Rapid diagnosis of bloodstream infections

Chapter 7

Summary

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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.