Real-time polymerase chain reaction with melting analysis of positive blood culture specimens in bloodstream infections: diagnostic value and turnaround time

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SUMMARY

A Real-time polymerase chain reaction (PCR) with melting analysis was devised to target bacterial and fungal genes together with the most prevalent antimicrobial resistance genes in 250 positive blood culture broths. This method allowed the blood culture cultivated pathogens to be classified into clinically relevant groups such as *Enterobacteriaceae*, oxidase-positive bacilli, oxidase-positive coccobacilli, *S. aureus* and yeast. Enterococci and streptococci could be distinguished from CoNS only by the Gram stain. Gram-positive bacilli were discriminated from Gram-positive cocci by Gram stain. Furthermore, the most important antimicrobial resistant genes such as *mecA*, *vanA*, bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ could be identified. All results were obtained with a turnaround time of three hours from the moment of blood culture positivity compared to 24-72 hours for phenotypic methods. In conclusion, the proposed approach can allow the clinician to implement proper early management of sepsis patients.

KEY WORDS: Real-time PCR, Melting analysis, Antimicrobial resistance genes, Sepsis.

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INTRODUCTION

Systemic inflammatory response syndrome (SIRS) is a systemic response of the innate immune response to inflammatory microbial and non microbial insults (1, 2, 3) (Castellheim *et al.*, 2009; Zhang *et al.*, 2010; Heffner *et al.*, 2010).
SIRS accompanying bloodstream infection (BSI) is referred to as "sepsis syndrome". The sepsis syndrome manifests along a disease continuum based on the occurrence of sepsis-related organ dysfunction, comprising severe sepsis (sepsis-induced organ dysfunction or tissue hypoperfusion) and septic shock (severe sepsis accompanied by hypotension, or need of vasopressors) (Bone *et al.*, 1992; Levy *et al.*, 2003; Lever & Mackenzie, 2007; Esper & Martin, 2009).
In the United States it has been estimated that about 750,000 patients develop bacterial or fungal BSI and their clinical evolution through sepsis and septic shock represents the 10th leading cause of death (Martin *et al.*, 2003). Recent population-based studies in Europe emphasized that sepsis could be recognized as the fourth leading cause of death in Europe (Raoult & Richet, 2011; Jensen *et al.*, 2011; Sogaard *et al.*, 2011; Harrison *et al.*, 2006; Blanco *et al.*, 2008; Reinhart *et al.*, 2010).
Inappropriate antimicrobial treatment represents an independent risk factor for mortality and/or microbiological failure in septic patients (Paul *et al.*, 2010; Fernandez-Hidalgo *et al.*, 2011; Suppli *et al.*, 2011). A correct identification of the invading microorganism and the related antimicrobial susceptibility testing (AST) plays a key

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role in allowing appropriate antimicrobial therapy and an optimal clinical management of BSI patients. The standard procedures for identification and susceptibility testing applied to blood culture require an overnight subculture on solid agar media, and 12-24 hours are needed for the definitive identification and antimicrobial susceptibility evaluation of the pathogen. As a consequence turnaround time (TAT) is an important parameter to consider in the evaluation of the microbiological response needed by clinical physicians and patients. Innovative approaches are required to overcome the delay in the diagnostic pathway to improve the turnaround time of microbiological diagnosis (Mancini *et al.*, 2010).
Recently, nucleic acid-based technologies (NAT) have been applied to sepsis diagnosis to shorten the time needed for identification. These assays are designed to detect and identify pathogens from blood culture bottles or directly from blood by different methods (Mancini *et al.*, 2010; Won *et al.* 2010; Reier-Nilsen *et al.*, 2009; Fujita *et al.*, 2011; Lamoth *et al.*, 2010; Wu *et al.*, 2008; Lucignano *et al.*, 2011). Semi-automated instruments have been commercially proposed (Lamoth *et al.*, 2010; Lucignano *et al.*, 2011), although they are very expensive. Moreover, most of the proposed molecular methods do not address the problem of prevalent antibiotic resistance mechanisms of infecting agents.
We devised an in-house real-time PCR with melting analysis that may overcome these drawbacks, by using gene targets able to identify broad groups of pathogens as in classical clinical bacteriology procedures (Ozenci *et al.*, 2008) and the most prevalent antibiotic resistance genes.

