infection	% of total number of infections
S.epidermidis	182	74	40,7%	75,5%
S.hominis	38	7	18,4%	7,1%
S.capitis	17	6	35,3%	6,1%
S.haemolyticus	10	7	70,0%	7,1%
S.warneri	4	1	25,0%	1,0%
S.cohnii	2	0	0,0%	0,0%
S.pettenkoferi	2	0	0,0%	0,0%
S.caprae	1	0	0,0%	0,0%
S.intermedius	1	0	0,0%	0,0%
S.saprophyticus	1	1	100,0%	1,0%
Micrococcus luteus	4	0	0,0%	0,0%
Rothia mucilaginosa	2	2	100,0%	2,0%
No ID	1	0	0,0%	0,0%
Total	265	98	37,0%	

Discussion

In this study, we examined the added value of identification of CoNS from positive blood cultures to species level and showed that identification to species level can be helpful in

discriminating between infection and contamination. *S. epidermidis* caused the majority of all bloodstream infections. *S. haemolyticus* strains were also often associated with bloodstream infections. Several species were always found to be contaminants: *S. warneri*, *S. pettenkoferi*, *S. cohnii*, *S. caprae* and *S. intermedius*.

Six strains were identified as not being staphylococci, but *Rothia* spp. or *Micrococcus* spp. It can be difficult to discern these strains from staphylococci by using only colony morphology, Gram-staining and catalase tests. MALDI-TOF MS thus has added value in their quick identification, especially since all *Micrococcus* strains represented contaminants and all *Rothia* strains were shown to cause clinically relevant bacteraemias.

One of the problems is that no gold standard exists for the diagnosis of CoNS bloodstream infections. Blood cultures with CoNS were significantly more likely to be considered infection by clinicians than by CDC-criteria. The clinical microbiologists and infectious diseases specialists in this study did not use a predefined set of objective criteria to determine whether or not a blood culture with CoNS represented true bloodstream infection, which could result in both over- and underestimation of the number of CoNS bloodstream infections. The CDC-criteria used in this study have the advantage of being a more objective set of criteria and are thus frequently used in studies. According to the CDC criteria, at least two positive blood culture sets are required for the diagnosis of CoNS bloodstream infections thereby excluding the possibility that a single positive blood culture set can represent infection[11,12]. Furthermore, not all patients with CoNS bloodstream infection present with fever or hypotension, which further limits the value of the CDC-criteria[11,13]. As a result, sensitivity and specificity may be as low as 67% and 56%, respectively[11]. Therefore, the clinicians' diagnosis is also frequently used as a 'gold standard' in many studies, due to the absence of a true gold standard[11,13,14].

Several studies have presented alternative criteria sets. Beekman *et al.*[11] and Elzi *et al.*[13] presented algorithms combining the number of positive blood cultures with clinical criteria, which showed better sensitivity and specificity than the CDC-criteria, but results were still suboptimal. García *et al.*[14] showed that both slime production and time-to-positivity of the blood culture were associated with CoNS bloodstream infection. Neither of these factors, nor the combination of the two, showed 100% sensitivity or specificity in diagnosing true CoNS bloodstream infections, but they might be a useful, simple addition to other sets of criteria. Of note, all these studies considered the clinical diagnosis the 'gold standard'. Additional objective criteria for diagnosing CoNS bloodstream infections are thus still necessary.

In our study, *S. epidermidis* was the most commonly isolated species in patients with infection. However, the vast majority (60-84%) of all *S. epidermidis* strains represented blood culture contamination, as did most *S. hominis* and *S. capitis* strains. Dependent on the definition (CDC criteria or clinical diagnosis), 40-70% of *S. haemolyticus* strains were shown to be infection. Little information about virulence factors of *S. haemolyticus* is available. *S. haemolyticus* is capable of biofilm formation, which allows it to adhere to foreign material and evade the immune system and antibiotics[15]. The genome is subject to frequent genetic rearrangements, which may contribute to the acquisition of antibiotic resistance and other

genes[16]. Indeed, they show a very high resistance rate for various antibiotics, including heteroresistance to glycopeptides[17-19]. Rapid ID of this species may therefore be helpful for early adjustment of antibiotic therapy.

S. saprophyticus is a well-known pathogen in urinary tract infections, but generally these are uncomplicated infections, without bloodstream infection[20]. In this study, we found one *S. saprophyticus* strain, which represented infection according to both the clinical microbiologist and CDC-criteria. *S. schleiferi* and *S. lugdunensis* are considered more virulent than other CoNS species[3]. However, in this study of 265 CoNS strains from 245 blood cultures we did not find any of these species, indicating that they are relatively rare in our country. CoNS species distribution in blood cultures varies between countries. In a small Indian study, *S. haemolyticus* was most commonly found in blood cultures[21], and in a Japanese study *S. epidermidis*, *S. haemolyticus* and *S. caprae* were the 3 most frequently isolated species[22]. In the 2 Dutch hospitals of our study, which are 150 kilometres apart, species distribution and the proportion of strains causing infection and contamination was similar, both by CDC-criteria and as judged by clinical microbiologists and infectious diseases specialists.

Thus, when MALDI-TOF ID shows *S. haemolyticus*, a bloodstream infection is significantly more likely than with *S. epidermidis*, S. *hominis*, and *S. capitis*. This might also be the case for. *S. saprophyticus*, although this species was rare in positive blood cultures in our population. *S. warneri* was always found to be a contaminant. It is difficult to draw a clear conclusion for other species in this study, due to their low numbers.

In other studies a similar species distribution of CoNS bloodstream infections was found, although different identification methods were used. Weinstein *et al*. used Microscan panels and showed that *S. epidermidis*, *S. hominis* and *S. haemolyticus* represented 98% of all CoNS bloodstream infections[23]. However, their ID method was not very reliable for species other than *S. epidermidis*[23]. Finkelstein *et al*. used the API Staph system for ID of CoNS and showed that *S. epidermidis*, *S. capitis* and *S. haemolyticus* were the most common CoNS causing bacteraemia, whereas *S. hominis* was always found to be a contaminant[24]. Our study is the first to show the value of MALDI-TOF MS ID, a more reliable ID method[7,23], in the diagnosis of CoNS bloodstream infection.

In conclusion, CoNS represent a large group of species, which vary considerably in pathogenicity. Discrimination between infection and contamination remains difficult as shown by the difference in diagnosis by clinicians and CDC criteria. This study shows that identification of CoNS species by MALDI-TOF MS has added value in the diagnosis of CoNS bloodstream infection.

