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1 **Acceleration of direct identification of *Staphylococcus aureus* versus**
2 **Coagulase Negative Staphylococci from blood culture material: a comparison**
3 **of six bacterial DNA extraction methods**

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26 **Running title:** Accelerated detection of *S.aureus* vs. CNS from blood culture material

27 **Keywords:** Blood culture material, bacterial DNA isolation, real-time PCR, Staphylococci

28 **Abstract**

29 To accelerate differentiation between *Staphylococcus aureus* and Coagulase
30 Negative Staphylococci (CNS), this study aimed to compare six different DNA
31 extraction methods from 2 commonly used blood culture materials, i.e. BACTEC and
32 Bact/ALERT. Furthermore, we analyzed the effect of reduced blood culture
33 incubation for detection of staphylococci directly from blood culture material.

34 A real-time PCR duplex assay was used to compare 6 different DNA isolation
35 protocols on two different blood culture systems. Negative blood culture material was
36 spiked with MRSA. Bacterial DNA was isolated with: automated extractor EasyMAG
37 (3 protocols), automated extractor MagNA Pure LC (LC Microbiology Kit M^{Grade}), a
38 manual kit MoYsis Plus, and a combination between MoYsis Plus and the
39 EasyMAG. The most optimal isolation method was used to evaluate reduced
40 bacterial incubation times.

41 Bacterial DNA isolation with the MoYsis Plus kit in combination with the specific B
42 protocol on the EasyMAG resulted in the most sensitive detection of *S.aureus*, with a
43 detection limit of 10 CFU/ml, in Bact/ALERT material, whereas using BACTEC
44 resulted in a detection limit of 100 CFU/ml.

45 An initial *S.aureus* or CNS load of 1 CFU/ml blood can be detected after 5 hours of
46 incubation in Bact/ALERT3D by combining the sensitive isolation method and the tuf
47 LightCycler assay.

48 **Introduction**

49 *S.aureus* is a pathogen which can cause both hospital- and community associated
50 infectious diseases, ranging from minor skin infections to endocarditis, bacteremia,
51 sepsis and septic shock [1]. Sepsis can result in high morbidity and mortality. In the
52 United States, blood stream infections develop in approximately 250.000 people
53 yearly [2]. In the Netherlands, the incidence of patients admitted to the ICU with
54 severe sepsis ranges around 8643 ± 929 per year [3]. Currently, blood culture is the
55 gold standard for identification of pathogens from suspected bacterial sepsis patients.
56 Unfortunately, blood culture is time-consuming taking at least 24-72 hours for final
57 determination of the bacteria causing the disease. Staphylococci are number one of
58 Gram positive organisms in blood cultures. Differentiating *S.aureus* from Coagulase
59 Negative Staphylococci (CNS) is important, because sepsis with *S.aureus* is
60 common and virulent with mortality rates in the range of 20-30% [4]. CNS are often
61 considered as being contaminants in blood cultures due to the fact that these species
62 are members of the normal skin flora and mucous membranes and can contaminate
63 the sample when it is taken. However, it is known that CNS infections are
64 increasingly recognized as clinically relevant infections and confirmation on the
65 presence of these species in blood culture is therefore important (reviewed in [5-8]).
66 Several molecular methods for rapid accurate detection of bacteria, from positive
67 blood culture material have been described, including (commercial) real-time PCR
68 based diagnostic tests [9-11], fluorescence in situ hybridization [12, 13], MALDI-TOF-
69 MS [14], but also DNA micro arrays [15, 16]. However, all these techniques are used
70 on positive blood culture material. Reduction in the time to result can be achieved by
71 applying molecular methods either directly on whole blood or on blood culture
72 material with reduced incubation times. Ideally usage of whole blood is preferred but
73 the techniques that are now available are often, clinically, not sensitive enough, as
74 has been shown by others investigating a commercial real-time PCR test available
75 [17-19]. Blood culture materials are known to contain inhibiting factors which can
76 reduce detection in a sensitive real-time PCR [20-23]. It is therefore important to
77 include a good isolation method, in the molecular diagnostic strategy, which is able to
78 efficiently remove inhibiting factors and one which still allows sensitive DNA detection
79 by PCR. In this study we compared 6 different, both manual and automated, bacterial
80 DNA isolation methods for 2 commonly used blood culture systems, i.e. BACTEC

