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Title: A new prototype system for the rapid diagnosis of bacterial and fungal sepsis

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Abstract: Fungal and bacterial infections can be identified more rapidly and sensitively with polymerase chain reaction (PCR)-based techniques than with conventional blood culturing techniques. There was recently been a surge in the number of molecular based infection identifications. There have been numerous reports of new real-time PCR-based pathogen identification, although the clinical practicability of such techniques is not yet clarified.
The present study focuses on the differentiation of the most common bacterial and fungal causative pathogens of bloodstream infections. A multiplex real-time PCR approach is introduced for the detection and differentiation of fungi, Gram-positive (G+) and Gram-negative (G-) bacteria. The Gram classification is performed with the specific fluorescence resonance energy transfer (FRET) probes recommended for LightCycler capillary real-time PCR. The novelty of this system is the use of a non-specific SYBR Green dye instead of labelled anchor probes or primers to excite the acceptor dyes on the FRET probes. In parallel, the use of an intercalary dye allows the detection of fungal amplicons. In this way, fungi, G+ and G- bacteria in the same reaction tube can be differentiated quickly (within an hour after the DNA preparation) via the melting temperatures of the amplicons and probes.
The identification of pathogen species with this modified FRET technique is specific and more rapid than with the gold-standard culture-based methods, and permits the rapid and early evidence-based management of bloodstream infections in clinical practice.

Dear Editor,

I enclose a manuscript entitled "A **new prototype system for the rapid diagnosis of bacterial and fungal sepsis**" by Ádám Horváth, Zoltán Pető, Edit Urbán, Csaba Vágvölgyi and Ferenc Somogyvári for consideration for publication in "Journal of Microbiological Methods".

By means of real-time PCR and melting point analysis, we have detected and identified the most frequent bacterial and fungal pathogens. Two primer pairs were used for the multiplex amplification of bacterial and fungal DNA, and two hybridization probes to differentiate Gram – and G+ bacteria. The novelty of this prototype system is the use of non-specific SYBR Green dye as a donor molecule instead of a labelled primer or other specific anchor probe.

Thank you in advance.

Yours sincerely,

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Dear Mr Volker Gurtler,

I enclose the corrected manuscript entitled “A **new prototype system for the rapid diagnosis of bacterial and fungal sepsis**” by Ádám Horváth, Zoltán Pető, Edit Urbán, Csaba Vágvölgyi and Ferenc Somogyvári for consideration for publication in "Journal of Microbiological Methods".

The methodological originality has amended in the “Introduction”, and the fungal ITS data have complemented in the “Table 1”. The whole ms have checked again by native English-speaking colleague.

Yours sincerely,

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***Highlights**

- We have detected and identified the most frequent bacterial and fungal pathogens.
- Two primer pairs were used for the multiplex amplification of bacterial and fungal DNA, and two hybridization probes to differentiate Gram – and G+ bacteria.
- The novelty of this prototype system is the use of non-specific SYBR Green dye as a donor molecule instead of a labelled primer or other specific anchor probe.

A new prototype system for the rapid diagnosis of bacterial and fungal sepsis

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1. Introduction

Bloodstream infections, which are among the most severe manifestations of bacterial diseases, are life-threatening infections that affect individuals with serious underlying conditions or an impaired immune system (Danai et al., 2006). There were 10,319,418 reported cases of bloodstream infections in the USA between 1979 and 2002, and during this period the number of cases demonstrated an annualized increase of 8.7% (Martin et al., 2003). In patients who require intensive care treatment, the majority of infections are caused by bacteria: 52.1% by Gram-positive (G+) and 37.6% by Gram-negative (G-) species. Fungal sepsis is likewise an important public health problem, but it account for only 4.6% of the overall infections (Martin et al., 2003). Mixed *Candida*/bacterial bloodstream infections have been reported to occur in >23% of all episodes of candidaemia (Klotz et al., 2007). Despite its comparatively low frequency, fungal sepsis can progress to severe sepsis and septic shock associated with a drastic rise in mortality. The early and appropriate treatment of such infections is critical, with the aim of interrupting the progression and improving the outcome (Vallés et al., 2003; Kumar et al., 2009).