MATERIALS AND METHODS

The study was performed on 250 consecutive positive blood cultures from patients with a clinical suspicion of sepsis, admitted to different medical and surgical units at the University Hospital Campus Bio-Medico of Rome, Italy. Each blood culture comprised three sets (time 0, time 30 and time 60) of one aerobic one anaerobic broth bottle (Bactec Plus Aerobic/F, Bactec Plus Anaerobic/F, Beckton Dickinson, Franklin Lakes, NJ USA) per patient drawn during a 1-h period from cases of clinically suspected BSI.
Blood culture vials were incubated in the Bactec 9240 automated system (Beckton Dickinson, Franklin Lakes, NJ USA). From positive broths, subcultures were prepared and, according to the appearance of colonies on subculture plates, the isolates were identified and the antimicrobial susceptibility test performed.

Identification and antimicrobial susceptibility testing (AST) by phenotypic methods

Isolates were identified by Vitek 2.0 compact instrument (bioMerieux sa, Marcy l'Etoile, France) or a Phoenix (Beckton Dickinson, Franklin Lakes, NJ USA) instrument with the support of some additional phenotypic tests (such as coagulase test, PYR test and oxidase test). AST was performed by the cited instruments following the manufacturer's recommendations. Vancomycin susceptibility for enterococci and ESBL phenotypes was confirmed with E-test (bioMerieux sa, Marcy l'Etoile, France). Methicillin resistance was confirmed phenotypically with the cefoxitin disk test. A total of 50 negative blood culture was tested as negative controls.
The positive broths were Gram-stained; the Gram stain guided the choice, when pertinent, of the PCR format to be used, according to the methods described below.

Preparation of bacterial DNA for real-time PCR

Eight millilitres of the positive broth specimens were transferred to a 15 ml sterile conical plastic tube. Samples were centrifuged first at 200 g (1000 rpm, Eppendorf 5810R centrifuge- Rotor A-4-62, Eppendorf AG Hamburg, Germany) x 10 min to pellet cells. The supernatant was collected into a new tube and centrifuged at 2300 g (3400 rpm) x 10 min to pellet bacteria. The bacterial pellet was suspended in 2 ml of sterile deionized water.
Bacterial DNA was extracted by the EZ1 DNA tissue kit (Qiagen, Dusseldorf, Germany) following the manufacturer's instructions starting from 200 µL of bacterial pellet suspension.

Real-time PCR

Real-time PCR assay and related specific primers pairs, as reported in Table 1, were set up to identify major groups of Gram-positive, Gram-nega-

tive bacteria, and yeasts, and to detect methicillin resistance (*mecA*) among staphylococcal isolates, vancomycin resistance (*vanA*) among enterococci and the most common beta lactamase and extended spectrum beta-lactamase (ESBL) resistance genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) among *Enterobacteriaceae* (Maiwald, 2011; Birkett *et al*., 2007; Kearns *et al*., 1999; Reisch *et al*., 2000; Vollmer *et al*., 2008, De Vos, *et al*., 1997).

Different sets of primers were devised with the two separate real-time PCR protocols indicated in table 2. In both real-time PCR protocols *16SrDNA* gene was used as internal control.

As regards Gram-positive bacteria, real-time PCR for coagulase gene and for a portion of *S. aureus* genome (Sa442) was performed to exactly identify *S. aureus* strains; real-time PCR for 23SrRNA was used to identify Gram-positive cocci or bacilli other than *S. aureus*. Moreover, *mecA* and *vanA* resistance genes were targeted.