81 (Becton Dickinson) and Bact/ALERT (bioMérieux), to be able to find the most
82 sensitive bacterial DNA isolation method. Additionally, we investigated the
83 occurrence of inhibition in PCR amplification after DNA isolation. A sensitive real-time
84 PCR assay was designed to be able to detect staphylococci and to differentiate
85 *S.aureus* from CNS [Loonen A.J.M. *et al.*, manuscript submitted]. Subsequently, this
86 real-time PCR was used in combination with the optimal DNA isolation method to
87 investigate the level of time reduction to identify staphylococci from blood culture
88 material. Results were compared with conventional blood culture techniques used in
89 diagnostic laboratories.

90 **Materials and Methods**

91 **Culture methods:** Negatively cultured blood culture bottles, derived from routine
92 diagnostics of both BACTEC (Becton Dickinson, the Netherlands) and Bact/ALERT
93 (bioMérieux, the Netherlands) systems, were used in this study for spiking
94 experiments (bottles have been cultured for 1 week and remained negative).
95 Additionally, healthy volunteers donated 10 ml blood as input for the Bact/ALERT
96 bottles. Bottles were incubated and handled according to manufactures protocol.

97 **DNA isolation methods:** Methicillin-resistant *S.aureus* ATCC 33592 was cultured
98 overnight on bloodagar. Fresh plates were used to make a 2 McF solution which
99 was subsequently serially diluted (1:10) in negative blood culture material from both
100 systems (BACTEC and Bact/ALERT). One hundred µl was plated on bloodagar
101 plates to check CFU per sample. Three different isolation protocols on the NucliSens
102 EasyMAG (bioMérieux) were tested (generic, specific A and specific B protocol: main
103 difference between protocols is the number of washing steps, with least steps in the
104 generic protocol and most washing steps in the specific B protocol), 1 isolation kit
105 (LC Microbiology Kit M^{Grade}) on the MagNA Pure LC (Roche Diagnostics), the
106 MolYsis Plus kit (Molzym GmBh, Bremen, Germany) was used according to Molzym
107 instructions and in combination with the EasyMAG. When MolYsis was used in
108 combination with the EasyMAG, manufacturer's instructions (Molzym) were followed
109 until the Buglysis step and subsequently bacterial DNA was extracted according to
110 the specific B protocol on the EasyMAG. Input volume in each isolation was 200µl,
111 only for the MagNA Pure LC 100µl was used, and samples were always eluted in
112 100µl elution buffer provided with each isolation method. Each extraction was
113 performed at least 2-3 times in independent experiments. Phocine herpes virus type
114 1 (PhHV-1) was added to each sample eluate as an amplification and inhibition
115 control. Samples were analyzed with the LightCycler assay described below.

116 **LightCycler assay:** A duplex real-time PCR, detecting the *tuf* gene of both
117 staphylococci and *S.aureus* specifically, is used as described in detail elsewhere
118 [Loonen A.J.M. *et al.*, submitted]. In brief, the positions of the primers and probes
119 were derived from the *S. aureus tuf* gene sequence (GenBank accession no.
120 AF298796). The sequences of the primers and probes were as follows;
121 *Staphylococcus* spp. forward primer ccaatgccacaaactcgtga (position 32-51),