There has been recently increasing interest in molecular diagnosis in sepsis cases, because this is more reliable and faster than the classical blood-culturing techniques (Leggieri et al., 2010). Molecular strategies such as PCR, ligase chain reaction, nucleic acid sequence based amplification, nested PCR, etc. allow nucleic acid-based differentiation (Carroll et al., 2000). Nevertheless, these molecular approaches are applied only following the positivity of the blood culture, and require a substantial amount of time as compared with the RT PCR-based method described below. The LightCycler PCR assay is relatively easy to perform and may be carried out in small laboratories.

A fluorescence resonance energy transfer (FRET) technique was earlier reported which involves a distance-dependent interaction between the electronic excited states of two dye molecules. The excitation is transferred from a donor (anchor) molecule to an acceptor (quencher) molecule without emission of a photon. This is an appropriate method for discriminating between the most common G+ and G- bacteria that cause sepsis (Klaschik et al., 2002). For better discrimination, subgroups were created within the G+ and G- stains, with differentiation *via* the melting temperature of the overall PCR product and the melting point of the probes (Klaschik et al., 2004). This system requires less than 4 h, including the time of the DNA preparation and the

evaluation of the PCR results. The parallel detection of fungal and bacterial infections in a real-time system is still an unresolved problem. Identification of the most common clinically relevant fungal infections is possible through a simple melting point analysis relating to the ITS2 (internal transcribed spacer) region. This non-coding region is a highly variable rRNA region that is adaptable for the identification of clinically relevant fungi in a broad range (Shoch et al., 2012). Measurments are made at 540 nm and require a non-specific intercalary dye (Somogyvári et al., 2005).

Real-time PCR detection can be performed by using free dyes or labelled sequence-specific probes. One kind of combination of the two techniques was so far being possible that unlabelled probes used for the amplicon detection and T_m determination (Zho et al., 2004). Another, parallel application was the combination of TaqMan chemistry and the very new aspecific dye, BOXTO, as a multiplex PCR (Lind et al., 2006). The novelty of our prototype system that we propose is the use of non-specific SYBR Green dye as a donor molecule instead of a labelled primer or other specific anchor probe. In this way it is possible to examine all three main groups of organisms potentially causing an infection in one tube multiplex PCR reaction.

1. Materials and methods

2.1. Reference strains

Strains of 17 clinically relevant bacterial species were collected, as typical of the main causative agents of bloodstream infections (Lucignano et al., 2011). Nine reference strains, *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC29212, *Listeria monocytogenes* ATCC 4701, *Bacteroides fragilis* ATCC 25285, *Pseudomonas aeruginosa* ATCC 27853, *Haemophilus influenzae* ATCC 49247, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were from the American Type Culture Collection (ATCC). *Streptococcus pyogenes* OKI 80002 was from the National Centre for Epidemiology, Hungary (OKI) and *Proteus vulgaris* HNCMB 60076 was from the Hungarian National Collection of Medical Bacteria (HNCMB). A further 6 clinical strains, *Enterococcus faecium, Stenotrophomonas maltophilia, Serratia marcescens, Enterobacter aerogenes, Enterobacter cloacae* and *Acinetobacter baumannii* were also included. The species identities of the clinical isolates were confirmed by conventional biochemical methods.

Ten fungal strains were examined. Five reference strains, *Candida albicans* ATCC 10231 and ATCC 14053, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *C. glabrata* ATCC 39316, were from the ATCC, *Cryptococcus neoformans* IFM 5844 and IFM 5855 were from IFM Quality Services Pty Ltd (IFM), and *Aspergillus fumigatus* SzMC 2486, *A. flavus* SzMC 2536 and *A. niger* SzMC 2761 were from the Szeged Microbiological Collection (SzMC).