For Gram-negative isolates *rpoC* and oxidase gene were used to distinguish between oxidase positive gram-negative bacteria and other gram-negative isolates. Furthermore, to exactly identify *Pseudomonas* spp. a specific primers pair corresponding to the beginning and end of the open reading frame of the *oprI* (outer membrane lipoprotein I) gene in fluorescent and non fluo-

TABLE1 - *Primers sequence for bacterial gene and antimicrobial resistance genes.*

Primers	*Sequences (5'-3')*	*Genes*	*Resistance*	*Primers reference*
27F 556R	AGAGTTTGATYMTGGCTCAG CTTACGCCCARTRAWTCCG	*16SrDNA*	-	Maiwald M, 2011
RpoCF RpoCR	TGAGAAGTGCGGTGTTGAAG ACGCTCCAGGTTGGTCATAC	*Gram negative RpoC*	-	This study
bla_{TEM}F bla_{TEM}R	GAGGACCGAAGGAGCTAACC TTGCCGGGAAGCTAGAGTAA	bla_{TEM}	Cephalosporins	This study
bla_{SHV}F bla_{SHV}R	TCAGCGAAAAACACCTTGC GCCTCATTCAGTTCCGTTTC	bla_{SHV}	Cephalosporins	This study
bla_{CTX-M}F bla_{CTX-M}R	ATGTGCAGYACCAGTAARGTKATGGC ATCACKCGGRTCGCCXGGRAT	bla_{CTX-M}	Cephalosporins	Birkett, *et al.*, 2007
Oxidase F Oxidase R	ACGCCAATGGGTACCATCTA TGCATCAGGTAGGCGTACAG	*Gram negative oxidase*	-	This study
PS1 PS2	ATGAACAACGTTCTGAAATTCTCTGCT CTTGCGGCTGGCTTTTTCCAG	*oprI*	-	De Vos, *et al.*, 1997
23SF 23SR	ACGCTCCAGGTTGGTCATAC CAGTGCTCTACCTCCATCATT	*Gram positive 23S rRNA*	-	This study
CoagF CoagR	GTA GAT TGG GCA ATT ACA TTT TGG AGG CGC ATC AGC TTT GTT ATC CCA TGT A	*Gram positive coagulase*	-	Kearns, *et al.*, 1999
Sa442-1 Sa442-2	GTCGGGTACACGATATTCTTCACG CTCTCGTATGACCAGCTTCGGTAC	*S. aureus genome*	-	Reisch, *et al.*, 2000
mecAF mecAR	GGCCAATTCCACATTGTTTC TTGATCGCAACGTTCAATTT	*mecA*	Methicillin	This study
vanAF vanAR	GGCTGCGATATTCAAAGCTC CCGGCTTAACAAAAACAGGA	*vanA*	Vancomicin	This study
NL1 260R	GCATATCAATAAGCGGAGGAAAAG TTAGCTTTAGATGRARTTTACCACC	*28SrRNA*	-	Vollmer, *et al.*, 2008

TABLE 2 - *Real-Time PCR reactions and Tm expected for each product.*

	Amplified genes	*Tm °C*
Gram Negative	*16SrDNA*	90.0-91.0
	RpoC	90.0-90.5*
	Oxidase	92.0-92.5†
	oprI	80-80.5
	bla_{TEM}	88.5-89.0
	bla_{SHV}	93.0-93.5
	bla_{CTX-M}	91.5-92.5
Gram Positive	*16SrDNA*	89.3-90.3
	23S rRNA	82.2-83.7
	Coagulase	81-81.5
	Sa442	78-78,5
	mecA	82.0-83.0
	vanA	85.0-85.5
Yeast	*28SrRNA*	87.8-89.2

*except for *Proteus spp*. (Tm 87.2-87.7); *Moraxella spp*. (Tm 86°C) and *Ochrobactrum anthropi* (Tm 86.5°C); †except for *Moraxella spp*. (Tm 87.3°C) and *Ochrobactrum anthropi* (Tm 85°C).

rescent *pseudomonas* was used. Moreover, real-time PCR was performed for the detection of the antibiotic resistance genes bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$.

For yeast isolates real-time PCR assay specific for fungal 28S (large-subunit) rRNA gene was performed as previously reported by other authors (De Vos *et al*., 1997).