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Chapter 7

Summary

General discussion

Conclusions

Rationale and goal

Bloodstream infections account for more deaths than any other type of infection[1]. The incidence is rising, and as a result also the number of deaths[2-6]. Rapid initiation of adequate antibiotic therapy is important to improve the patients' prognosis[7]. This initial treatment should ideally cover all, most likely, causative microorganisms until identification of the causative microorganism. Therefore, empirical therapy usually consists of one or more broad-spectrum antibiotics. However, excessive use of broad-spectrum antibiotics increases the risk of side effects, *Clostridium difficile*, fungal and yeast infections, costs and ultimately an increase of antibiotic resistant microorganisms[8-12]. Also, although these antibiotics cover a wide range of microorganisms, the causative microorganism may not be susceptible to the chosen antibiotic(s), resulting an inadequate treatment of the infection, which is associated with a poorer prognosis[13,14]. The risk of inadequate treatment is rising due to the increased incidence of infections with antibiotic resistant microorganisms[15,16]. The sooner the causative microorganism and its antibiotic susceptibility pattern are known, the earlier antibiotic therapy can be adjusted.

In order to culture the causative microorganism, blood cultures are drawn. On average, 15 hours (range 2.6-127 hours) of incubation is required before growth is detected[10]. Identification (ID) and antibiotic susceptibility testing (AST) usually require an additional 24-48 hours. This means that currently 48-72 hours are required before antibiotic therapy can be adjusted.

Many efforts have been made to reduce time to ID and AST. The recent introduction of MALDI-TOF MS has resulted in earlier ID results. However, for AST, developments have been few.

Therefore, the goal of this thesis was to explore new methods for more rapid ID and AST. Furthermore, we aimed to investigate their impact on the diagnosis, treatment and outcome of patients with bloodstream infections.

Rapid methods for identification (ID)

An important step in the diagnosis of bloodstream infections is ID of the causative microorganism, since it provides information on the original source of the infection and provides some information on the optimal treatment of the infection. Both MALDI-TOF MS and systems like BD Phoenix and Vitek 2 require bacteria from an overnight subculture on agar before ID can be performed according to the manufacturers' guideline. For Vitek 2, several studies determined inoculating the system with bacteria harvested directly from the positive blood culture using a Serum Separator Tube (SST), a procedure that only requires a 10-minute centrifugation step, thereby significantly reducing time to results[17-21]. Very little data were available on the same procedure for harvesting bacteria for direct inoculation of the Phoenix system. Therefore a study investigating this procedure for Gram-negative rods (GNRs) was performed (this thesis, **Chapter 2**). Very good results were found for

Enterobacteriaceae, only two errors occurred in this group: one strain was not identified and one strain of *E. coli* was misidentified as *S. choleraesuis*. Results of ID for *Pseudomonas* species were less reliable. Both errors in this group were *P. aeruginosa* strains that were identified as *P. fluorescens*, a rare cause of bloodstream infections. These misidentifications did not lead to errors in interpretation of AST, but rare or unlikely results of ID should be dealt with carefully and be confirmed using additional tests.
Other studies also showed that ID of non-fermenting GNR was less reliable than that of Enterobacteriaceae[22,23]. This may be due to the lower growth rate of non-fermenters, which could result in weaker fluorescent biochemical reactions in the Phoenix ID panel. Errors in ID with the direct method could also be caused by traces of blood culture components in the ID broth. This however seems less likely, since with Enterobacteriaceae, errors in ID were rare. Thus, by only a small change in daily laboratory routine, reliable results of ID of GNRs from blood cultures can be obtained up to a day earlier than with the standard method.

To obtain results of ID even sooner, a real-time PCR was developed based on amplification of a conserved region of the 16S rRNA gene combined with genus- and species specific probes (this thesis, **Chapter 3**). The assay was designed for use on blood culture material without the need for an extensive DNA-extraction procedure and results can be obtained within 2 hours. We found an overall agreement of 97% between conventional ID results and those of the multiprobe assay. Analysis of the conflicting results showed that, in some cases, our assay was in accordance with sequencing results. The sequences derived from the remaining discrepant clinical isolates, all coagulase-negative staphylococci, only showed three or even fewer mismatches with the *S. aureus* sequence. Within the group of staphylococci thus very homologous sequences exist that could cause false-positives because of the less efficient binding capacity of the *S. aureus*-specific probe. Since the assay uses the 16S rRNA gene for identification, the assay can be extended for the identification of more bacterial genera and species by adding more probes.

After these two studies were performed, in many laboratories, MALDI-TOF MS was introduced for the ID of bacteria. This requires a subculture of bacteria on agar. Alternatively, a commercial kit is available for isolation of bacteria directly from positive blood cultures. Turnaround time is approximately 30 minutes. Results of ID using this kit are shown to be reliable. However, in up to 20-36% of all blood cultures, no result is obtained[24], in which case a subculture on agar is still required. The methods presented in this thesis perform better and do not have this disadvantage.

Even more rapid ID could be obtained by using an assay that can be applied directly on blood. However, the detection limit of currently available methods probably is too high to be able to detect all bloodstream infections[25], resulting in false-negative results. On the other hand, bacterial DNA may be detected in blood that is negative in blood cultures. This may represent a bloodstream infection that was undetected by blood culture[25-31], for example due to the

presence of an antibiotic, or because the microorganism is difficult to culture. But it may also represent a false-positive result, due to contamination or due to the presence of bacterial DNA in the blood without the presence of a bloodstream infection (DNAemia).
It is difficult to discern a true bloodstream infection from contamination or DNAemia, since no gold standard exists for the diagnosis of bloodstream infections, so the value of a positive test result in molecular assays performed directly on blood is not yet completely clear.
Because of the risk of false-positive and -negative results, and because of the need of antibiotic susceptibility testing (AST), these assays will not be able to replace conventional blood culture (yet). Since they are still costly to perform, as well as labour-intensive and have a relatively high turnaround time, they are not likely to be introduced on a large scale soon.

In conclusion, the methods presented in **chapter 2** and **3** produce reliable ID results more rapidly than conventional methods, thereby potentially significantly reducing turnaround time for ID. Furthermore, both methods can be performed with equipment available in the majority of labs, and they are both easy to perform, with a limited hands-on time.

Rapid methods for antibiotic susceptibility testing (AST)

In some cases, ID provides sufficient information on the AST of the causative microorganism and can be used to switch the patient to a more appropriate antibiotic drug. However, in most cases antibiotic susceptibility testing is required to make an informed decision on the antibiotic treatment of a bacteraemic patient, especially in this era of increasing antibiotic resistance. Currently, AST can require up to 48 hours, and in recent years few new more rapid methods have been developed.

