Applications of LightCycler Staphylococcus M\textsuperscript{GRADE} assay to detect \textit{Staphylococcus aureus} and coagulase-negative staphylococci in clinical blood samples and in blood culture bottles

Applicazioni del saggio LightCycler Staphylococcus M\textsuperscript{GRADE} per l’individuazione di \textit{Staphylococcus aureus} e stafilococchi coagulasi-negativi in campioni clinici di sangue ed emocolture

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\section*{INTRODUCTION}

A rapid and specific way for detection of bacteremias represents one of the most important aims in clinical microbiology laboratory. Systemic infections still cause high rate of morbidity and mortality: a recent study reported on 750,000 septic patients per year and more than 210,000 of them died [1]. To further complicate this multi-factorial challenge, sometimes negative results from blood cultures can occur during the follow up of septic patient. \textit{Staphylococcus aureus} is one of the most frequent human pathogens involved in both skin and soft tissue infections, as well as in nosocomial sepsis originated from infected surgical wounds and contaminated intravascular catheters [2]. Also primary bacteremia, endocarditis and sepsis with severe medical \textit{sequelae} are often caused by \textit{S. aureus} especially in debilitated patients [3]. Moreover, epidemiological data show an increase of coagulase-negative staphylococci (CoNS) infections, generally associated with medical devices as catheters, artificial joints and prosthetic heart valves [4].

Given the high percentage (19.7\% at one month and 37.6\% at one year) of fatal outcome of staphylococcus systemic infections, there is the need of methods for an earlier diagnosis followed by antimicrobial therapy to decrease death rate [5]. New assays based on real-time PCR represent improved methods able to amplify and quantify sequences from microorganism genome directly in specimens or pure cultures [6]. For this reason, we tested a rapid and reproducible method, the LightCycler \textit{Staphylococcus M\textsuperscript{GRADE}} assay, for the detection and differentiation of \textit{S. aureus} and coagulase-negative staphylococci (CoNS) in clinical samples by using Fluorescence Resonance Energy Transfer (FRET) probes developed for LightCycler instrument. Differentiation is based on melting curve analysis subsequent to PCR amplification.

\section*{MATERIALS AND METHODS}

We first evaluated the applicability of this assay by adding known amount of \textit{S. aureus} and/or
CoNS cells to blood samples, extracting the DNA and demonstrating reproducibility, specificity and sensitivity. Then we evaluated the assay testing blood samples, and blood culture samples from 31 intensive care unit hospitalized patients with suspected staphylococcus systemic infections. The results were compared with those of standard blood culture and identification methods.

Bacterial strains
Four different strains of S. aureus, 4 different strains of Staphylococcus epidermidis and 2 strains of Staphylococcus haemolyticus used in this study were obtained from a collection of blood culture isolates previously identify in our laboratory.

Artificially spiked blood specimens: detection limit and specificity assay evaluation
In order to evaluate the detection limit of our method, an amount of 5 ml of whole blood from healthy volunteers was artificially spiked in titration experiments with three different S. aureus strains to a final concentrations of $10^4$, $10^3$, $10^2$, 10 CFU/ml. In order to test assay specificity, 5 ml of whole blood from healthy volunteers were artificially spiked with 4 different strains of S. aureus, 4 different strains of S. epidermidis and 2 strains of S. haemolyticus to a final concentration of $10^5$ CFU/ml. DNA was extracted from samples and analyzed in triplicate.

Standard blood culture system
All blood cultures from 31 intensive care unit hospitalized patients were evaluated in the BACTEC 9120 blood culture system (Becton-Dickinson Diagnostic Instrument System, Sparks Md.) which detected microbial growth by continuous monitoring. Blood samples (10 ml) were inoculated into two types of culture bottles (BACTEC Plus aerobic/F and BACTEC Plus anaerobic/F for aerobic and anaerobic cultures, respectively). BACTEC Plus aerobic/F bottles that signaled positive were identified, Gram-stained and inoculated onto 5% sheep blood agar; if Gram-positive cocci in clusters were seen, a 100 µl aliquot for DNA extraction was removed.

Patient whole blood specimens
EDTA-anticoagulated whole blood specimens were taken in parallel from each patients. An aliquot of 100 µl was used for DNA extraction.