In the case of polymicrobial blood cultures, real-time PCR assay was applied according to the Gram staining indication. If both Gram-positive and Gram-negative were present all reactions were performed, as described in Table 3.

If the Gram stain failed to detect a microorganism or gave equivocal results all described protocols were simultaneously applied.

Eva Green real-time PCR reaction was performed in a 25 µL reaction volume containing 12,5 µL 2x Rotor gene-probe PCR master mix, 0.6 µL (10 µM) each primers, 1.3 µL Eva Green Dye 20X solution (Biotium, Hayward, CA), 5uL DNA template. The reactions were performed on a Rotor Gene 6000 instrument (Corbett Life Science, Sidney, Australia) at the following conditions: 5 minutes incubation period at 95°C followed by 35 cycles of PCR, each cycle consisting of 20 seconds at 95°C, 20 seconds at 50°C and 20 seconds at 72°C and by a final step of 7 minutes at 72°C.

After the end-point analysis, melting curve analysis was performed to check that the correct fragments were amplified. Melting analysis was performed by raising the temperature a fraction of a degree and the change in fluorescence was measured to produce a melt curve. Melting conditions were as follows: ramp form 65°C to 95°C rising by 1 degree each step, wait for 90 seconds of pre-melt conditioning on first step; wait for 5 seconds for each step afterwards to acquire the green channel fluorescence signal. RT- PCR results were analysed by Rotor-gene software (version 6.0). For each PCR product specific Tm was determined by testing well characterized bacterial cultures along with the following standard strains *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC29213 and *E.faecalis* ATCC29212: their values are reported in table 2.

Real-time PCR sensitivity for the various genes was estimated by counting bacterial cultures of ATCC strains and strains from our collection by the plate count method.

The sensitivities for the methods for the various genes were as follows: for *16SrDNA* gene: 20-120 c.f.u./100 µL; for *23S rDNA* gene *E. faecalis* ATCC 29212: 450 c.f.u./100 µL; for *coagulase* gene and *SA442* sequence in *S. aureus* ATCC29213: $9x10^3$ c.f.u./100 µL; for *rpoC* gene in *E.coli* ATCC 25922: $5.5 x10^3$ c.f.u./100 µL; for *oxidase* gene in *P.aeruginosa* (a strain from our collection): 4000 c.f.u./100 µL; for bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ resistance genes in *K. pneumonia*e (a strain from our collection): 2000 c.f.u./100 µL; for *mecA* gene in *S. aureus* (a strain from our collection): 2500 c.f.u./100 µL.

The study was approved by the Ethics Committee of the University Campus Bio-Medico, Rome, Italy. All authors involved in the study complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

RESULTS

Among the 250 positive blood cultures the isolates included 124/250 (49.6%) Gram-negative species with 98/250 (39.2%) *Enterobacteriaceae*, 25/250 (10%) non fermenter isolates and 1/250 anaerobe; 83/250 (33.2%) Gram-positive species,

11/250 (4.4%) yeasts, 32/250 (12.8%) polymicrobial isolates (Table 3).
Among Gram-positive microorganisms the most frequent isolates were: *S. epidermidis* 34/83 (40.9%); *S. aureus* 18/83 (21.7%); *S. hominis* 5/83 (6%); *E. faecium* 5/83 (6%) and 5/83 (6%) *E. faecalis*; among Gram-negative microorganisms the most frequent isolates were: *E. coli* 65/124 (52.4%), *P. aeruginosa* 19/124 (15.3%), *K. pneumoniae* 11/124 (8.8%), *E. cloacae* 6/124 (4.8%), *S.*

TABLE 3 - *Real-Time PCR results from positive monomicrobic and polymicrobic blood cultures and their concordance with standard cultures and antimicrobial susceptibility test results.*