122 Staphylococcus spp. reverse primer caccgaccagtgattgagaatac (position 371-349),
123 *S.aureus* forward primer tcctgggtcaattacaccacatactg (position 583-608), *S.aureus*
124 reverse primer ggaaatagaattgtggacgatagttga (position 698-671), Staphylococcus
125 spp. probe VIC-ccattcatgatgccagttg-BHQ1 (position 326-344) , *S.aureus* probe FAM-
126 tgataatacgtataacttctgc-BHQ1 (position 637-617). Oligonucleotide primers were
127 obtained from Eurogentec (Liège, Belgium), Staphylococcus XS probe and the
128 *S.aureus* XS probe were obtained from Biolegio (Nijmegen, the Netherlands). PhHV-
129 1 primers and VIC labeled probe [24] were obtained from Applied Biosystems
130 (Nieuwerkerk aan den IJssel, the Netherlands). PCR mixtures consisted of 5x
131 LightCycler TaqMan Master mixture (Roche Diagnostics), 0.9µM concentration of
132 each primer, 0.2µM concentration of each probe, and aquadest in a volume of 15µl to
133 which 5µl DNA sample was added, leading to a final reaction volume of 20µl in each
134 capillary. Primers and probe targeting PhHV had a concentration of 0.1µM each and
135 were used in a separate single real-time PCR. All samples were amplified on the
136 LightCycler v2.0 (Roche Diagnostics) according to the following cycling parameters:
137 an initial incubation step for 10 min at 95°C for enzyme activation and DNA
138 denaturation, subsequently 45 cycli of 15 sec 95 °C followed by 1 min 60°C. Results
139 were analyzed with LightCycler Software version 4.05 (Roche Diagnostics).

140

141 **Results**

142 **Evaluation of different bacterial DNA extraction methods**

143 In table 1 the results are presented of the comparison of 6 different bacterial DNA
144 extraction methods on *S.aureus* spiked blood culture material. The MagNA Pure LC
145 (Roche Diagnostics) resulted in a detection limit of 10^4 CFU/ml for both culture
146 systems. On the EasyMAG 3 protocols were evaluated to isolate bacterial DNA from
147 spiked clinical material, generic, specific A and specific B. We tested all 3 protocols
148 and usage of the specific B protocol resulted in the best detection limit of 10^2 CFU/ml
149 of *S.aureus* in Bact/ALERT material. Although MoYsis Plus did not result in a low
150 detection limit, we tested this manual kit in combination with the specific B protocol
151 on the EasyMAG. By combining both methods all human DNA was removed and
152 bacteria were lysed by the MoYsis Plus kit, and subsequently the EasyMAG was
153 used for optimal washing and elution of bacterial DNA. It was possible to reduce
154 hands-on time and avoid possible contamination due to skipping of manual
155 handlings. This combination of methods resulted in the most sensitive detection limit,
156 i.e. 10 CFU/ml, in Bact/ALERT material. Time to result for this combined method
157 followed by real-time PCR is approximately 4-5 hours, but strongly depending on the
158 number of samples. No inhibition was observed in the real-time PCR, all samples
159 and controls showed similar C_p values in one run ($C_p \pm 0.5$). All experiments were
160 performed at least 2-3 times.

161 **Reduced blood culture times**

162 To investigate the effect of reduced blood culture incubation for pathogen detection
163 by PCR a combination between the sensitive *tuf* LightCycler assay and the sensitive
164 isolation method, the MoYsis Plus Kit in combination with the specific B protocol on
165 the EasyMAG, was analyzed on *S.aureus* (ATCC 33592) spiked Bact/ALERT blood
166 culture bottles containing fresh blood of healthy volunteers. First it was investigated
167 how many CFU *S.aureus* per ml needed to be present to be able to detect a signal
168 with the LightCycler assay, therefore 1000, 100 and 10 CFU were spiked in different
169 bottles, both SA (Standard Aerobic) and SN (Standard Anaerobic) Bact/ALERT
170 bottles. BACTEC bottles were not investigated due to poorer results obtained with all
171 isolation methods analysed. The results of this experiment are depicted in figure 2
172 and indicate that 1000 CFU of *S.aureus* (100 CFU/ml blood) could be detected by