2.2. Bacterial DNA purification

The bacterial strains were grown on Columbia CNA agar base under aerobic circumstances, except that *Bacteroides fragilis* was grown under anaerobic conditions. The bacterial DNA was extracted with the QIAamp® DNA Blood Mini Kit (QuiaGene Inc, Chatsworth, Calif., USA), following the manufacturer's instructions in "Protocols for Bacteria". 1 ml of log-phase culture suspension at a concentration of 10^7 CFU/mL was used for the preparation. For determination of the sensitivity of the reaction, 100 μL of the serially diluted *S. aureus* reference strain was used

for DNA extraction. The number of bacterial cells was determined by plating aliquots of serially diluted samples onto Columbia CNA agar base.

For lysis of the rigid multilayered G+ bacterial cell wall, we used a pre-incubation step with 20 mg/mL lysozyme (in 20 mM Tris·HCl, pH 8.0, 2 mM EDTA, 1.2% TritonX100). The spin protocol for "DNA Purification from Tissues" was followed after incubation at 30 ^{o}C for 30 min. The final concentration of DNA was 2.0-13.8 ng/μL with a ratio A260/A280 = 1.6-1.8 after purification.

2.3. Fungal DNA purification

All the clinically important fungi were grown on Sabouraud agar. The fungal DNA was extracted from 1 mL of a log-phase culture suspension contains 9.6 x 10^7 of fungal cells. For determination of the sensitivity of the reaction, 100 μL of the serially diluted *C. albicans* reference strain was used for DNA extraction. The number of fungal cells was determined by plating aliquots of serially diluted samples onto Sabouraud-glucose agar.

We followed the QIAamp® DNA Mini Kit Protocol for Yeasts. In this case, additional reagents were required for elimination of the complex fungal cell wall structure: sorbitol buffer (1 M sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol) (Lim et al., 2008) was used and the samples were incubated with lyticase for 30 min at 30 ^{o}C. Efficient and complete lysis was achieved in 1.5 hour on a shaking water bath. This purification yielded 2.0–25 μg of DNA in 100 μL of water (2.0–13.8 ng/μL), with A260/A280 = 1.6–1.8.

2.4. Bacterial and fungal primer design, FRET probes

Two primer pairs were used for multiplex amplification of bacterial and fungal DNA.

The bacterial primer pair was PLK1 (TAC GGG AGG CAG CAG) forward and PLK2 (TAT TAC CGC GGC TGC T) reverse, which are highly conserved in different groups of eubacteria (Klaschik et al., 2002) and amplify the 16S rRNA sequence. The PLK2 reverse primer was modified and used without the inner fluorescence labelling. Originally the labelled primer excited the Gram specific probes. We applied the non-specific SYBR Green dye for excitation; it also

serves for visualization of the fungal amplicons. This primer pair produces a 187 bp fragment in each species.

The previously described hybridization probes were used for the Gram classification (Klaschik et al., 2004). ISN2 (5'-CCG CAG AAT AAG CAC CGG CTA ACT CCG T-3') labelled with LCRed 640 was specific for G-, and ISP3 (5'-CCT AAC CAG AAA GCC ACG GCT AAC TAC GTG-3') labelled with Cy5.5 was specific for G+ bacteria. The originally used (Klaschik et al., 2004) ISP2 probe was labelled with LCRed705 at the 5' end. The producers offered Cy5.5 dye instead of LCred705. This modified probe can serve as a template in further experiments.

The ITS86 (GTG AAT CAT CGA ATC TTT GAA C) forward and ITS 4 (TCC TCC GCT TAT TGA TAG C) reverse primers were used for detection of the medically important yeasts. These primers are located in the ITS2 region, which is a highly variable sequences between the 5.8S and 28S rRNA sequence and amplifies 192-494 bp (Lott et al., 1993).

2.5. Mastermixes/excitation dyes

Different non-specific intercalary dyes are used for real-time PCR investigations. Most of them are accessible in ready-to-use mastermix formulas. Our goal was to choose the best dye for excitation of the labelled probes. The tested dyes were LCGreen "LightCycler® 480 High Resolution Melting Master" (Roche Diagnostic GmbH, Mannheim, Germany); SybrGreen "LightCycler® 480 DNA Master SYBR Green I", (Roche); "IQ™ SYBR® Green Supermix" (Bio-Rad Laboratries, Inc., Hercules, CA, USA) and "Maxime™ SYBR Green qPCR Master Mix no ROX" (Fermentas, Vilnius, Lithuania) and EvaGreen ("LC-FastStart DNA Master Hybridization Probes" (Roche) combined with EvaGreen dye (Biotium Inc., Hayward, CA, USA) and "Sso Fast™ EvaGreen® Supermix" (BioRad). All mastermixes were used according to the manufacturer's instructions.