Blood cultures isolates (n°/total)	*Gene target*	*Gene target identified by Real-Time PCR method*	*Concordance (n° of positive Real-Time PCR test/phenotypic test)*
Gram-positive isolates (83/250)	*16S rRNA*	83/83	83/83
	23S rRNA	65/83	65/65
	Coagulase	18/83	18/18
	Sa442	18/83	18/18
	mecA	39/83	39/30
	vanA	0/83	0/0
Enterobacteriaceae isolates (98/250)	*16S rRNA*	98/98	98/98
	RpoC	98/98	98/98
	Oxidase	0/100	0/0
	bla_{TEM}	28/98	28/17
	bla_{SHV}	11/98	11/5
	$bla_{CTX\text{-}M}$	41/98	41/34
Non fermenter gram negative isolates (25/250)	*16S rRNA*	25/25	25/25
	RpoC	2/25	2/2
	Oxidase	23/25	23/23
	oprI	19/25	na
	bla_{TEM}	0/25	na
	bla_{SHV}	0/25	na
	$bla_{CTX\text{-}M}$	10/25*	na
Gram negative anaerobes (1/250)	16S rRNA	1/1	1/1
	RpoC	1/1	1/1
	Oxidase	0/1	0/0
	bla_{TEM}	0/0	0/0
	bla_{SHV}	0/0	0/0
	$bla_{CTX\text{-}M}$	0/0	0/0
Yeast (11/250)	*28S*	11/11	11/11
Polymicrobial (32/250)	*16S rRNA*	32/32	32/32
	23S rRNA	20/32	20/20
	Coagulase	2/32	2/2
	Sa442	2/32	2/2
	mecA	9/32	9/7
	vanA	0/32	0/0
	RpoC	19/32	19/19
	Oxidase	5/32	5/5
	bla_{TEM}	6/32	6/5
	bla_{SHV}	9/32	9/6
	$bla_{CTX\text{-}M}$	10/32	10/9
	28S	3/32	3/3

na: not applicable; *8 *P. aeruginosa* in which ESBL phenotype was not determined; 8/8 CTX-M positive; 7/8 carbapenem resistant); 2 *Ochrobactrum anthropic* ESBL phenotype positive and CTX-M positive.

marcescens 5/124 (4%) and *P. mirabilis* 3/124 (2.4%). The most frequent associations recovered from polymicrobial blood cultures in more than one case were: *E. coli/K. pneumoniae* in 3/32 (9.3%), *E.coli/E.faecalis* in 2/32 (6.2%), *E. coli/E.faecium* in 2/32 (6.2%), *E. coli/C.albicans* in 2/32 (6.2%) and *K.pneumoniae/S. malophilia* in 2/32 (6.2%) (Table 3).

Real-time PCR results and definitive identification of isolates

The results of real-time PCR compared to the definitive phenotypic characterization of the isolates are as follows.

Real-time PCR identified *S. aureus* at the species level with the following results: Gram-positive cocci arranged in clusters by staining the blood culture broth; coagulase gene positive, Sa442 sequence positive; 23SrDNA negative.

CoNS were characterized by the following results: Gram-positive cocci arranged in clusters; coagulase gene negative; Sa442 sequence negative; 23S rDNA positive.

Enterococci and streptococci: Gram-positive cocci in chains; coagulase gene negative; Sa442 negative; 23S rDNA positive.

Bacillus spp and *L. monocytogenes* resulted as: Gram-positive rods at Gram staining; coagulase gene and Sa442 negative; 23S rDNA positive.

Enterobacteriaceae, *S. maltophilia, Bacteroides uniformis* gave the same results by real-time PCR: Gram negative bacilli at Gram stain; *rpoC* positive; oxidase gene negative, *oprI* negative.

P. aeruginosa and *S. paucimobilis* gave the following results in real-time PCR: Gram negative bacilli at Gram stain of culture broths; *rpoC* negative; oxidase gene positive, *oprI* positive.

Moraxella spp and *O. anthropi:* Gram negative coccobacilli; *rpoC* positive; oxidase gene positive, *oprI* negative.

Yeasts: Gram positive yeasts at Gram stain of broths and 28S rDNA gene positive.

The results obtained with the proposed method in the 207 monomicrobic bacterial BSI are summarized in the attached flowchart (Figure 1).