For AST, bacteria can also be harvested directly from the positive blood culture with the help of serum separator tubes (SST). However, for the BD Phoenix system, as for ID, for AST there was little information on this topic, especially on Gram-positive cocci. Therefore, this method was examined for both Gram-positive cocci (GPC) and Gram-negative rods (GNR) (this thesis, **Chapter 3**).
Direct AST for *Staphylococcus* species and *Enterococcus* species showed good agreement with conventional methods, comparable to results of the standard method, but with fewer very major errors. Categorical agreement for most tested antibiotics in this study, including oxacillin and vancomycin, was well over 90% and the percentage of major and very major errors is low, meeting the standards proposed by Jorgensen[32]. Only erythromycin and trimethoprim-sulfamethoxazole showed lower agreements. The majority of errors for erythromycin were minor errors, but also some major errors occurred. Trimethoprim-sulfamethoxazole was the only antibiotic for GPC showing very major errors. This was also observed in some other studies on direct methods for AST[17,19,20,22]. Therefore, the conclusion is that the direct Phoenix method using SSTs can be used to reliably report results of AST for GPC, except for trimethoprim-sulfamethoxazole and erythromycin.

For Enterobacteriaceae and *Pseudomonas* species, results of the direct method also showed very good agreement with the routinely used method, with essential and categorical agreements of over 95% for all antibiotics tested. Only two very major errors occurred, both with trimethoprim-sulfamethoxazole in *Pseudomonas aeruginosa* strains, which would never be considered an adequate treatment, due to intrinsic resistance. These errors thus would have no clinical consequences. A similar study by Funke *et al.* also showed >95% agreement for all antibiotics, with only 0.1% very major errors[22]. Therefore, we conclude that also for GNR, results of the direct Phoenix method for AST can be used to guide antibiotic therapy in bloodstream infections.
Using this method, turnaround time can potentially be reduced with up to 18 hours.

For an even shorter turnaround time, a new method for AST was developed (this thesis, **Chapter 4**), combining culture and PCR, RAMAST (RApid Molecular Antibiotic Susceptibility Testing), based on a proof-of-principle study by Rolain *et al*.[33]. Bacteria were harvested from the blood culture bottle using a SST. They were incubated in wells containing various antibiotics, after which quantitative PCR was used to test for bacterial growth in the presence of an antibiotic. It was shown that only 6 hours of incubation is required for sufficient growth.
For *S. aureus*, all antibiotics showed a 100% agreement, except for two errors for amoxicillin. This was shown to be comparable with the results of the conventional method of Phoenix inoculation, which also showed errors for amoxicillin in *S. aureus* in this study.
For *Enterococcus* species, agreement was 95% for amoxicillin and 100% for vancomycin.
In Gram-negative rods, all but one of the antibiotics showed an agreement of at least 93% with routine methods. Only piperacillin showed a high percentage of errors, which was also found in other methods for antibiotic susceptibility testing. RAMAST results for piperacillin should thus not be reported.
Overall, results of RAMAST were comparable to those of conventional methods[34-36] and met the criteria of Jorgensen *et al*.[32].
The PCR-assay that was used for RAMAST did not require an extensive DNA-isolation[37]. Instead, a simple centrifugation and washing step of the incubated diluted blood culture was sufficient, saving time and money. This method has also proven useful for the identification PCR-assay discussed above and may prove useful for other PCR-assays on positive blood cultures.
In the study by Rolain *et al.*[33], bacteria were harvested from a fresh culture of reference strains on agar, which required only 2–4 hours of incubation time before sufficient growth was obtained, whereas for RAMAST 6 hours of incubation was required, probably because bacteria were harvested directly from the positive blood culture. These bacteria may be in a stationary state because nutrients in the blood culture broth are depleted due to the high bacterial load[38], therefore requiring more incubation time for sufficient growth.
The procedure of incubation, DNA extraction and the PCR assay required 9 hours in total for all tested species, which is faster than routine methods. It can be used for most antibiotics

used to treat bloodstream infections and can easily be extended to other antibiotics if desired, which is not the case for more rapid molecular techniques, such as *mec*A PCR.
Thus, this study shows that RAMAST can provide accurate results for antibiotic susceptibility testing for the majority of clinically relevant blood culture isolates, in less time than other available methods for antibiotic susceptibility testing.

Currently, for AST, there are more rapid techniques in development, as described in **Chapter 1**. Genotypic AST has become available for many genes in recent years, such genes that code for MRSA, VRE, ESBL-production, carbapenemase production and ampC-production. Molecular techniques have become easier to perform and cheaper over the years. However, they will not be able to completely replace phenotypic techniques, since they test only for the genes included in the panel, and each gene codes for resistance against one antibiotic or a group of antibiotics. To offer a complete panel of antibiotics to test for, a large amount of genes will have to be combined in one assay. Techniques are now available to achieve this, such as micro-array, Luminex, or whole genome sequencing. However, there would still be the risk of missing mechanisms of antibiotic resistance because of genes that are not in the panel because they are yet unknown. Another risk is missing resistance mechanisms that rely on overproduction of normal molecules, such as a thicker cell wall in glycopeptide intermediate *S. aureus*, beta-lactamase hyperproducing *S. aureus* or an overexpression of porines in for example *P. aeruginosa*. Phenotypic antibiotic susceptibility testing would thus still be important. A similar problem arises with techniques that have been developed for AST using MALDI-TOF MS: each test identifies only one resistance mechanism, so many different tests would have to be combined to test for a complete set of antibiotic susceptibility tests.
Directly observing the effect of an antibiotic on single cell level might overcome these issues described for MALDI-TOF MS and genotypic tests, since potentially any change in bacterial morphology or viability can be observed, instead of testing for changes in individual proteins or genes. Such a technique would allow for more rapid antibiotic susceptibility testing than current techniques, since it would not require a large number of cell divisions before any effect of an antibiotic can be observed. However, the techniques described in Chapter 1, such as O-Cell-O-Scope and Accelerate are still in an early developmental stage and few results are currently available. So, none of these techniques will be able to replace currently used AST methods any time soon.

Impact of MALDI-TOF MS on the diagnosis of CoNS bloodstream infections

Currently, in the majority of laboratories, conventional methods for bacterial ID have been replaced by MALDI-TOF MS. Conventional methods such as API, Vitek 2 or Phoenix, have shown to be unreliable in the ID of coagulase-negative *Staphylococcus* species (CoNS), therefore, in the past usually no further ID was performed. MALDI-TOF MS has proven to be a reliable method for further ID of CoNS[39], allowing for routine ID of these species. The clinical

value of this extensive ID has not yet been determined. Therefore, the added value of identification of CoNS from positive blood cultures to species level was examined (this thesis, **Chapter 6**). The study showed that identification to species level can be helpful in discriminating between infection and contamination.

No gold standard exists for the diagnosis of CoNS bloodstream infections. The clinical microbiologists and infectious diseases specialists in this study did not use a predefined set of objective criteria to determine whether or not a blood culture with CoNS represented true bloodstream infection, which could result in both over- and underestimation of the number of CoNS bloodstream infections. The CDC has defined a more objective set of criteria, which is frequently used in studies. But sensitivity and specificity may still be as low as 67% and 56%, respectively[40]. Therefore, the clinicians' diagnosis is frequently used as a 'gold standard' in many studies, in the absence of a true gold standard[40-42]. In this study, both methods were used. Blood cultures with CoNS were significantly more likely to be considered infection by clinicians than by the CDC criteria.