173 PCR immediately after spiking indicating that no incubation step was required. The
174 bottles containing 100 CFU of *S.aureus* (10 CFU/ml blood) resulted in positive real-
175 time PCR signals after 1 hour incubation in the Bact/ALERT 3D. The 10 CFU bottles
176 (1 CFU/ml blood) needed a maximum of 5 hours incubation in the Bact/ALERT3D to
177 be able to detect the *S.aureus* bacteria present using a subsequent PCR. The
178 experiment with 10 CFU *S.aureus* per bottle was repeated 8 times with both aerobe
179 and anaerobe bottles and showed reproducible results. Standard deviations (SD) of
180 the Cp values were calculated for the 10 CFU/bottle experiments (*aureus* probe).
181 Three hour time point (2/8 positive) showed SD of 3.44, 4hr timepoint (5/8 positive)
182 indicated SD of 1.09, and the 5 hr time point (8/8 positive) resulted in SD of 1.09.
183 Clinical strains of *S.aureus* and *S.epidermidis* were also spiked in Bact/ALERT blood
184 culture bottles (aerobe (SA) and anaerobe (SN)) containing blood of healthy
185 volunteers. The results of these experiments were similar as the results presented for
186 the *S.aureus* ATCC 33592 strain. With conventional detection methods, results of the
187 bottles containing 10 CFU *S.aureus* were available after at least 28 hours.

188

189 **Discussion**

190 In this study, 6 different bacterial DNA extraction protocols were evaluated, by using
191 a sensitive and specific *tuf* gene based real-time PCR detection assay, which
192 enabled the differentiation between *S.aureus* and CNS. From this study it can be
193 concluded that the MoLYsis Plus kit (Molzym) in combination with the specific B
194 protocol on the EasyMAG (bioMérieux) resulted in the most optimal isolation of
195 bacterial DNA from Bact/ALERT blood culture material. A detection limit of 10
196 CFU/ml was obtained in the *tuf* LightCycler assay for Bact/ALERT material. By using
197 the *tuf* LightCycler assay 1 CFU/ml *S.aureus* or CNS could be detected from
198 Bact/ALERT blood culture material when an incubation step of a maximum of 5 hours
199 was applied. By using this reduced incubation time it was possible to identify
200 *S.aureus* versus CNS within one working day.

201 Six different bacterial DNA extraction methods were compared on two commonly
202 used blood culture systems (BACTEC and Bact/ALERT). The specific B protocol on
203 the EasyMAG contains most washing steps compared to the generic and specific A
204 protocol, possibly explaining the better results obtained with this isolation protocol.
205 The bacterial DNA isolation performed with the MoLYsis Plus kit resulted in a low
206 detection limit, for both culture systems. Gebert *et al.*, published a study in 2008 in
207 which they show that the MoLYsis Plus kit performs well in their hands [25]. They
208 used 500µl input material, and were able to conclude that PCR analysis in
209 combination with MoLYsis Plus DNA isolation resulted in accelerated detection of
210 pathogens in blood culture samples. Inhibiting factors were not found to be present in
211 the samples in the study of Gebert *et al* [25]. In our study, no PCR inhibition was
212 observed (all samples and controls showed similar $C_p \pm 0,5$ values in one run). The
213 detection limit in the LightCycler assay, after MoLYsis Plus isolation, was low i.e., 10^4
214 CFU/ml. The MoLYsis Plus kit eliminates human background DNA from the samples
215 and enrichment of bacterial DNA takes place. Complete isolation with the MoLYsis
216 Plus kit takes 3 hrs, of which 2 hours hands-on time. By using the EasyMAG specific
217 B protocol after completion of the MoLYsis Plus Buglysis step, the total isolation time
218 is not significantly reduced. However, hands-on time and the chance for
219 contamination are drastically reduced, due to the fact that column handling is
220 skipped. A comparison between both culture systems resulted in a difference in
221 bacterial DNA isolation efficiency. Usage of Bact/ALERT blood culture material

222 led in most isolation methods to better results compared to BACTEC material.
223 We experienced difficulties in the bacterial DNA isolations due to the beads present
224 in BACTEC bottles, but we can not conclude that the beads led to the weaker
225 performance of BACTEC material compared to Bact/ALERT material. However, due
226 to this finding all experiments were continued with Bact/ALERT material.

