

Acceleration of the direct identification of *Staphylococcus aureus* versus coagulase-negative staphylococci from blood culture material: a comparison of six bacterial DNA extraction methods

A. J. M. Loonen · A. R. Jansz · H. Kreeftenberg ·
C. A. Bruggeman · P. F. G. Wolffs ·
A. J. C. van den Brule

Received: 23 June 2010 / Accepted: 1 October 2010 / Published online: 24 October 2010
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Abstract To accelerate differentiation between *Staphylococcus aureus* and coagulase-negative staphylococci (CNS), this study aimed to compare six different DNA extraction methods from two commonly used blood culture materials, i.e. BACTEC and BacT/ALERT. Furthermore, we analysed the effect of reduced blood culture incubation for the detection of staphylococci directly from blood culture material. A real-time polymerase chain reaction (PCR) duplex assay was used to compare the six different DNA isolation protocols on two different blood culture

systems. Negative blood culture material was spiked with methicillin-resistant *S. aureus* (MRSA). Bacterial DNA was isolated with automated extractor easyMAG (three protocols), automated extractor MagNA Pure LC (LC Microbiology Kit M^{Grade}), a manual kit MolYsis Plus and a combination of MolYsis Plus and the easyMAG. The most optimal isolation method was used to evaluate reduced bacterial incubation times. Bacterial DNA isolation with the MolYsis Plus kit in combination with the specific B protocol on the easyMAG resulted in the most sensitive detection of *S. aureus*, with a detection limit of 10 CFU/ml, in BacT/ALERT material, whereas using BACTEC resulted in a detection limit of 100 CFU/ml. An initial *S. aureus* or CNS load of 1 CFU/ml blood can be detected after 5 h of incubation in BacT/ALERT 3D by combining the sensitive isolation method and the *tuf* LightCycler assay.

A. J. M. Loonen · A. R. Jansz · A. J. C. van den Brule
PAMM Laboratory for Medical Microbiology,
Eindhoven/Veldhoven, The Netherlands

A. J. M. Loonen · A. J. C. van den Brule
Centre for Medical Molecular Diagnostics,
Fontys University of Applied Science,
Eindhoven, The Netherlands

A. J. M. Loonen · C. A. Bruggeman · P. F. G. Wolffs
Department of Medical Microbiology,
Maastricht University Medical Centre,
Maastricht, The Netherlands

H. Kreeftenberg
Department of Intensive Care, Catharina Hospital,
Eindhoven, The Netherlands

A. J. C. van den Brule (✉)
Department of Molecular Diagnostics,
Catharina Hospital/PAMM Laboratory,
Michelangelolaan 2,
5623 EJ Eindhoven, The Netherlands
e-mail: a.van.den.brule@pamm.nl

Introduction

Staphylococcus aureus is a pathogen which can cause both hospital- and community-associated infectious diseases, ranging from minor skin infections to endocarditis, bacteraemia, sepsis and septic shock [1]. Sepsis can result in high morbidity and mortality. In the United States, bloodstream infections develop in approximately 250,000 people annually [2]. In the Netherlands, the incidence of patients admitted to the intensive care unit (ICU) with severe sepsis is in the range of around 8,643±929 per year [3]. Currently, blood culture is the gold standard for the identification of pathogens from suspected bacterial sepsis patients. Unfor-

tunately, blood culture is time-consuming, taking at least 24–72 h for the final determination of the bacteria causing the disease. Staphylococci are the most common Gram-positive organisms in blood cultures. Differentiating *S. aureus* from coagulase-negative staphylococci (CNS) is important, because sepsis with *S. aureus* is common and virulent, with mortality rates in the range of 20–30% [4]. CNS are often considered as being contaminants in blood cultures due to the fact that these species are members of the normal skin flora and mucous membranes, and can contaminate the sample when it is taken. However, it is known that CNS infections are increasingly recognised as clinically relevant infections and confirmation on the presence of these species in blood culture is, therefore, important (reviewed in [5–8]). Several molecular methods for the rapid and accurate detection of bacteria from positive blood culture material have been described, including (commercial) real-time polymerase chain reaction (PCR)-based diagnostic tests [9–11], fluorescence in situ hybridisation [12, 13], matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) [14] and also DNA micro-arrays [15, 16]. However, all of these techniques are used on positive blood culture material.