The species of CoNS found in this study were shown to vary considerably in pathogenicity. Most CoNS bloodstream infections were shown to be caused by *S. epidermidis*. However, the vast majority of *S. epidermidis* strains were contaminants. Of *S. haemolyticus* strains, 40-70% was shown to represent true bloodstream infections. This percentage was significantly higher than that of the other species. Several species were always found to be contaminants: *S. warneri*, *S. pettenkoferi*, *S. cohnii*, *S. caprae* and *S. intermedius*. Six strains were identified as not being staphylococci, but *Rothia* spp. or *Micrococcus* spp., of which the first species was always associated with infection, and the latter was always shown to be contamination.

Early recognition of blood culture contamination can reduce overuse of antibiotics, since very often the patient is treated (unnecessarily) with antibiotics targeted at the cultured contaminant[43,44]. This study shows how difficult it still is to distinguish between a contaminated blood culture and a true bloodstream infection. It would even be more efficient to prevent blood culture contamination. Careful disinfection of both skin and blood culture bottles when drawing the blood culture, and blood culture drawing by dedicated teams only, are measures that have shown to significantly reduce blood culture contamination[45,46]. But it is highly unlikely that blood culture contamination can ever be completely prevented[45,46].

Therefore, efforts to improve recognition of blood culture contamination remain important. The species of CoNS found in this study were shown to vary considerably in pathogenicity, and identification of CoNS species by MALDI-TOF MS thus has added value in the diagnosis of CoNS bloodstream infection.

Impact of rapid ID and AST on the treatment and prognosis of bloodstream infections

To assess the clinical impact of rapid ID and AST on the treatment and prognosis of patients with bloodstream infections, a randomised controlled trial was performed, combining the previously described the multiplex PCR-assay for ID and RAMAST for AST (together: Fast AST,

or FAST) (this thesis, **Chapter 5**). Time to appropriate therapy in patients randomised for the rapid tests was compared to that of patients in the standard-of-care (SOC) group, in which only routine methods for ID and AST were used.

It was shown that results of FAST were available on average 15.6 hours earlier than SOC tests, and results were available the same working day the blood culture signalled positive. Although therapy could have been switched in the majority of patients as a result of FAST, only twelve patients were actually switched to another antibiotic after results became available. This resulted in a significant reduction in patients receiving inappropriate therapy at the time results of SOC tests became available. However, due to the small number of switches after FAST, no reduction in time to appropriate therapy was found, and as a result, also no effect was seen on mortality or length of hospital stay.

Few randomised studies have assessed the clinical impact of rapid diagnosis of infections, with varying results. All of these studies, except for one[47], showed a reduction in the use of inappropriate antibiotics. One of study[48] also found a reduction in mortality. Only one study focussed on bloodstream infections exclusively[49]. In this study it was demonstrated that more antibiotic switches occurred after rapid ID and AST, but the effect on patient outcome was very limited. Results thus vary greatly between these studies, as well do setting, methods and patient population. The methods for rapid AST used in these studies are now routinely used in most labs and have thus become our current standard-of-care.

There may be several explanations for the lack of impact of rapid ID and AST in our study. First of all, the implementation rate of the results of FAST was low for several reasons. It is not always possible to narrow down antibiotic therapy for example when a second infection is suspected, or when a source of infection is suspected with multiple microorganisms involved, for example in the case of an intraabdominal infection or an abscess. Several other studies also showed that implementation of results on standard-of-care (SOC) ID and AST is suboptimal[50,51], thereby limiting their clinical impact. However, implementation of FAST results was lower than that of SOC test results. This may be explained by the reluctance of the medical microbiologist or attending physicians to change therapy after 2-3 days in patients with improving status on the basis of a newly introduced test. A similar result was reported by Oosterheert *et al.*[52], showing that PCR results were rarely implemented for antibiotic switches in respiratory infections, in spite of being more sensitive than the routine method. In addition, several implementation research studies have shown that implementation in a controlled setting such as during an randomised controlled trial, does not fully represent “real-life” implementation, and as such will not fully represent the impact of clinical usage of a novel test[53,54].

Furthermore, in the Netherlands, antibiotic resistance is less common than in other countries[55], resulting in a lower percentage of patients receiving inadequate therapy. Also, a lower percentage of patients receiving inappropriate antibiotic therapy at the time of FAST results may have been due to the fact that results of the Gram-stain (and an advice for targeting of antibiotic therapy) were reported to the attending physician before FAST results were known[56,57].

Thirdly, although time to results was significantly reduced with FAST tests, at least still one working day is required before a blood culture signals positive. Implementation of antibiotic targeting has been shown to be directly associated with the time required to obtain results; switching is less likely when time to results is longer[49,56]. Thus, results of ID and AST may have to be available even earlier to significantly impact antibiotic switching and patient outcome. However, to date, the methods used in this study are among the most rapid methods that are currently available, and no method for AST is yet available to routinely be performed directly on blood instead of blood cultures.

General discussion

This thesis presents several new rapid methods for both ID and AST directly on blood cultures, which were shown to be highly reliable.
For a new method to be successfully implemented in clinical practise, reliability is an important factor, but not the only one. Every lab should be able to perform the technique, with a low hands-on time, and it should be affordable, especially in these times of increasing health care costs. The use of Serum Separator Tubes to harvest bacteria from positive blood cultures meets all these criteria and requires only a small adjustment to the daily lab routine to obtain a large reduction in time-to-results.
The materials and devices required for the molecular methods for ID and AST presented in this thesis are available in every molecular diagnostics laboratory. Also, they are easy to perform, using laboratory techniques that are used in many other tests. The ID and AST panels include the most commonly isolated bacterial species and most often used antibiotics, which could easily be extended if desired. However, molecular techniques are generally more expensive than currently used techniques for ID and AST, although the described methods do not require expensive commercial kits. Since these techniques have become increasingly common, their costs have lowered in the past years and may even become lower. Rapid ID and AST require also a higher hands-on time than currently used techniques. There is, however, room for improvement: many steps allow for automation, which would reduce hands-on time. Also, higher throughput would reduce hands-on time and costs per test. Although the presented methods thus show some disadvantages when compared with currently used techniques, they offer a significant improvement in turnaround time, as was shown in **Chapter 5** of this thesis.

Since this study, in many labs MALDI-TOF MS was introduced. It was almost an overnight success, since it meets almost all aforementioned criteria: it is reliable, easy to perform, cheap and far more rapid than most other routinely used ID methods. It does require equipment and techniques that were not present in most laboratories, but the low cost per test and easy to perform techniques allowed for an easy introduction in laboratory routine. However, MALDI-TOF MS requires a subculture on agar. It is possible to skip this subculture by performing a short routine of incubation and centrifugation steps. However, this does not

always result in an ID, as shown earlier, in which case a subculture still is required. This was never necessary with the molecular ID assay.