227 By applying a 5 hour incubation step for blood culture bottles containing
228 approximately 10 CFU of *S.aureus* or CNS it was possible to detect these bacteria by
229 the *tuf* LightCycler assay. These results could be produced within 1 working day
230 when starting early in the morning. Compared to conventional culture techniques for
231 staphylococci, results were available at least 19hrs faster. Gebert *et al.*[25], also
232 showed in their paper that by combining a sensitive real-time PCR and a sensitive
233 isolation method, culture times can be reduced. They showed a 5.0-8.7 hours time
234 gain in spiking experiments and an average time gain of 10.7 hours was obtained
235 when the method was applied on blood culture bottles of septic patients. Maximum
236 time gain can be reached when starting either immediately in the morning or when
237 the microbiology laboratory has 24 hours service. Laboratories with a 24 hours setup
238 will always benefit from this method.

239 The experiments described in this paper all focus on accelerated detection of
240 staphylococci, especially on detection of *S.aureus*. Staphylococci were chosen as a
241 model system because Gram positive bacteria are frequently detected in blood
242 culture bottles, making it a relevant group of micro-organisms for such a study [26-
243 28]. Infections are, however, caused by a variety of pathogens. Staphylococci are
244 fast growing bacteria and in this study a proof-of-principle is shown. Accelerated
245 detection, of other pathogens causing blood stream infections (i.e. *E.coli*), directly
246 from blood culture material in combination with reduced culture time and with this
247 sensitive isolation method should be investigated in future experiments. It is of
248 clinical importance in the future to focus especially on fastidious pathogens or fungi,
249 since those pathogens are difficult to culture and for these pathogens time to result
250 can than hopefully be drastically reduced. A similar approach as presented in this
251 study can be applied to investigate reduced incubation times for other pathogens.

252 It is known from literature that staphylococci are the most frequently causative agent
253 of medical device related and surgical-site infections [7],. Therefore it is interesting to

254 apply the *tuf* LightCycler duplex assay in these patient groups using whole blood
255 samples. Preliminary results showed that the MoLYsis Complete kit (Molzylm) has
256 already proven to be successful in our hands (data not shown) as well as in the
257 hands of others [29].

258 In conclusion, by combining the MoLYsis Plus kit and the specific B protocol on the
259 EasyMAG 10 CFU/ml could be detected from *S.aureus* spiked Bact/ALERT blood
260 culture material. By applying this isolation method followed by the sensitive *tuf*
261 LightCycler duplex assay, using 5 hours incubated spiked blood culture material, 1
262 CFU *S.aureus* or CNS per 1 ml blood could be detected. Future studies are needed
263 to address the clinical application of this approach.

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267

268 **Conflict of interest**

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

270

271 **References**

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341

342

343

344 **Figures and Tables**

345 **Table 1. Evaluation of 6 different DNA isolation protocols on blood culture**
 346 **material spiked with 10-fold dilution series of *S.aureus***

347

DNA isolation protocol	Detection limit in CFU/ml					
	10 ⁵	10 ⁴	10 ³	10 ²	10	1
EasyMAG generic	+*	+*	+	-	-	-
EasyMAG specA	+*	+*	+*	-	-	-
EasyMAG specB	+*	+*	+*	+	-	-
MPLC	+*	+*	-	-	-	-
MolYsis Plus	+*	+*	-	-	-	-
MolYsis Plus + EasyMAG specB	+*	+*	+*	+*	+	-

