Detection and differentiation of the eukaryotic and prokaryotic pathogenes

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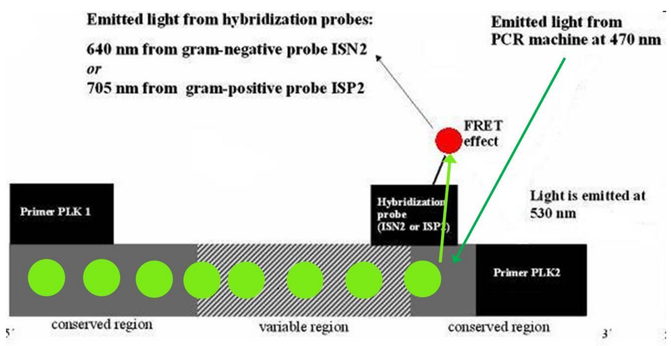
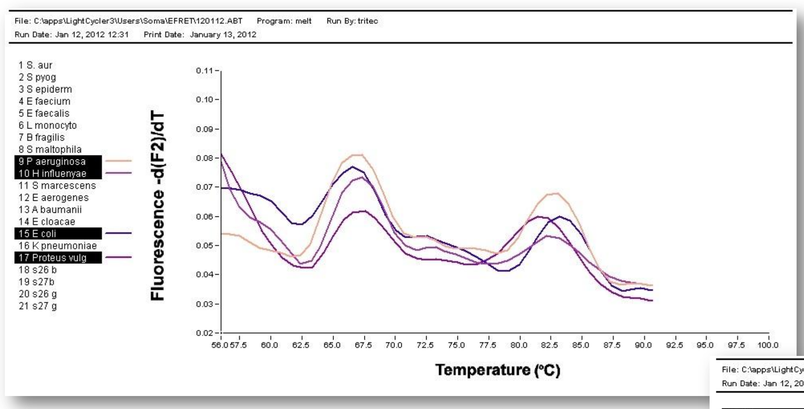
CROSSROADS IN BIOLOGY
4th International Symposium

INTRODUCTION

Early detection of bacterial and fungal infections are critical for successful outcomes for patients with systemic infections. The application of blood culturing techniques are time consuming and the identification of fungal pathogens are difficult. Our aim was to find a rapid molecular diagnostic tool to detect and to differentiate the most common pathogens in intensive care units in one single experiment. The identification and the differentiation took place in one real-time PCR reaction. We used two specific primer pairs to differentiate the fungal and bacterial amplicons by capillary real-time PCR. Our idea was to separate these causative microorganisms with the help of melting point analysis. As regard to fungi it was easy to separate each species with the help of the aspecific EvaGreen dye melting point analysis of the amplicons due to the extreme variability of the ITS2 region. We wanted to separate the most common bacterial pathogens with the help of the procariotic specific 16S rDNA region. In order to detect and differentiate Gram-positive (G+) and Gram-negative (G-) bacteria we used a G+ and a G- specific F-RET probes, which are the recommended probes of the LightCycler system. The novelty of the system was the use of a nonspecific EvaGreen dye instead of labeled anchor probes to excite the acceptor dyes on probes.Thus we used the upper channels (640 and 705 nm) of the LC for the bacterial detection while the fungal detection happened on the lower one (580 nm). The melting point analysis following the amplification was appropriate to discriminate the seven most frequent *Candida* species. We could also differentiate G+ and G- bacteria in the same tube with the help of the melting temperatures of the probes.