The role of AST is expected to become increasingly important in the future, since antibiotic resistance in bacteria is on the rise. This reduces adequacy of empiric antibiotic regimens. In clinical practise, this is compensated for by using more broad-spectrum antibiotics empirically. However, the use of more (broad-spectrum) antibiotics increases the risk of side effects, *Clostridium difficile*, fungal and yeast infections and costs in the individual patient[8-11]. But more importantly, it promotes the development and spread of antibiotic resistant microorganisms[12], resulting in a downward spiral. In the Netherlands, antibiotic resistance is low when compared to most other countries[55], due to the combination of restrictive use of antibiotics, isolation of patients carrying resistant microorganisms and search-and-destroy measures. On the other hand, antibiotic resistant microorganisms are very common in livestock, which is a large potential reservoir for introduction antibiotic resistant microorganisms in humans[58,59]. Also, tourists and patients treated in hospitals abroad can bring home with them resistant microorganisms from countries with much higher rates of antibiotic resistance[60,61]. As a result, antibiotic resistance is rising also in the Netherlands. MRSA infections are still very rare[15], but ESBL-producing Enterobacteriaceae have become very prevalent[15] and outbreaks of carbapenamase-producing bacteria have already been described[16,62].
More rapid ID and AST could not only allow for earlier adequate therapy, but also for earlier narrowing down antibiotic therapy, thereby reducing the amount of (broad-spectrum) antibiotics used and thus the risk of antibiotic resistance development. Therefore, efforts to develop and implement more rapid techniques have become increasingly important.

It was shown in this thesis that the use of more rapid ID and AST methods can indeed significantly reduce time-to-results in clinical practice. However, implementation rate of the rapid test results was low and little effect on antibiotic switching and outcome was observed.
Similar studies combining rapid ID and AST have been performed in the past[47-49,63,64], with varying but generally favourable results, as described earlier. However, they were testing 'rapid' methods that have become more or less standard-of-care tests nowadays. Studies on the clinical impact of early adequate antibiotic therapy are all observational in nature, since a randomised controlled trial on the impact of delayed adequate therapy would be unethical. Their results are conflicting: many studies show a beneficial effect of early adequate antibiotic therapy on mortality and morbidity[13,65], but many other studies fail to show this effect[13,66,67]. The variation in results may be due the observational nature of the studies, since this allows for many confounding factors. However, pooled analyses show that early adequate therapy is probably beneficial for the patients' prognosis[13,65]. And if more rapid ID and AST indeed result in earlier adequate therapy, this might thus improve the patients' prognosis. However, this remains to be proven in a randomised design, since the trial presented in this study did show

an effect of rapid ID and AST on the timing of antibiotic switching, but this was insufficient to affect the patients' prognosis.

Results of rapid ID and AST in this trial showed a low implementation rate, which has probably negatively affected the outcome. It is thus important not only to develop new more rapid methods for ID and AST, but to also work on a higher implementation rate of ID and AST results. Studies have shown that results on ID and AST that are available sooner, are more likely to result in an antibiotic switch[56,57]. Early involvement of a clinical microbiologist or an infectious diseases specialist in the treatment of the bacteraemic patient has also been shown to improve the appropriateness of antibiotic treatment[57,68]. The establishment of antibiotic stewardship teams, or A-teams, could also promote the implementation of results of ID and AST. Other measures to promote a higher compliance with results of ID and AST may be: more education of health care professionals on antibiotic treatment, and improving availability of ID and AST results to the attending physician by improving electronic systems.

Besides more rapid ID and AST, other measures should also be taken to improve early appropriate antibiotic treatment in bloodstream infections. It has been shown that improvements in laboratory logistics may reduce time to results and possibly also outcome, for example by decreasing transportation time of blood culture bottles from patient to incubator[69] or by extending laboratory opening times[70,71]. Also, early involvement of a clinical microbiologist or an infectious diseases specialist in the treatment of the bacteraemic patient has been shown to improve the appropriateness of antibiotic treatment[57,68]. The establishment of antibiotic stewardship teams, or A-teams, in the Netherlands and other countries, may thus be a very good tool to establish earlier appropriate treatment.

Conclusions

This thesis shows that it is possible to reduce time-to-results in patients with positive blood cultures. It also offers a useful rapid tool to improve recognition of contaminated blood cultures. But the simple introduction of a more rapid technique does not automatically reduce time to appropriate treatment. A combined approach of a rapid method and other measures to reduce turnaround time and improve implementation of ID an AST results is necessary to improve the clinical impact of the rapid diagnostic techniques.

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Nederlandse samenvatting

Dit proefschrift beschrijft enkele snelle technieken voor identificatie en antibiotica gevoeligheidsbepaling van bacteriën geïsoleerd uit bloed, alsmede de invloed van deze technieken op de behandeling van patiënten met infecties van het bloed.

Infecties van het bloed zijn verantwoordelijk voor meer overlijdens dan iedere andere infectieziekte. De prognose van deze infecties is in de loop der jaren verbeterd, maar als gevolg van het groeiende aantal patiënten met risicofactoren voor het oplopen van dergelijke infectie, neemt het aantal infecties toe, en daarmee ook het absolute aantal doden als gevolg hiervan.

Het vaststellen van de infectie, de verwekker en de antibiotica gevoeligheid hiervan duurt enkele dagen: de verwekker heeft tenminste 1 dag nodig om te groeien in een bloedkweek, en vervolgens duurt het nog 1-2 dagen voordat de resultaten van identificatie en gevoeligheidsheidsbepaling van de verwekker bekend zijn.

Het is van belang zo snel mogelijk te starten met adequate antibiotische therapie, daar dit de prognose van de patiënt verbetert. Daarom wordt reeds gestart met antibiotica zodra een infectie vermoed wordt. Omdat op dat moment nog niet bekend is wat de verwekker is van de infectie, wordt doorgaans gekozen voor een of meer breed-spectrum antibiotica, om zoveel mogelijk potentiële verwekkers te dekken. Overmatig gebruik van deze antibiotica heeft echter ook negatieve gevolgen: meer bijwerkingen, hogere kosten, een verhoogd risico op infecties met *Clostridium difficile*, gisten en schimmels, en op de lange termijn toename van resistente micro-organismen. Bovendien komt het voor dat de verwekker niet gevoelig is voor de gekozen middelen, wat zou kunnen resulteren in een slechtere prognose voor de patiënt.

Identificatie en antibiotica gevoeligheidsbepaling van de verwekker zijn dus van groot belang, want zodra de resultaten hiervan bekend zijn, kan de behandeling van de patiënt worden aangepast naar een meer adequaat middel en/of een middel met een smaller spectrum. Als de resultaten hiervan sneller bekend zouden zijn, dan zou deze aanpassing eerder plaats kunnen vinden, waardoor patiënten eerder adequate behandeling zouden kunnen ontvangen en het onnodig gebruik van (breed-spectrum) antibiotica verminderd kunnen worden.