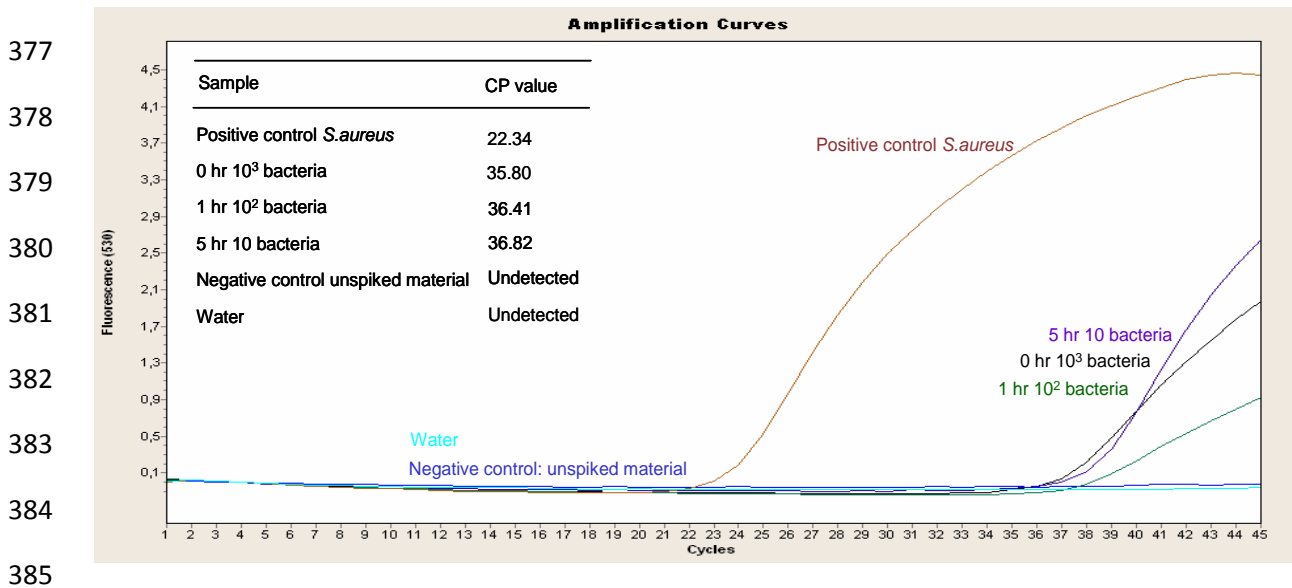
357 * BACTEC; + Bact/ALERT; - not detected

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371 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

376 B



386 **Figure 2. *S.aureus* detection in relation to reduced bacterial culture times.**

387 A, Overview of detection of *S.aureus* in relation to reduced culture times.

388 Bact/ALERT blood culture bottles with blood of healthy volunteers were spiked with
389 *S.aureus* and samples were taken every hour. The experiments with 1000 and 800
390 bacteria per bottle were performed once. Thousand bacteria/bottle did not need a
391 pre-culture step, a *tuf* real-time PCR signal could be detected immediately after
392 spiking (0 hr). Hundred bacteria/bottle needed a pre-culture step of 1 hour in the
393 Bact/ALERT 3D to obtain a positive signal in the real-time PCR. The experiment with
394 10 bacteria per bottle (10 ml blood) was repeated 8 times. Real-time PCR signal
395 could be detected after a pre-culture step of 3-5 hours. All bottles, 8 of 8, were
396 positive after 5 hours pre-culture. Standard deviations (SD) for 10 CFU/bottle were
397 calculated; 3 hr SD 3.44, 4 hr SD 1.09, and 5 hr SD 1.09. B, LightCycler *tuf* real-time
398 PCR result in the 530nm fluorescence channel (*S.aureus* probe).