For polymicrobial blood cultures Gram staining guided the identification of polymicrobic blood cultures when there were gram positive and gram negative bacteria. In these cases, both real-time PCR for Gram positive and Gram negative were performed and interpreted as indicated above. The concomitant presence of two Gram negative

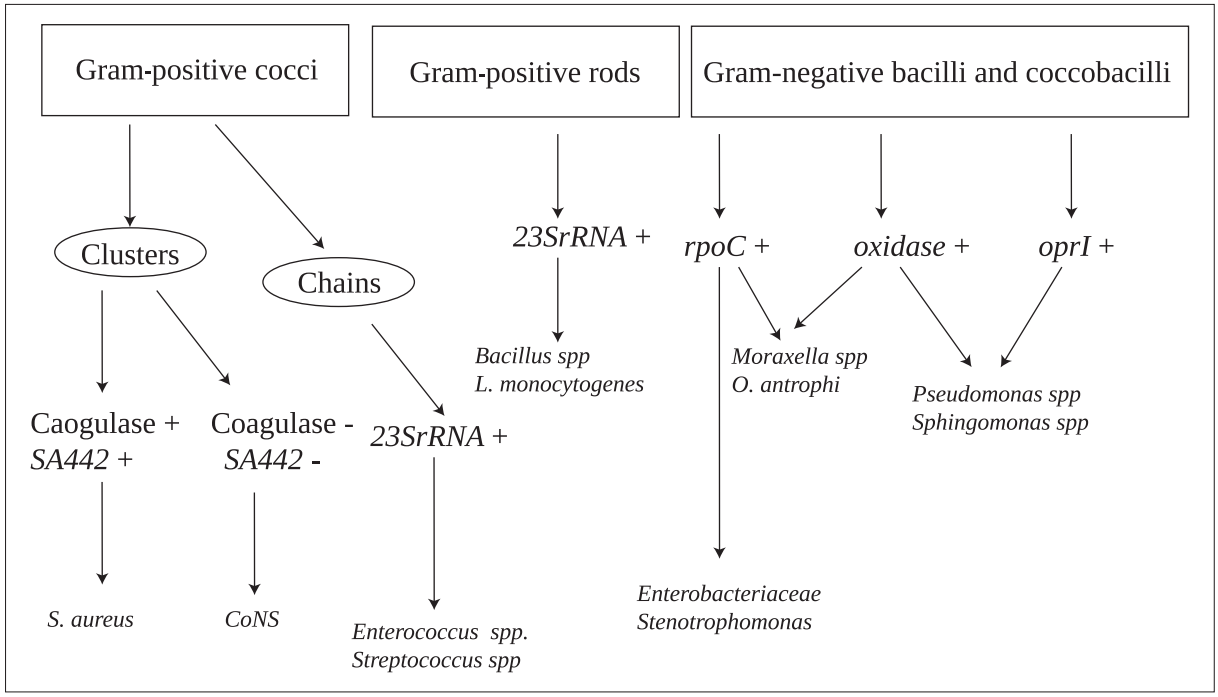

FIGURE 1 - *Flowchart of the results obtained with the proposed method in 207 monomicrobic bacterial BSI.*

species could be revealed because results of the *rpoC* gene showed two peaks at different Tm.

Antimicrobial resistance genes

Staphylococcus mecA was detected by real-time PCR in 39/83 (47%) strains while methicillin resistance was detected phenotypically in 30/39 (77%) strains (Table 4). In 28/98 (28.6%) *Enterobacteriaceae* isolates bla_{TEM} was detected by real-time PCR while ESBL phenotypes were detected in 17/28 (60.7%); in 11/98 (11.2%) *Enterobacteriaceae* isolates bla_{SHV} was detected by real-time PCR, while phenotypic methods were positive in 5/11 (45.5%); in 41/98 (41.8%) *Enterobacteriaceae* isolates $bla_{CTX\text{-}M}$ was detected by real-time PCR while phenotypic ESBL were positive in 34/41 (82.3%) (Table 4). In polymicrobic blood cultures *mecA* was detected in 9/32 (28%) strains while phenotypic methods detected 7/9 (77.8%); ESBL phenotype was not identified in 1/6 (16.7%) isolates bla_{TEM}-positive, in 3/9 (33.3%) bla_{SHV}-positive and 1/10 (10%) $bla_{CTX\text{-}M}$ positive (Table 4).