In **hoofdstuk 2** wordt beschreven hoe een bestaande methode voor determinatie en gevoeligheidsbepaling, waarbij gebruik wordt gemaakt van het BD Phoenix systeem, kan worden aangepast om sneller resultaten te verkrijgen. Normaal gesproken wordt de positieve bloedkweek overgeënt op een agarplaat, om zo de bacteriën te verkrijgen waarmee het apparaat kan worden geïnoculeerd. Om deze stap over te slaan kunnen bacteriën worden geïsoleerd uit de bloedkweek middels een centrifugeerstap in een bloedbuis. Deze methode is voor een groot aantal gegroeide bloedkweken getest. Hieruit bleek dat de resultaten van determinatie met de snelle methode even betrouwbaar waren als die van de conventionele

methode, met uitzondering van de determinatie van *Pseudomonas aeruginosa*. Ook voor gevoeligheidsbepaling bleek de snelle methode zeer betrouwbaar, met uitzonderding van erythromycine en trimethoprim-sulfamethoxazol in Gram-positieve kokken. Met een kleine, goedkope aanpassing in een reeds gebruikte methode voor identificatie en gevoeligheids-bepaling kunnen resultaten hiervan dus aanzienlijk eerder beschikbaar zijn.

Met de conventionele methodes voor identificatie van bacteriën wordt gebruik gemaakt van diverse biochemische reacties om het micro-organisme te determineren, waarvoor de micro-organismen enige tijd geïncubeerd moeten worden. Met behulp van moleculaire technieken zouden deze resultaten sneller verkregen kunnen worden, omdat hiervoor geen incubatietijd nodig is. In **hoofdstuk 3** wordt een PCR beschreven die gebruik maakt van 2 universele primers waarmee een deel van het 16S rRNA gen geämplificeerd wordt, en een set van diverse species-specifieke probes die aan dit stuk kunnen hechten, waarmee de stam vervolgens geïdentificeerd kan worden. Deze PCR kan na een verdunningsstap direct op de positieve bloedkweek worden toegepast. De bepaling is getest op 248 positieve bloedkweken, waarna de resultaten werden vergeleken met die van conventionele identificatie. Alle geteste species toonden 100% overeenkomst tussen beide methoden, met uitzondering van de *Staphylococcus aureus* probe, welke weliswaar ook een sensitiviteit had van 100%, maar een specificiteit van 93%. Met deze test kan dus een zeer betrouwbare identificatie verkregen worden in een kortere tijd dan met conventionele technieken.

PCR kan ook gebruikt worden voor snellere gevoeligheidsbepaling, middels een methode die in **hoofdstuk 4** wordt beschreven. Hiervoor worden bacteriën direct uit de gegroeide bloedkweek geïsoleerd middels een centrifugeerstap zoals beschreven in hoofdstuk 2. Een suspensie hiervan wordt gedurende 6 uur geïncubeerd met de te testen antibiotica. Vervolgens wordt een PCR verricht op iedere suspensie. De cycle threshold (Ct) waardes worden vergeleken met die van een controlesuspensie waarin geen groei heeft plaats-gevonden. Als de Ct-waarde van een suspensie geïncubeerd met een antibioticum lager is dan die van de controlesuspensie, betekent dit dat er groei heeft plaatsgevonden in de aanwezigheid van het antibioticum, de bacterie is dus resistent. Als er geen groei wordt geconstateerd, dan is de stam gevoelig. De totale procedure, beginnende met een gegroeide bloedkweek, duurt 9 uur. De methode is getest op 114 positieve bloedkweken. Voor Gram-negatieve staven kwamen de resultaten van de test in 96.7% van de geteste antibioticum-bacteriecombinaties overeen met die van conventionele methodes (het BD Phoenix systeem en, in geval van een discrepantie, microbroth dilution). Voor *Staphylococcus aureus* was dit 97.9% en voor *Enterococcus* species 95.0%. Deze semi-moleculaire test bleek dus net zo betrouwbaar als de methode die op dit moment in de kliniek wordt gebruikt, maar de resultaten ervan zijn eerder beschikbaar.

In **hoofdstuk 5** wordt een gerandomiseerde gecontroleerde studie beschreven met als doel te bepalen of snellere diagnostiek ook leidt tot een snellere aanpassing van therapie in

patiënten met infecties van het bloed. Patiënten met een gegroeide bloedkweek werden gerandomiseerd voor de snelle (FAST) groep, waarin de testen beschreven in hoofdstuk 3 en 4 werden gebruikt voor identificatie en gevoeligheidsbepaling, of de standard-of-care (SOC) groep, waarin alleen de reguliere testen werden verricht. Er werden 250 patiënten geïncludeerd, 129 in de FAST groep en 121 in de SOC groep. Resultaten van FAST-diagnostiek bleken opnieuw zeer betrouwbaar en waren gemiddeld 15.9 uur eerder beschikbaar dan die van SOC-testen. In totaal hadden 78 patiënten naar aanleiding van FAST-testen over kunnen stappen naar een ander antibioticum, echter, dit gebeurde slechts in 16 patiënten. Deze aanpassing van therapie vond significant eerder plaats dan bij patiënten in wie SOC-diagnostiek de aanleiding was voor een aanpassing in therapie. Door de FAST-diagnostiek ontvingen significant minder patiënten inadequate of te brede antibiotica op het moment dat de resultaten van SOC-diagnostiek beschikbaar werden. Maar door de suboptimale implementatie van de snelle diagnostiek werden er geen effecten gevonden op klinische uitkomstmaten.

In de afgelopen jaren is in de meeste laboratoria matrix assisted laser desorption/ionisation time-of-flight mass-spectrometry (MALDI-TOF MS) ingevoerd als standaard methode voor identificatie van bacteriën. Deze methode is zeer betrouwbaar, goedkoop en snel. Daarnaast kunnen hiermee routinematig bacteriën gedetermineerd worden waarvan dat eerder met de routine diagnostiek niet mogelijk was, zoals de groep van coagulase-negatieve staphylokokken (CoNS). CoNS worden meestal beschouwd als contaminant als ze in een bloedkweek worden gevonden, maar ze kunnen ook infecties van het bloed veroorzaken. Het is erg moeilijk om dit onderscheid te maken, en daarmee om te bepalen of de patiënt behandeling behoeft voor de gekweekte stam. In **hoofdstuk 6** wordt beschreven hoe de identificatie van CoNS in bloedkweken met behulp van MALDI-TOF MS kan helpen in het maken van het onderscheid tussen CoNS-infectie en contaminatie van de bloedkweekfles. 265 stammen van 245 patiënten uit twee ziekenhuizen werden met MALDI-TOF MS geïdentificeerd. Van deze patiënten werd bepaald of zij een infectie van het bloed hadden of dat de stam een contaminant betrof, hiervoor werd zowel gebruik gemaakt van een in de literatuur veel gebruikte set van criteria (CDC-criteria) als van de klinische inschatting van de arts-microbioloog danwel de internist-infectioloog. De meest voorkomende stam was *Staphylococcus epidermidis*, deze bleek in 83.5% van de gevallen een contaminant als gekeken werd naar de CDC-criteria, en in 59.3% als gebruik werd gemaakt van de klinische inschatting. *Staphylococcus haemolyticus* bleek relatief het vaakst geassocieerd met een infectie van het bloed: in 40% (CDC-criteria) tot 70% (klinische inschatting) van de gevallen. Sommige species, zoals *Staphylococcus warneri* bleken altijd een contaminant te zijn. Deze studie laat zien dat in geval van een CoNS in de bloedkweek het onderscheiden van een infectie van het bloed en contaminatie van de bloedkweek inderdaad zeer lastig is, en dat de determinatie van de stam middels MALDI-TOF MS hierbij kan helpen.