DNA extraction
DNA extraction from blood samples and blood cultures was performed by using the MagNA Pure LC Microbiology MGRADE combined to Magnetic Particle Separator (MGP's) by means of a manual procedure. Briefly, in a pre-incubation step, in order to achieve optimal sensitivity, 100 µl of clinical specimens were added to 130 µl of Bacteria lysis buffer and 20 µl of Proteinase K, mixed with vortex, incubated for 10 min at 65°C, incubated again for 10 min at 95°C and restored at room temperature. Then 300 µl of Lysis/Binding buffer and 1 µl (in each sample) of Internal Control (IC) were added to pre-processed samples and mixed with vortex. Special buffer (150 µl) containing chaotropic salt and magnetic glass particles (MGP's) were added and vials vortexed. In this process, DNA binds to the silica surface of the added MGP's due to the salt conditions and the high ionic strength of the Lysis/Binding buffer. During separation of DNA binding-MGP's by Magnetic Particle Separator, unbound substances such as proteins, cell debris and PCR inhibitors, were removed by three washing-vortexing steps using 450 µl of three different washing buffers. Then purified DNA was eluted using elevated temperature (80°C) and an elution buffer (low salt conditions). A kit-specific IC (Roche Diagnostics, Mannheim, Germany) has been performed and associated to LightCycler Staphylococcus MGRADE assay in order to reduce inhibitors which can reduce PCR efficiency and interfere with PCR amplification. IC is a synthetic double-stranded DNA molecule with primer binding sites identical to the Staphylococcus target sequence, which contain a unique hybridization probe binding region that differentiates IC from target specific amplicon. We added IC directly into the Lysis/Binding buffer to co-extract together with sample. IC would co-amplify by the same primer pair as the analyte, but would be detected by a second set of hybridization probes (included in the detection mixture). This process allows to control the entire procedure.

Real-time PCR procedure
The real-time PCR were carried out with LightCycler Staphylococcus MGRADE assay developed for Light Cycler instrument (Roche Diagnostics, Mannheim, Germany). Specific primers directed to the internal transcribed spacer (ITS) regions of the rRNA operon were used. Amplification reactions were performed in glass cap-
illaries, which ensure quick equilibration between air and components due to the high ratio surface: volume. For amplicon detection the LightCycler *Staphylococcus* MGRADE kit was used as described by manufacturer. Briefly, a volume of 20 µl containing 5 µl of DNA template and 15 µl of master mixture were mixed, centrifuged at 700 x g for 5s and transferred into the carousel of Light Cycler instrument.

Amplification mixture contained FastStart Taq polymerase, FastStart Taq polymerase buffer, a deoxynucleoside triphosphate mixture (with dUTP instead of dTTP), primer and hybridization probe mixture specific for Staphylococcus DNA and the Staphylococcus IC (previously described).

Hybridization probes consist of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase. One probe is labeled at the 5' end with LightCycler Red 640 and to avoid extension modified at the 3' end by phosphorylation. The other probe is labeled at the 3' end with fluorescein. After specific hybridization, the two probes come in close proximity resulting in fluorescence resonance energy transfer between the two fluorophores. During FRET, fluorescein is excited by light source of the Light Cycler instrument; the excitation energy is transferred to the acceptor fluorophore, LightCycler Red 640, and the emitted fluorescence is measured by such an instrument.

The reaction protocol was as follows: an initial FastStart DNA Taq polymerase activation and denaturation of the template DNA at 95°C for 10 min, a 45-cycles amplification consisting of a 95°C segment for 10 s, a 50°C segment for 15 s and a 72°C segment for 10 s. The assay, including PCR setup and PCR run on Light Cycler instrument, was completed within 90 min. A melt phase from 40 to 80°C with a temperature transition rate of 0.1°C/s and a rapid cooling phase were performed to determine melting curves.

**RESULTS**

**Melting curve analysis**

Result interpretation is based on melting curve analysis subsequent to PCR amplification, as

![Figure 1 - Melting curves analysis for the evaluation of detection limit and specificity of LightCycler *Staphylococcus* MGRADE assay on blood samples. Different batches of the same blood samples were spiked with *S. aureus* and *S. epidermidis* at different concentrations (10^4 - 10^2 CFU/ml). Left and right groups of curves refer to different melting points for *S. aureus* (including its positive control) and *S. epidermidis*.](image-url)
provided for by the LightCycler software. The melting temperatures of DNA from *S. aureus*, IC and CoNS are 62.1°C ± 2°C, 56.6°C ± 2°C, ranging from 45.4°C ± 2°C to 57.3°C ± 2°C respectively. Preliminary experiments carried out to evaluate blood as useful sample to apply this method show the applicability and reproducibility of the assay.

Artificially spiked blood specimens: detection limit and specificity assay evaluation
To determine the detection limit of LightCycler *Staphylococcus MGRADE* on whole blood, samples from healthy volunteers was artificially mixed with *S. aureus* (10⁴ to 10 CFU/ml). The genomic DNA extracted was used as described previously. To evaluate the specificity of LightCycler *Staphylococcus MGRADE* in the differentiation between *S. aureus* and CoNS, whole blood samples from healthy volunteers were artificially mixed with *S. aureus*, *S. epidermidis*, and/or *S. haemolyticus* at different concentrations (10⁴ to 10 CFU/ml). Figure 1 shows results from a representative experiment: it can be seen the melting curves obtained in triplicate from the same extracts of blood samples spiked with different concentrations of *S. aureus* and *S. epidermidis* previously identify in our laboratory. Melting temperatures differentiating the two species are evident, as well as positive control melting temperature. The detection limit of LightCycler Staphylococcus MGRADE on whole blood samples was of 10² CFU/ml for both strains.

Clinical samples
Concerning clinical samples, seven patients were negative in all tested procedures, nine patients were positive for *S. aureus* by LightCycler instrument both from blood and blood bottles; these results were confirmed by blood cultures. Fourteen patients were positive for CoNS by LightCycler Instruments both from blood and blood bottles, whereas one patient resulted positive for CoNS by LightCycler instrument only from blood bottles (Table 1).