Melting analysis of real-time PCR products

For each PCR product specific Tm was determined by testing well-characterized bacterial cultures, to check that the correct fragments were amplified. Their values are reported in table 2. Melting temperatures are reported as temperature range for each amplification product. Some variations of melting peaks and their corresponding temperature values were observed during isolate testing and are reported (Table 2). In polymicrobic gram negative blood culture a double peak in the *rpoC* amplification product was observed with a first peak at about 87.5°C and a second one at 91°C. Variation in Tm of *rpoC* prod-

TABLE 4 - *Blood cultures isolates.*

Blood cultures (n=250)			
Gram positive isolates (83)	*Gram negative isolates (124)*	*Fungi isolates (11)*	*Polimicrobial isolates (32)*
S. epid (34)	*E. coli (65)*	*C.albicans (7)*	*E. coli + E. faecalis (2)*
S. aureus (18)	*P. aeruginosa (19)*	*C. tropicalis (1)*	*E.coli + E. faecium (2)*
E. faecium (5)	*Sphingomonas (1)*	*C. parapsilosis (3)*	*E. coli + E. raffinosus (1)*
S. hominis (5)	*S. maltoph (2)*		*E. coli + C. albicans (2)*
S. pneumoniae (1)	*K. pneumoniae (11)*		*S. epidermidis + C. albicans (1)*
S. sanguinis (1)	*K. oxytoca (2)*		*K. pneumoniae + E.coli (3)*
E. faecalis (5)	*R. planticola (1)*		*S. bovis, S. gallinarum, S. epidermidis (1)*
E. raffinosus (1)	*Pantoea agglomerans (2)*		*E. faecalis + S. epid (1)*
E. gallinarum (1)	*E. cloacae (6)*		*K. pneumoniae + A. baumanni (1)*
Bacillus spp. (1)	*E. aerogenes (1)*		*E. coli + Aeromonas (1)*
S. capitis (1)	*S. marcescens (5)*		*E. coli + K. pneumoniae (1)*
S. equi (1)	*Serratia odorifera (1)*		*P. aeruginosa + E. Faecium (1)*
S. agalactiae (1)	*C.freundii (1)*		*P. aeruginosa + S. haemoliticus (1)*
S. oralis (1)	*P. mirabilis (3)*		*E. coli + M. morganii (1)*
S. haemolyticus (2)	*Moraxella (1)*		*E. coli+ P. aeruginosa (1)*
S. saprophyticus (1)	*B. uniformis (1)*		*P. aeruginosa + E. faecalis (1)*
S. mitis (1)	*Ochrobactrum anthropi (2)*		*E. coli + S. salivarius (1)*
S. anginosus (1)			*K. Pn e S. maltoph (2)*
S. bovis (1)			*E. coli, Citrobacter koseri, E. faecalis E. casseliflavus/gallinarum (1)*
L. monocytogenes (1)			*E. coli e di S. haemolyticus (1)*
			E. coli, K oxyt, Eavium. (1)
			S. aureus+ S. haemolitcus (1)
			S.aureus + E. faecalis (1)
			S. epidermidis+ S. haemolitcus (1)
			S. epidermidis+ S. capitis (1)
			Bacillus licheniformis+s epidermidis (1)

uct was also found in *Proteus spp.* isolates testing. In fact, *rpoC* melting temperature was lower (Tm range = 87.2-87.7°C) in *Proteus spp* than in other *Enterobacteriaceae*. *RpoC* melting temperature in *Moraxella spp* and *Ochrobactrum anthropi* isolates was 86°C and 86.5°C, respectively (Table 2). A lower Tm for oxidase gene PCR product than in *P. aeruginosa* isolates was observed in *Moraxella spp*. isolates and *Ochrobactrum anthropi* isolates that were 87.3°C and 85°C, respectively, as reported in Table 2.
Real-time PCR assay was always positive when an isolate was identified by a phenotypic test or a resistance was detected by the AST method.
No amplification product was obtained from real-time PCR performed in the 50 negative blood culture included in the study as negative controls.
The turnaround time for characterization of pathogens causing BSIs by our molecular method was 3 hours.