2.6. PCR conditions

PCR was performed on a LightCycler real-time PCR instrument (Roche). The reaction volume of 10 μL contained 1 μL of DNA, 1 μM of each of the primers, 0.7 μM of each of the probes, an

appropiate amount of mastermix and 0.2 mM BSA in the cases of the Fermentas and BioRad mastermixes.

The PCR conditions were as follows: initial denaturation at 95 ^{o}C for 600 s, followed by 40 cycles of denaturation (95 ^{o}C for 0 s, 20 ^{o}C/s), annealing (55 ^{o}C for 15 s, 20 ^{o}C/s), and extension (72 ^{o}C for 20 s, 2 ^{o}C/s). The emitted fluorescence was measured after the annealing steps. The melting curve analysis procedure consisted of 1 cycle at 95 °C for 10 s, 40 °C for 120 s, followed by an increase of the temperature to 95 °C at 0.2 °C/s. The fluorescence signal (F) was monitored continuously during the temperature ramp and plotted against temperature (*T*).

2.7. Data analysis

The melting peaks were evaluated with the LightCycler Software V 3.5. The melting peaks were determined with the manual T_m option on the three detection channels (F1, F2 and F3).

The standard deviation (SD) of the melting points was calculated from five parallel experiments. The fungal or bacterial infection was verified by gel electrophoresis on 1.5% agarose gel with the help of a low-range DNA ladder.

2. Results and discussion

3.1. Discrimination of the fungal, G+ and G- bacterial pathogens

DNA samples from all species studied were prepared and amplified successfully with the SYBR Green dye-based method in the LightCycler instrument. Species-specific T_m-s were obtained by melting point analysis on three detection channel and all pathogens were identified correctly as fungi or G- or G+ bacteria (Table 1). On the F1 channel (540 nm), the melting points of all the amplicons (T_m A) were visible due to the fluorescent signal of the SYBR Green non-specific intercalary dye. On the F2 (640 nm) and F3 (705 nm) channels, the G- and the G+ probes (T_m P), respectively, gave fluorescence signals. After the discrimination of the G- and G+ strains, the fungal pathogens could be screened because the fungal strains gave no signal on the other two channels.

3.2. Determination of the bacterial pathogens

Four G+ and nine G- bacterial subgroups could be distinguished through a joint consideration of the melting points of the probes and the melting point of the overall PCR product (Figure 1). *S. aureus* and the *S. epidermidis* have very close-lying melting temperatures and their species-specific differentiation is not possible *via* this 16S rRNA sequence (Figure 2). A comparison of the Gene Bank sequences (*S. aureus* and *S. epidermidis* NCBI Taxonomy ID: NC_009782.1 and JF_799903.1) of these species revealed a variance of only three base pairs, none of them in the region where the probe is associated with the DNA. Thus, determination of the clinically relevant *Staphylococcus* species requires other gene sequences whereby the antibiotic resistance can be detected (Kilic and Basustaoglu, 2011). The situation is the same with the two *Enterococcus* species (Maheux et al., 2011). At the same time, *S. pyogenes* and *L. monocytogenes* are clearly differentiable.

Among the G- bacteria, *E. coli* is one of the main causative agents of bloodstream infections (Bo et al., 2011). Unfortunately, it has almost the same T_m as those of *E. cloacae* and *S. marcescens*.

Other bacterial strains, such as *H. influenza*, are clearly differentiable through the melting temperature of the probe (Figure 3) or amplicon.

Antibiotic resistance cannot be determined directly with our prototype system. The susceptibility testing of resistant *E. coli* strains can be performed with a PCR-based technique with other 16S rRNA specific primers (Waldeisen et al., 2011). Unfortunately, these investigations require a PCR after the identification of the bacteria.