Reduction in the time to obtaining results can be achieved by applying molecular methods either directly on whole blood or on blood culture material with reduced incubation times. Ideally, usage of whole blood is preferred but the techniques that are now available are often not sensitive enough, clinically, as has been shown by others investigating a commercial real-time PCR test currently available [17–19]. Blood culture materials are known to contain inhibiting factors which can reduce detection in a sensitive real-time PCR [20–23]. It is, therefore, important to include a good isolation method in the molecular diagnostic strategy, which is able to efficiently remove inhibiting factors and one which still allows sensitive DNA detection by PCR.

In this study we compared six different, both manual and automated, bacterial DNA isolation methods for two commonly used blood culture systems, i.e. BACTEC (Becton Dickinson) and BacT/ALERT (bioMérieux), to be able to find the most sensitive bacterial DNA isolation method. Additionally, we investigated the occurrence of inhibition in PCR amplification after DNA isolation. A sensitive real-time PCR assay was designed to be able to detect staphylococci and to differentiate *S. aureus* from CNS (Loonen et al., manuscript submitted). Subsequently, this real-time PCR was used in combination with the optimal DNA isolation method to investigate the level of time reduction to identify staphylococci from blood culture material. The results were compared with conventional blood culture techniques used in diagnostic laboratories.

Materials and methods

Culture methods

Negatively cultured blood culture bottles, derived from routine diagnostics of both BACTEC (Becton Dickinson, The Netherlands) and BacT/ALERT (bioMérieux, The Netherlands) systems, were used in this study for spiking experiments (bottles have been cultured for 1 week and remained negative). Additionally, healthy volunteers donated 10 ml blood as the input for the BacT/ALERT bottles. The bottles were incubated and handled according to the manufacturer's protocol.

DNA isolation methods

Methicillin-resistant *S. aureus* (MRSA) ATCC 33592 was cultured overnight on blood agar. Fresh plates were used to make a 2-McF solution which was subsequently serially diluted (1:10) in negative blood culture material from both systems (BACTEC and BacT/ALERT). One hundred μ l was plated on blood agar plates to check the number of CFU per sample. Three different isolation protocols on the NucliSens easyMAG (bioMérieux) were tested (generic, specific A and specific B protocol: the main difference between the protocols is the number of washing steps, with the least steps in the generic protocol and the most washing steps in the specific B protocol), one isolation kit (LC Microbiology Kit M^{Grade}) on the MagNA Pure LC (Roche Diagnostics), the MolYsis Plus kit (MolzYM GmbH, Bremen, Germany) was used according to MolzYM's instructions and in combination with the easyMAG. When MolYsis Plus was used in combination with the easyMAG, the manufacturer's instructions (MolzYM) were followed until the BugLysis step and, subsequently, bacterial DNA was extracted according to the specific B protocol on the easyMAG. The input volume in each isolation was 200 μ l, except for the MagNA Pure LC, where 100 μ l was used, and samples were always eluted in 100 μ l elution buffer provided with each isolation method. Each extraction was performed at least 2–3 times in independent experiments. Phocine herpes virus type 1 (PhHV-1) was added to each sample eluate as an amplification and inhibition control. Samples were analysed with the LightCycler assay described below.

LightCycler assay

A duplex real-time PCR, detecting the *tuf* gene of both staphylococci and *S. aureus* specifically, is used as described in detail elsewhere (Loonen et al., submitted). In brief, the positions of the primers and probes were derived from the *S. aureus tuf* gene sequence (GenBank

accession no. AF298796). The sequences of the primers and probes were as follows: *Staphylococcus* spp. forward primer ccaatgccacaaactcgtga (position 32-51), *Staphylococcus* spp. reverse primer cacgaccagtgattgagaatacg (position 371-349), *S. aureus* forward primer tctcgtgtaattacaccaca tactg (position 583-608), *S. aureus* reverse primer ggaaata gaattgtggacgatagtttg (position 698-671), *Staphylococcus* spp. probe VIC-ccattcatgatgccagttg-BHQ1 (position 326-344), *S. aureus* probe FAM-tgataatcgtatctctctgc-BHQ1 (position 637-617). Oligonucleotide primers were obtained from Eurogentec (Liège, Belgium), *Staphylococcus* XS probe and the *S. aureus* XS probe were obtained from Biolegio (Nijmegen, The Netherlands). PhHV-1 primers and VIC-labelled probe [24] were obtained from Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). PCR mixtures consisted of 5× LightCycler TaqMan Master mixture (Roche Diagnostics), 0.9 μM concentration of each primer, 0.2 μM concentration of each probe and Aquadest in a volume of 15 μl to which 5 μl DNA sample was added, leading to a final reaction volume of 20 μl in each capillary. Primers and probe targeting PhHV had a concentration of 0.1 μM each and were used in a separate single real-time PCR assays. All samples were amplified on the LightCycler v2.0 (Roche Diagnostics) according to the following cycling parameters: an initial incubation step for 10 min at 95°C for enzyme activation and DNA denaturation, then, 45 cycles of 15 s at 95°C followed by 1 min at 60°C. The results were analysed with LightCycler Software version 4.05 (Roche Diagnostics).