### DISCUSSION
As the rate of mortality due to staphylococci systemic infections, especially in high risk patients, is over 19.7% at 1 month and clinical signs are often non-specific, a PCR-based method for detection/differentiation of *S. aureus* and CoNS must be rapid to be of clinical benefit [5].

Up to date molecular diagnostic assays based on the detection of the mecA gene encountered difficulty in discriminating MRSA from methicillin-resistant CoNS because the mecA gene is widely distributed in *S. aureus*, as well as in CoNS [7]. Other investigators used 16S rRNA sequences as target of their PCRs. However, interpretation of results from such experiments should be more complicated by closely related species that have similar or identical 16S rRNA sequences or because different 16S rRNA sequences (genic polymorphism) can be found within a single organism [8].

On the contrary, spacer regions separating the two bacterial rRNA genes, are well known for their high degree of sequence and length variation at both the genus and species levels. The reported assay represents a rapid and powerful method for detection of *S. aureus* directly from clinical specimens containing also a mixture of staphylococci differentiating *S. aureus* from CoNS. Particularly, specificity cer-

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**Table 1 - LightCycler Staphylococcus MGRADE assay from blood samples and blood culture bottles.** As gold standard, data produced by BACTEC 9120 blood culture system are also reported

<table>
<thead>
<tr>
<th>Organism</th>
<th>Whole blood samples</th>
<th>Blood Culture bottles</th>
<th>Blood culture system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>CoNS</td>
<td>14</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>No isolates</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

Sensitivity = 100% and specificity = 100% for detecting *S. aureus* in both blood culture bottles and in blood samples.
Sensitivity = 93.33% and specificity = 100% for detecting CoNS in blood samples. Sensitivity = 100% and specificity = 100% for detecting CoNS in blood culture bottles.
tified by the producer and our data consistently indicate that although the test is very sensitive, the specificity is warranted based on the difference between melting curves. This method can be applied on blood samples, it is reproducible starting from the same sample of extracted DNA and allows the detection of S. aureus and the differentiation from CoNS with a sensitivity limit of 10^2 CFU/ml.

We confirm that use of IC (see Material and Methods), during extraction and LightCycler Staphylococcus M^GRADE assay, is very important to control both the extraction and amplification procedures.

Only few recent articles were dealing with a real-time PCR assay detecting S. aureus in blood culture bottles [7, 9-11]. Often the methods used did not correctly detect 100% of S. aureus strains from blood bottle samples. Such a correct identification of the causative organism offers an indirect information on the antimicrobial susceptibility features of the bacterium. Thus the physician might limit the use of broad-spectrum antibiotics, and reduce the cost of the therapy, as well as the emergence of antibiotic resistance [11]. Furthermore, since few years a number of relevant papers appeared dealing with the molecular methods aiming to detect MRSA strains, as well as other strains that developed other types of antibiotic resistance [12, 13].

The tested method improves S. aureus diagnosis in patients with suspected systemic infection, giving results earlier than blood cultures in terms of both positivity and identification of species (S. aureus). Difference in carrying over time between conventional methods and our test is obvious, due to the necessity of a 1-2 days for growth and another 1-2 days for identification. On the other hand all PCR types, but real-time PCR, need more than one step (e.g. amplification and then probing of the amplicons). On the contrary, our method includes both amplification and detection within the same step, with significant saving of time and elimination of post-amplification contaminants.

Melting curves can be considered of extreme utility as diagnostic parameters. The low detection limit, obtained in our study, might be improved using an automated extraction system (as described by the manufacturer).

**Key words:** real-time PCR, Staphylococcus aureus, CoNS, blood samples

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**SUMMARY**

We evaluated the applicability of the LightCycler Staphylococcus M^GRADE assay on artificially infected blood samples from healthy donors and on clinical specimens of 31 hospitalized patients. The sensitivity and specificity of the assay for detecting Staphylococcus aureus was 100% in blood samples, and 100% in blood culture bottles, when data from the BACTEC 9120 blood culture system were taken as gold standard. The same specificity and sensitivity was found during the search for CoNS (Coagulase Negative Staphylococci) in blood culture bottles, whereas a 93.33% sensitivity and 100% specificity was observed for detecting CoNS directly in blood clinical specimens.

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**RIASSUNTO**

Abbiamo valutato l’applicabilità del saggio LightCycler Staphylococcus M^GRADE su campioni di sangue di volontari sani infettati in vitro e su campioni clinici di 31 pazienti ospedalizzati. La sensibilità e la specificità del saggio nell’individuare Staphylococcus aureus è stata del 100% nei campioni di sangue e del 100% nei flaconi delle emocolture, considerando come gold standard i dati ottenuti dal sistema automatizzato per emocolture BACTEC 9120. La stessa specificità e sensibilità è stata dimostrata nella ricerca per CoNS (Coagulase Negative Staphylococci) in flaconi da emocolture, mentre una sensibilità del 93.33% ed una specificità del 100% sono state osservate per l’individuazione di CoNS direttamente da campioni clinici di sangue intero.
REFERENCES