3.3. Determination of fungal pathogens

Fourteen frequently encountered fungal pathogens could be distinguished. The highly variable ITS 2 target sequence allowed correct identification of all of the clinically relevant fungal strains through the T_m points on the F1 channel (Gutzmer et al 2004., Somogyvári et al., 2005). There was no signal on the F2 or F3 channel. The sensitivity of the reaction was 5 colony forming units (CFU) per reaction.

The correct differentiation between bacteria and fungi was verified by means of gel electrophoresis with the help of the amplicon length (fungal amplicons 192-494 bp, bacterial 187 bp).

2.4 Calibration of the multiplex PCR

All three non-specific dyes (LCGreen, EvaGreen and SybrGreen) excited all of the labelled probes (LCRed640, LCRed705 and Cy5.5). The best results were obtained with the SybrGreen dye.

The determination of T_m is very sensitive to the composition of the PCR reaction mixture, and primarily to the ionic strength. To avoid T_m bias originating from pipetting errors between PCR runs, the application of mastermixes is advisable. One limitation of the method is that the various forms offered by different suppliers differ in reagent composition, which may influence the T_m values.

Naturally, repeated runs with a given mastermix yield reproducible data. In the event of a change of mastermix, however, calibration is necessary to establish the new T_m data on the fungal strains.

The data determined in the present work were obtained with the use of “Fermentas Maxima SybrGreen, no ROX” and five parallel experiments.

3. Conclusions

Real-time PCR is one of the fastest diagnostic methods currently available. The use of rRNA genes for the detection is based on the conserved 16S rRNA sequences of the bacteria. As regards fungi, the ITS sequence refers to a segment of non-functional RNA situated between 5.8S and 28S rRNAs. To reproduce the results, it is possible to differentiate between fungi and bacteria, or between fungal species by electrophoresis (Ferrer et al., 2001, Turenne et al., 1999) or melting analysis (Khan et al., 2009). The Roche LightCycler PCR was specially constructed to amplify amplicons under 500 bp. The amplicons amplified by PLK1/PLK2 comprised 187 bp, while the fungal amplicons amplified by ITS86/ITS4 primer pair varied between 192 bp (*Geotrichum candidum*) and 494 bp (*Malassezia furfur*), values is perfectly suitable for this instrument profile. We took advantage of the LC system when we used the FRET technique to detect and differentiate the bacterial pathogens. We excited the fluorescent probes with the help of a non-specific intercalary dye, which is an uncommon procedure in real-time investigations. This allows the detection of fungal pathogens in parallel with bacteria. We made use of a multiplex PCR in combination with FRET probes and melting point analysis for the broad-range identification of the most frequent causative agents of bloodstream infections. Among the limitations of the method, neither the G+ *S. aureus* and *S. epidermidis* nor the G- *E. coli*, *E. cloacae* and *S. marcescens* can be distinguished. Additional species-specific probes or primers are needed for the further differentiation of these species, if necessary. In spite of the limitations, the rapidly available information on the bacterial species permits targeted therapy with narrow-spectrum antibiotics instead of empirically administered broad-spectrum antibiotics.

The pathogen load in sepsis is generally below 10 CFU/mL (Loeffler et al., 2000). As the sensitivity of this PCR is 5 CFU per reaction, in combination with a correct preparation method it is suitable for the detection of bloodstream infections.

The incidence of sepsis has continuously increased in recent decades, and the early detection of the pathogens present can have a great impact on the clinical outcome of the infection (von Lilienfeld-Toal et al., 2009, Vince et al., 2008, Wallet et al., 2010, Bauer and Reinhard 2010). The molecular diagnostic systems allow species identification in less than 24 h, which is a drastic

improvement relative to the gold standard culture-based method and Gram staining-based identification methods that yield results in 24 to 72 h (Tissari et al., 2010, Cleven et al., 2010).
In summary, the causative agents of the infection can be detected in 2 h without DNA preparation. This was accomplished by the multiplex PCR with the new combination of aspecific dyes and labelled probes. This system is advantageous to traditional FRET based assays by accurate detecting both the T_m of the probes and amplicons.