Results

Evaluation of different bacterial DNA extraction methods

In Table 1, the results are presented of the comparison of six different bacterial DNA extraction methods on *S. aureus* spiked blood culture material. The MagNA Pure LC (Roche Diagnostics) resulted in a detection limit of 10⁴ CFU/ml for both culture systems. On the easyMAG, three protocols were evaluated to isolate bacterial DNA from spiked

clinical material, generic, specific A and specific B. We tested all three protocols and usage of the specific B protocol resulted in the best detection limit of 10² CFU/ml of *S. aureus* in BacT/ALERT material. Although MolYsis Plus did not result in a low detection limit, we tested this manual kit in combination with the specific B protocol on the easyMAG. By combining both methods, all human DNA was removed and bacteria were lysed by the MolYsis Plus kit, and, subsequently, the easyMAG was used for the optimal washing and elution of bacterial DNA. It was possible to reduce the hands-on time and avoid possible contamination due to skipping of manual handlings. This combination of methods resulted in the most sensitive detection limit, i.e. 10 CFU/ml, in BacT/ALERT material. The time to obtaining results for this combined method followed by real-time PCR is approximately 4–5 h, but it strongly depends on the number of samples. No inhibition was observed in the real-time PCR, and all samples and controls showed similar Cp values in one run (Cp±0.5). All experiments were performed at least 2–3 times.

Reduced blood culture times

To investigate the effect of reduced blood culture incubation for pathogen detection by PCR, a combination between the sensitive *tuf* LightCycler assay and the sensitive isolation method, the MolYsis Plus Kit in combination with the specific B protocol on the easyMAG, was analysed on *S. aureus* (ATCC 33592)-spiked BacT/ALERT blood culture bottles containing fresh blood from healthy volunteers. First, it was investigated how many CFU of *S. aureus* per ml needed to be present to be able to detect a signal with the LightCycler assay, therefore, 1,000, 100 and 10 CFU were spiked in different bottles, both standard aerobic (SA) and standard anaerobic (SN) BacT/ALERT bottles. BACTEC bottles were not investigated due to the poorer results obtained with all of the isolation methods analysed. The results of this experiment are depicted in Fig. 1 and indicate that 1,000 CFU of *S. aureus* (100 CFU/ml blood) could be detected by PCR immediately after spiking, indicating that no incubation step was required. The bottles

Table 1 Evaluation of six different DNA isolation protocols on blood culture material spiked with ten-fold dilution series of *Staphylococcus aureus*

DNA isolation protocol	Detection limit in CFU/ml					
	10 ⁵	10 ⁴	10 ³	10 ²	10	1
easyMAG generic	+	*	+	–	–	–
easyMAG specific A	+	*	+	–	–	–
easyMAG specific B	+	*	+	+	–	–
MagNA Pure LC	+	*	–	–	–	–
MolYsis Plus	+	*	–	–	–	–
MolYsis Plus + easyMAG specific B	+	*	+	+	+	–

*BACTEC; + BacT/ALERT; – not detected

A

Bacterial load CFU/bottle	Timepoints (hours)					
	0	1	2	3	4	5
1000	1/1	1/1	1/1	1/1	1/1	1/1
100	0/1	1/1	1/1	1/1	1/1	1/1
10	0/8	0/8	0/8	2/8	5/8	8/8