Samenvattend kunnen uit dit proefschrift de volgende conclusies getrokken worden:

- Het is mogelijk om met een kleine aanpassing aan het protocol, namelijk isolatie van bacteriën direct uit de bloedkweek door een centrifugeerstap in plaats van een tijdrovende incubatie op agar, sneller betrouwbare resultaten te verkrijgen van identificatie en gevoeligheidsbepaling in patiënten met een positieve bloedkweek.
- Een real-time PCR-assay levert snel en betrouwbare resultaten van identificatie van bacteriën in een bloedkweek, waarbij een overenting op agar of een uitgebreide DNA-isolatie niet nodig is.
- Door kweek en PCR te combineren kunnen resultaten van antibiotica gevoeligheids-bepalingen van bacteriën uit een positieve bloedkweek binnen 9 uur beschikbaar zijn.
- Door gebruik te maken van moleculaire technieken zijn resultaten van identificatie en gevoeligheidsbepaling eerder beschikbaar dan met conventionele technieken, en kunnen daardoor resulteren in een vroegere switch naar een smaller of meer adequaat antibioticum in patiënten met een infectie van het bloed. Echter, implementatie van de resultaten was beperkt, waardoor de invloed van de snellere technieken op klinische uitkomstmaten beperkt is.
- Determinatie van CoNS met behulp van MALDI-TOF MS kan helpen bij het maken van onderscheid tussen patiënten met een infectie van het bloed en contaminatie van de bloedkweek.

Valorisation of the thesis

Background

Bloodstream infections are the most common form of infections. Mortality is high, between 15 and 35%[1-5]. As a result, bloodstream infections in the top 7 of most common causes of death, and account for approximately 157.000 deaths in Europe annually[6]. The incidence is rising, and as a result, the total number of deaths attributed to bloodstream infections has risen[7,8].

Treatment of bloodstream infections has two pillars: symptomatic treatment, by fluid resuscitation, and treatment of the cause of infection with antibiotics. Empirically one or more broad-spectrum antibiotics are administered, but since no 'one size fits all' antibiotic exists, therapy will likely be too narrow or too broad. In the past years antibiotic resistance has increased substantially in Gram-negative bacteria[9]. Thus, empirical therapy is more likely to be inadequate, which is associated with increased mortality. It is therefore increasingly important to know the antibiotic susceptibility pattern of the causative microorganism. Data on the antibiotic susceptibility of a microorganism currently are not available until at least 2 days after initiation of antibiotic therapy, since a positive blood culture requires more than one working day to grow and results of antibiotic susceptibility testing will not become available until another day later. More rapid identification and antibiotic susceptibility testing potentially enable earlier switching to adequate therapy and reduce the use of broad-spectrum therapy.

Conclusions of this thesis

In this thesis more rapid methods for ID and AST are presented, combining techniques and devices that are already available in the majority of microbiological labs (**Chapters 2**,**3** en **4**). Furthermore, it was shown that by using these methods, results of ID and AST are available significantly earlier than standard-of-care methods (**Chapter 5**). Also, significantly more patients received appropriate therapy on the same working day the blood culture had signalled positive, due to the rapid tests. However, due to lack of implementation of test results this did not result in a reduction of total time too-broad or inadequate therapy was used (**Chapter 5**). In addition it was shown that MALDI-TOF MS can be a useful tool in discerning blood culture contamination from true bloodstream infection, possibly leading to less use of antibiotic therapy (**Chapter 6**).

Target groups

The methods and results presented in this thesis are important for many groups of professionals involved in the healthcare system.

For clinical microbiologists and molecular medical microbiologists, the methods presented in this thesis offer new approaches to the diagnosis of bloodstream infections. It was shown that

more rapid bacterial ID and AST are possible, using techniques that they very likely already have available. The DNA-extraction techniques used in this thesis were very simple to perform, cheap and reliable, showing that PCR-assays on positive blood cultures can be performed without the need of expensive and extensive DNA-extraction kits. Additionally, it was shown that MALDI-TOF MS, which nowadays has become standard equipment in almost every lab, can help in determining whether a blood culture was contaminated or not. Such test results may help clinicians in deciding whether or not to prescribe antibiotic therapy.
For clinical microbiologists, as well as for infectious diseases specialists, the thesis shows how important implementation of test results is, and that focus on successful implementation strategies is essential in gaining full benefit from technical advances.

Ultimately, and most importantly, the results of the thesis are important for the patient. It shows that rapid ID and AST did not result in an overall time reduction of administering inadequate or too broad-spectrum antibiotic therapy. However, for a small group of patients it did result in significantly earlier appropriate antibiotic therapy. When implementation rates are higher, earlier overall appropriate therapy may be achieved with these new techniques. Earlier adequate antibiotic therapy and early recognition of a clinically relevant blood culture can improve the patients' prognosis[10,11]. Narrowing down antibiotic therapy earlier can also be beneficial for the patient, since using less antibiotics means a lower risk of side effects, and more narrow-spectrum antibiotics carry a lower risk of *Clostridium difficile* infections[12,13].
The benefits of narrowing down antibiotic therapy earlier will be more profound on the population level, since it reduces the total use of antibiotics. The more antibiotics are being used, the higher the levels of antibiotic resistance are[14]. In some countries, carbapenem resistant Enterobacteriaceae (CRE) and MRSA have already become endemic, making treatment of infections with these bacteria much more difficult, and sometimes even impossible[15,16]. In the Netherlands, this is not (yet) the case, infections with CRE and MRSA are still rare. However, infections with ESBL-producing Enterobacteriaceae seem to become more and more common[9], and outbreaks of infections caused by CRE have already been described[15]. These data indicate that in the future we may also face more serious resistance problems if no preventive measures are implemented. Reducing the use of antibiotics could result in lower rates of development and selection of resistant microorganisms, and more rapid ID and AST could aid in the reduction of antibiotic use, if properly implemented.