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6. Glossary

RT PCR	real-time PCR
FRET	fluorescence resonance energy transfer
LC	LightCycler
ICU	Intensive Care Unit
ITS	internal transcribed spacer
ATCC	American Type Cell Culture
IFM	IFM Quality Services Pty Ltd
SzMC	Szeged Microbiological Collection
T_m	melting temperature
SD	standard deviation
T_m A	melting peak of the amplicon
T_m P	melting peak of the probe

Table(s)

Table 1. Melting points of bacterial and fungal amplicons and probes. All the amplicons T_m measured at the F1 channel (540 nm). The signal generated by aspecific SybrGreen dye. The G+ specific probes got the signal at the F2 channel (640 nm) the G- probes at the F3 channel (705 nm). The signal induced by the help of special FRET technique.

Microbial strains	T_m P (°C)		T_m A (°C)	
Gram positive (G+)	mean	SD	mean	SD
Enterococcus faecalis	67.94	0.07	84.14	0.36
Enterococcus faecium	67.84	0.21	84.59	0.78
Listeria monocytogenes	67.80	0.19	86.01	0.36
Staphyloccus aureus	64.85	0.21	83.91	0.54
Staphyloccus epidermidis	64.50	0.30	83.60	0.36
Streptococcus pyogenes	46.54	0.56	84.38	0.78
Gram negative (G-)				
Acinetobacter baumannii	66.09	0.15	82.90	0.16
Bacteroides fragilis	48.65	0.18	84.47	0.84
Enterobacter aerogenes	63.95	0.34	83.47	0.48
Enterobacter cloacae	64.98	0.09	84.38	0.24
Escherichia coli	64.69	0.44	84.74	0.54
Haemophilus influenzae	61.99	0.35	84.28	0.30
Klebsiella pneumoniae	65.13	0.23	84.57	0.20
Proteus vulgaris	64.58	0.18	82.87	0.24
Pseudomonas aeruginosa	53.32	0.33	83.00	0.34
Serratia marcescens	64.01	0.30	84.17	0.30
Stenotrophomonas maltophilia	58.10	0.07	84.42	0.15
Fungi				
Candida albicans	-	-	87.1	0.33
Candida dubliniensis	-	-	85.5	0.50
Candida quillermondii	-	-	85.1	0.70
Candida krusei	-	-	89.8	0.02
Candida parapsilosis	-	-	85.4	0.88
Candida tropicalis	-	-	84.5	0.75
Aspergillus fumigatus	-	-	91.0	0.38

Figure(s)

Figure 1. Differentiation of the bacterial pathogens. The group temperatures indicate the entire T_m of the pathogens. The subgroup temperatures are the melting temperatures of the hybridization probes.