B

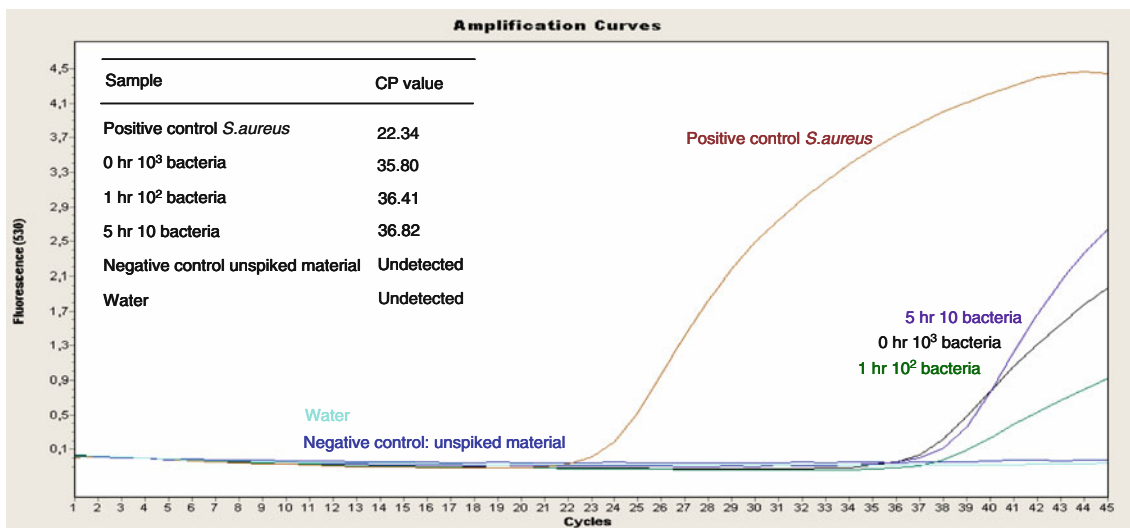


Fig. 1 *Staphylococcus aureus* detection in relation to reduced bacterial culture times. **a** Overview of the detection of *S. aureus* in relation to reduced culture times. BacT/ALERT blood culture bottles with the blood of healthy volunteers were spiked with *S. aureus* and samples were taken every hour. The experiments with 1,000 and 100 bacteria per bottle were performed once. The 1,000 bacteria/bottle experiment did not need a pre-culture step; a *tuf* real-time polymerase chain reaction (PCR) signal could be detected immediately after spiking (0 h). The 100 bacteria/bottle needed a pre-culture step of 1 h

in the BacT/ALERT 3D to obtain a positive signal in the real-time PCR. The experiment with 10 bacteria/bottle (10 ml blood) was repeated eight times. Real-time PCR signal could be detected after a pre-culture step of 3–5 h. All bottles, 8 of 8, were positive after 5 h of pre-culture. Standard deviations (SD) for 10 CFU/bottle were calculated as follows: 3 h SD 3.44, 4 h SD 1.09 and 5 h SD 1.09. **b** LightCycler *tuf* real-time PCR result in the 530-nm fluorescence channel (*S. aureus* probe)

containing 100 CFU of *S. aureus* (10 CFU/ml blood) resulted in positive real-time PCR signals after 1 h of incubation in the BacT/ALERT 3D. The 10-CFU bottles (1 CFU/ml blood) needed a maximum of 5 h of incubation in the BacT/ALERT 3D to be able to detect the *S. aureus* bacteria present using a subsequent PCR. The experiment with 10 CFU of *S. aureus* per bottle was repeated eight times with both aerobic and anaerobic bottles and showed reproducible results. Standard deviations (SD) of the Cp values were calculated for the 10 CFU/bottle experiments (*aureus* probe). The 3-h time point (2/8 positive) showed an SD of 3.44, the 4-h time point (5/8 positive) indicated an SD of 1.09 and the 5-h time point (8/8 positive) resulted in an SD of 1.09. Clinical strains of *S. aureus* and *S. epidermidis* were also spiked in BacT/ALERT blood culture bottles (aerobic [SA] and anaerobic [SN]) containing the blood of healthy volunteers. The results of these experiments were similar to

the results presented for the *S. aureus* ATCC 33592 strain. With conventional detection methods, the results of the bottles containing 10 CFU of *S. aureus* were available after at least 28 h.

Discussion

In this study, six different bacterial DNA extraction protocols were evaluated, by using a sensitive and specific *tuf* gene-based real-time PCR detection assay, which enabled the differentiation between *S. aureus* and CNS. From this study, it can be concluded that the MoLYsis Plus kit (Molzym) in combination with the specific B protocol on the easyMAG (bioMérieux) resulted in the most optimal isolation of bacterial DNA from BacT/ALERT blood culture material. A detection limit of 10 CFU/ml was

obtained in the *tuf* LightCycler assay for BacT/ALERT material. By using the *tuf* LightCycler assay, 1 CFU/ml of *S. aureus* or CNS could be detected from BacT/ALERT blood culture material when an incubation step of a maximum of 5 h was applied. By using this reduced incubation time, it was possible to identify *S. aureus* versus CNS within one working day.