Financial implications

In 2014, expenses on antibiotics in the Netherlands were 35,6 million euros, which was an increase of 0.6 million euros when compared to 2011. The total number of Defined Daily Doses (DDD) of antibiotics had decreased with 6% between 2011 and 2014, and prices of most antibiotics have also decreased over the past years[17]. That the total expenses on antibiotics have risen nevertheless can be attributed to the increased use of more expensive broad-spectrum antibiotics, such as 3rd generation cefalosporins, linezolid, vancomycin and

meropenem[9,17]. Prices for these antibiotics range from 14.82 euros/dose (for vancomycin) to 60.84 euros per dose (linezolid), whereas the more frequently used amoxicillin, amoxicillin-clavulanate and cefuroxime are relatively inexpensive, ranging from 1.37 to 5.04 euros/dose[9,18].
Earlier results of ID and AST can lead to an earlier switch to a more narrow-spectrum antibiotic and thus a reduction of use of these expensive broad-spectrum antibiotics. However, as a result of the relatively low antibiotic resistance rates in the Netherlands, expensive broad-spectrum antibiotics are used relatively infrequent. The most widely used broad-spectrum antibiotics are 3rd generation cefalosporins, with 5 DDD per 100 patient-days, on a total amount of antibiotics used in hospitals of 74.7 DDD per 100 patient-days (6.7%)[9]. But the other expensive very broad-spectrum antibiotics combined represent only a very small fraction of the total of amount of antibiotics used in hospitals, with 3.3 DDD per 100 patient-days (4.4%)[9]. Although antibiotic costs are only a small fraction (<1%) of the total costs of pharmaceuticals in the Netherlands, which are >4 billion euros[17,19], earlier ID and AST can result in a reduction of direct costs of antibiotics.

But earlier adequate therapy results in a better prognosis for the patient[10,11], which results in a reduction in health care costs, for instance by shortening length of hospital stay. And since reducing the amount of antibiotics used could reduce antibiotic resistance development, it would also reduce the additional healthcare costs that are generated by infections with antibiotic resistant microorganisms, which are estimated to be 1,5 billion euros per year in Europe[20] and even 20 billion dollars per year in the USA[21].

Future references

The assays presented in this thesis can easily be adjusted, by adding or replacing microorganisms or antibiotics. Automation of the tests potentially allows for more rapid assays, reduced hands-on time, higher throughput and lower costs. And in the future, more rapid PCR techniques could be used to reduce PCR time.

The assays could be combined with other measures to reduce time-to-results, such as extended laboratory opening hours and improved hospital and laboratory logistics.
Implementation of test results can be improved by the formation of Antibiotic Stewardship Teams. The rapid assays could be of assistance in their goal of earlier adequate therapy and reduction of unnecessary broad-spectrum antibiotic therapy.

Possibly, currently available methods are not sufficient in reducing broad-spectrum antibiotic use and time to appropriate therapy. Development of more rapid techniques is required, especially since studies have shown that the rate of antibiotic switching is inversely related to the time to results[22,23]. Since the incubation of the blood culture bottle is the most time consuming step, this would be the most likely target for improvement. It is possible to reduce

incubation time and still obtain reliable ID results, as was shown by Loonen *et al.* in 2012[24]. Ideally, the blood culture step can be eliminated completely, by using PCR- and sequencing techniques that can be used directly on blood. However, the possibility that, in the near future, these techniques can replace culture based techniques is low, for reasons described in **Chapter 1**. At this moment, most effort is invested in new, more rapid assays and techniques that are performed on positive blood cultures or subcultures on agar.

Additionally, better techniques and tools to discern contaminated blood cultures from those representing true bloodstream infection are needed with the goal to reduce, or, ideally, eliminate blood culture contamination.

It is important to limit the costs of newly developed techniques, because although a more rapid diagnosis can save money and lives, healthcare costs continue to rise and more patients will require diagnosis and treatment of bloodstream infections, since their incidence is rising. Cost effectiveness studies should thus be part of the introduction of new tests.

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Curriculum vitae

Judith Wilhelmina Aleida Beuving werd geboren op 20 mei 1983 te Veghel. Na het behalen van haar VWO-diploma aan het Zwijsen College aldaar verhuisde ze in 2001 naar Maastricht voor haar studie Geneeskunde. In 2005 behaalde ze cum laude haar doctoraaldiploma. In 2006-2007 doorliep zij haar 18-weekse klinische stage op de afdeling Infectieziekten in het azM, onder begeleiding van prof. dr. Annelies Verbon. In 2007 doorliep zij haar 18-weekse wetenschapsstage in het azM op de afdeling Medische Microbiologie, met een onderzoek naar de waarde van de bepaling van sTREM-1 in BAL-vloeistof in de diagnostiek van ventilator-associated pneumonia, onder begeleiding van prof. dr. Annelies Verbon, dr. Guy Oudhous en dr. Catharina Linssen. In 2007 behaalde zij haar arts-examen. Vervolgens begon zij in augustus 2007 in het azM met haar promotie-onderzoek naar methoden voor snelle diagnostiek op bloedkweken en de klinische waarde daarvan, onder begeleiding van prof. dr. Verbon, dr. Petra Wolffs en prof. dr. Cathrien Bruggeman. In oktober 2011 verhuisde zij naar Tilburg om te starten met de opleiding tot arts-microbioloog in het St. Elisabeth Ziekenhuis te Tilburg en het Amphia Ziekenhuis te Breda, onder begeleiding van prof. dr. Jan Kluytmans, dr. Anton Buiting en drs. Peter van Keulen. In 2015 is zij getrouwd met Dennis Hoogenboom.

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List of abbreviations

AST	Antibiotic susceptibility testing
cfu	Colony-forming units
CoNS	Coagulase-negative staphylococci
CRE	Carbapenem-resistant Enterobacteriaceae
DNA	Deoxyribonucleic acid
DOBBI	Diagnosis of bacterial bloodstream infections
DDD	Defined daily doses
ESBL	Extended-spectrum beta-lactamase
FAST	Fast Antibiotic Susceptibility Testing
GPC	Gram-positive cocci
GNR	Gram-negative rods
ID	Identification
MALDI-TOF MS	Matrix-assisted laser/desorption ionisation time-of-flight mass spectrometry
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MUMC	Maastricht University Medical Center
PCR	Polymerase chain reaction
PCR-ESI	PCR-Electrospray Ionization Mass Spectrometry (PCR-ESI
RAMAST	RApid Molecular Antibiotic Susceptibility Testing
Rpm	Rounds per minute
16S rRNA	16S ribosomal ribonucleic acid
SIRS	Systemic inflammatory response syndrome
SOC	Standard-of-care
SST	Serum separator tube
VRE	Vancomycin-resistant *Enterococcus* species