Figure 1. Primer and probe composition of bacterial PCR

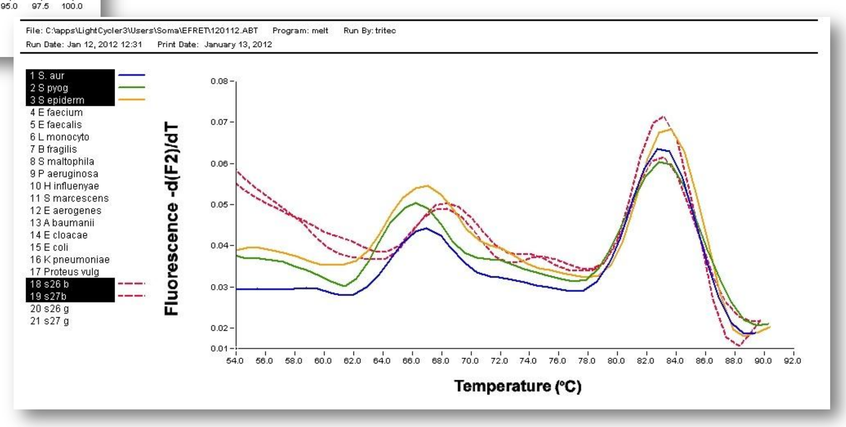




F2 channel G-

Figure 2. Differentiation of G+ and G- bacteria in different wavelengths

F3 channel G+



MATERIALS AND METHODS

Microbial samples

6 reference fungal- (*C. albicans* (ATCC 10231, *C. tropicalis* (ATCC 4893; CBS 94), *C. glabrata* (ATCC 90030; CBS 138), *C. parapsilosis* (ATCC 22019; CBS 604), *C. krusei* (CBS 573) and *A. fumigatus* (clinical isolate) and *Staphylococcus epidermidis* (OKI 80001) as G+ and *Escherichia coli* (ATCC 25922) as G- strains were analyzed.

DNA preparation

DNA extraction was carried out as reported by Liu et al. [4].

Primers and DNA amplification

Primers and probes are listed in Table 1. The construction af bacterial primers and probes are depicted in Fig. 1. SsoFast EvaGreen (BioRad Laboratories) was used according to the manufacturer instructions. DNA has been amplified by the LightCycler (Roche) real-time PCR as described previously [3].

Table 1. Primers and probes

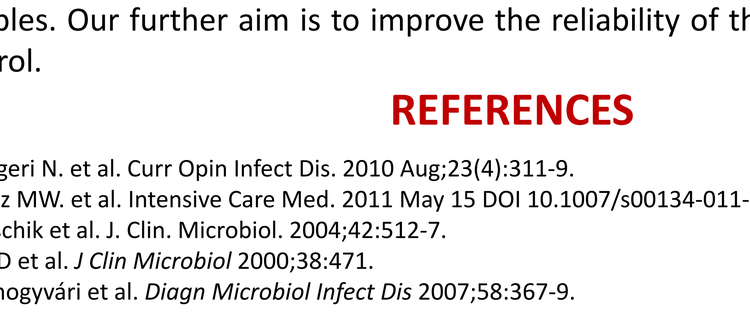
Fungal primers are described anywhere [5]. The bacterial primers and probes described by Klaschik et al. [3]. The PLK2 primer have modified (no internal fluorescein).

Primer			Sequence (5'-3')
ITS86	Between 5.8S rRNS and 28S rRNS	Fungal forward	gTg AAT CAT CgA ATC TTT gAA C
ITS4	Between 5.8S rRNS and 28S rRNS	Fungal reverse	TCC TCC gCT TAT TgA TAg C
PLK1	16S rRNS	Bact. forward	TAC ggg Agg CAg CAg
PLK2	16S RNS	Bact. reverse	TAT TAC CgC ggC TgC T
ISN2		G- spec. probe	CCG CAG AAT AAG CAC CGG CTA ACT CCG T - LCRed640
ISP2		G+ spec. probe	CCT AAC CAG AAA GCC ACG GCT AAC TAC GTG - LCRed705

The Project named „TÁMOP-4.2.2/B-10/1-2010-0012 – Creating the Center of Excellence at the University of Szeged" is supported by the European Union and co-financed by the European Regional Development Fund.

RESULTS

We could differentiate the G+ and G- bacteria and the fungi in the same tube with the analysis of the melting temperature of the probes and the aspecific EvaGreen dye. The analysis take place in different channels. The species and the melting points were depicted in Fig. 2. The melting point analysis on F1 channel was appropriate to discriminate the seven clinically most significant *Candida* species (Fig. 3). These differences are sufficient for the accurate differentiation.



F1 channel Fungi

Figure 3. Differentiation of fungi.

C. albicans **black; *C. tropicalis* violet; *C. glabrata* red; *C. parapsylosis* green; *C. krusei* braun; Asp. fumigatus grey.**

CONCLUSIONS

We have created an easy-to-perform and rapid diagnostic tool to detect the clinically most relevant G+ and G- bacteria and *Candida* species in a single tube. The sensitivity of the PCR reaction is suitable for the detection of the blood-stream infection. This method is not adaptable as a general identification method but useful when some of the bacterial and fungal species are expectable from clinical samples. Our further aim is to improve the reliability of the reaction with internal control.

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