Six different bacterial DNA extraction methods were compared on two commonly used blood culture systems (BACTEC and BacT/ALERT). The specific B protocol on the easyMAG contains the most washing steps compared to the generic and specific A protocol, possibly explaining the better results obtained with this isolation protocol. The bacterial DNA isolation performed with the MoLYsis Plus kit resulted in a low detection limit for both culture systems. Gebert et al. published a study in 2008 in which they show that the MoLYsis Plus kit performs well in their hands [25]. They used 500 µl of input material and were able to conclude that PCR analysis in combination with MoLYsis Plus DNA isolation resulted in accelerated detection of pathogens in blood culture samples. Inhibiting factors were not found to be present in the samples in the study of Gebert et al. [25]. In our study, no PCR inhibition was observed (all samples and controls showed similar Cp ± 0.5 values in one run). The detection limit in the LightCycler assay, after MoLYsis Plus isolation, was low, i.e. 10⁴ CFU/ml. The MoLYsis Plus kit eliminates human background DNA from the samples and enrichment of bacterial DNA takes place. Complete isolation with the MoLYsis Plus kit takes 3 h, of which 2 h is hands-on time. By using the easyMAG specific B protocol after completion of the MoLYsis Plus Buglysis step, the total isolation time is not significantly reduced. However, the hands-on time and the chance for contamination are drastically reduced, due to the fact that column handling is skipped. A comparison between both culture systems resulted in a difference in bacterial DNA isolation efficiency. The use of BacT/ALERT blood culture material led, in most isolation methods, to better results compared to BACTEC material. We experienced difficulties in the bacterial DNA isolations due to the beads present in BACTEC bottles, but we cannot conclude that the beads lead to the weaker performance of BACTEC material compared to BacT/ALERT material. However, due to this finding, all experiments were continued with the BacT/ALERT material.

By applying a 5-h incubation step for blood culture bottles containing approximately 10 CFU of *S. aureus* or CNS, it was possible to detect these bacteria by the *tuf* LightCycler assay. These results could be produced within one working day when starting early in the morning. Compared to conventional culture techniques for staphylococci, the results were available at least 19 h faster. Gebert et al. [25] also showed in their paper that, by combining a

sensitive real-time PCR and a sensitive isolation method, culture times can be reduced. They showed a 5.0–8.7-h time gain in spiking experiments and an average time gain of 10.7 h was obtained when the method was applied on blood culture bottles of septic patients. The maximum time gain can be reached when starting either immediately in the morning or when the microbiology laboratory has a 24-h service. Laboratories with a 24-h setup will always benefit from this method.

The experiments described in this paper all focus on the accelerated detection of staphylococci, especially on the detection of *S. aureus*. Staphylococci were chosen as a model system because Gram-positive bacteria are frequently detected in blood culture bottles, making it a relevant group of micro-organisms for such a study [26–28]. Infections are, however, caused by a variety of pathogens. Staphylococci are fast-growing bacteria and, in this study, a proof-of-principle is shown. The accelerated detection of other pathogens causing bloodstream infections (i.e. *Escherichia coli*) directly from blood culture material in combination with reduced culture time and with this sensitive isolation method should be investigated in future experiments. It is of clinical importance in the future to focus especially on fastidious pathogens or fungi, since those pathogens are difficult to culture and, for these pathogens, the time to obtaining results can then, hopefully, be drastically reduced. A similar approach as presented in this study can be applied to investigate reduced incubation times for other pathogens.

It is known from the literature that staphylococci are the most frequent causative agent of medical-device-related and surgical-site infections [7]. Therefore, it is interesting to apply the *tuf* LightCycler duplex assay in these patient groups using whole blood samples. The preliminary results showed that the MoLYsis Complete kit (Molzylm) has already proven to be successful in our hands (data not shown), as well as in the hands of others [29].

In conclusion, by combining the MoLYsis Plus kit and the specific B protocol on the easyMAG, 10 CFU/ml could be detected from *S. aureus*-spiked BacT/ALERT blood culture material. By applying this isolation method followed by the sensitive *tuf* LightCycler duplex assay, using 5-h-incubated spiked blood culture material, 1 CFU of *S. aureus* or CNS per 1 ml of blood could be detected. Future studies are needed in order to address the clinical application of this approach.

Acknowledgements We thank Wendy Hansen, Jitske Stalpers, Marielle Bierma, Christel van Herk, Paul van Kaathoven, Gitta Stienen and Jan Michielsse for their technical support.

Conflict of interest The authors declare that they have no conflict of interest.

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