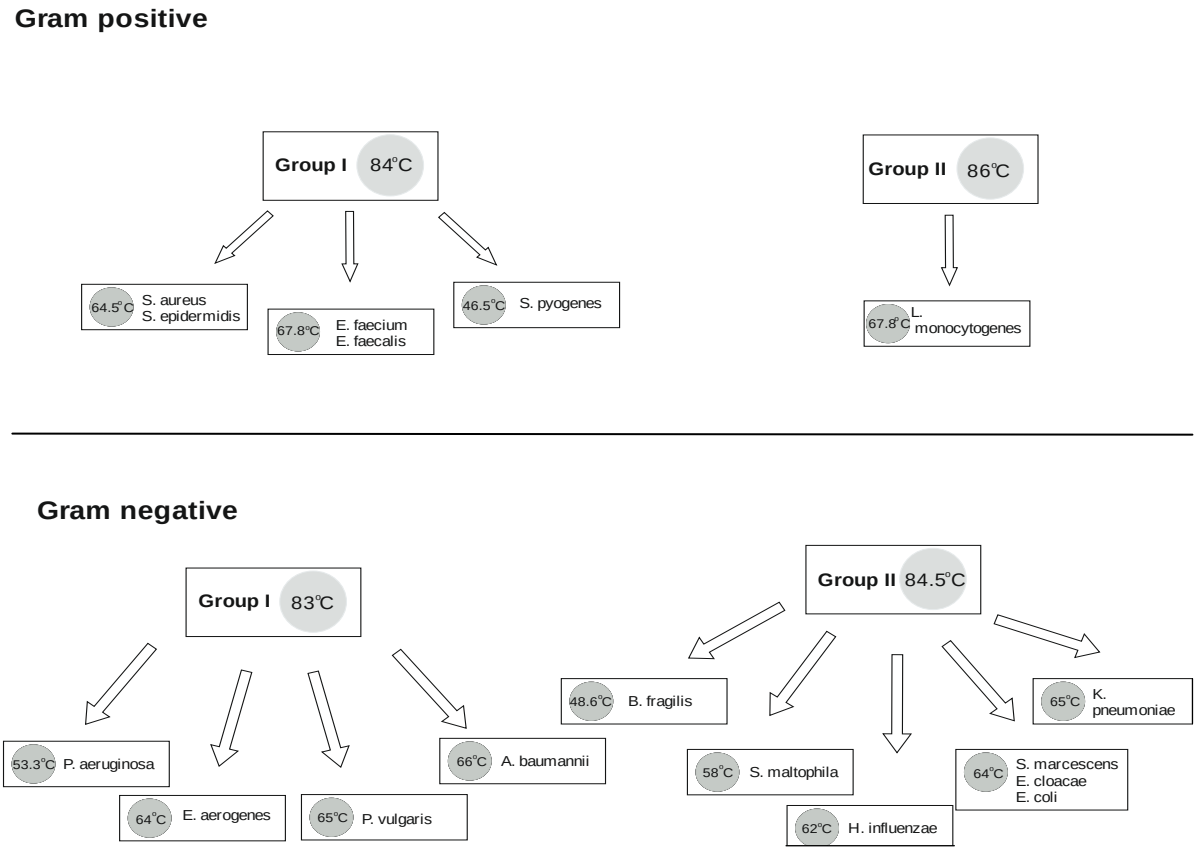

Figure 2. Melting peaks of *Staphylococcus aureus* and *Staphylococcus epidermidis*, revealing that it is impossible to differentiate these *Staphylococcus* species *via* the T_m data of the amplicons or probes.

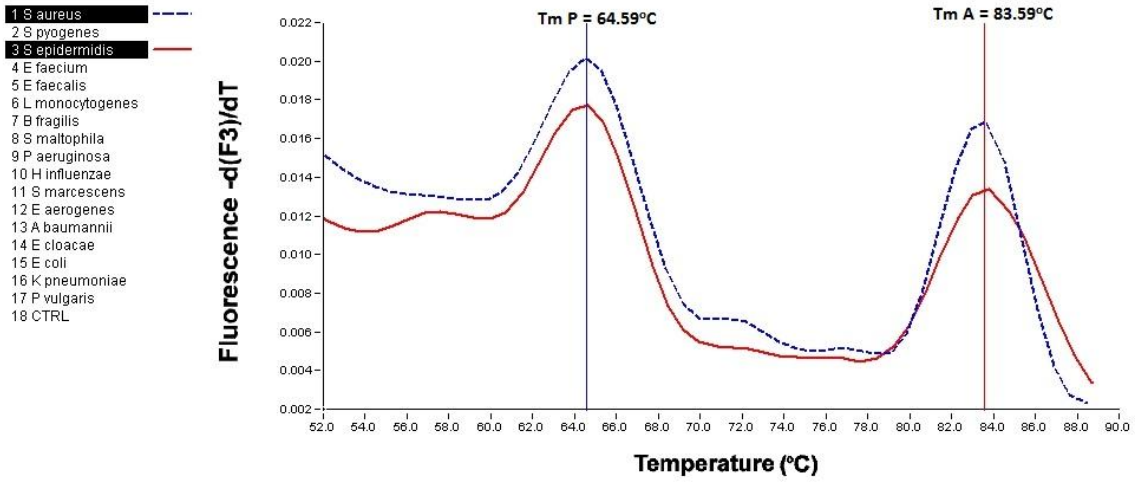

Figure 3. Differentiation of *Escherichia coli* from *Haemophilus influenzae*. Although these pathogens have very similar T_m-s in the 16S rRNA region, the T_m-s of the probes are clearly different.