DISCUSSION

The isolation and identification of a causal microorganism is an essential step in the workup of BSI and in the management of septic patients.
The current standard method for BSI/sepsis diagnosis is blood culture performed by automated systems. The practical value of blood culture in the diagnosis of sepsis, however, is impaired by the delay in the time to results and the fact that positive blood cultures can be found for only approximately 30% of these patients. Furthermore, it is known that the sensitivity for many slow-growing and fastidious organisms is low (Reinhart *et al.*, 2012)
A rapid detection and microbiological diagnosis of BSIs with the detection of the most important antimicrobial resistance genes may have an impact on the morbidity, mortality and length of hospitalization of patients. The development of reliable techniques designed to provide fast isolates identification and antimicrobial susceptibility testing of the causative BSI pathogen represents one of the main tasks of the clinical microbiology laboratory.
During recent years real-time PCR-based molecular assays have been developed for bacterial and fungal detection from clinical samples with a substantial gain in shortening the detection time of microorganisms grown in blood culture bottles (in about 4-6 hours a precise microorganism identification can be achieved) (Mancini *et al.*, 2010).
Most of the molecular assays commonly performed for the diagnosis of sepsis do not include antimicrobial resistance genes testing of the identified microorganism, and hence do not support clinicians in their therapeutic choices (Mancini *et al.*, 2010; Won *et al.* 2010; Reier-Nilsen *et al.*, 2009; Fujita *et al.*, 2011; Lamoth *et al.*, 2010; Wu *et al.*, 2008; Lucignano *et al.*, 2011).
The present study applied an in-house real-time PCR assay to positive blood culture broths also with the detection of the most important antibiotic resistance genes. The results of real-time PCR were compared to the traditional identification and AST methods used for the characterization of the isolates detected in positive blood cultures to evaluate whether this molecular method could be routinely used to reduce turnaround time allowing clinicians to start prompt more appropriate antibiotic therapy.
By applying the reported real-time PCR method, the blood culture cultivated pathogens could be classified into clinically meaningful groups such as *Enterobacteriaceae*, oxidase-positive bacilli, oxidase-positive coccobacilli, *S. aureus* and yeast. Nevertheless, enterococci and streptococci could be distinguished from CoNS only by the Gram stain and the possible presence of *mecA*. Gram-positive bacilli were discriminated from Gram-positive cocci only by Gram stain. This method also detect edthe presence of yeast in blood culture broths with a turnaround time of 3 hours. The diagnosis of fungemia by blood culture, the current "gold standard," is slow and insensitive, as yeasts are not detected in 50% of patients with candidemia and molds are rarely recovered. In addition, almost two days are necessary for isolation. The early initiation of targeted antifungal therapy is essential to improve patient outcomes. The application of this molecular protocol is very important in critically ill patients (Garey *et al.*, 2006; Lau *et al.*, 2010).
The methicillin resistance determinant *mecA* for staphylococci was missed by phenotypic methods in 9 isolates.
Bla_{TEM} and bla_{SHV} genes were detected by real-time PCR in 39 *Enterobacteriaceae* strains, while phenotypic methods detected ESBL in 22 of these

strains: some of the detected sequences may not have belonged to an ESBL TEM or SHV-mutated beta-lactamase; *bla*$_{CTX-M}$ gene was detected in 41 *Enterobacteriaceae* while phenotypic methods classified 34 as ESBL. The detected resistance genes may not have been expressed, resulting in a negative ESBL phenotype, but it seems more prudent to think that most of them were, and to use this information for the antibiotic therapy.

In conclusion, applying this real-time PCR assay to positive blood culture broths, the blood culture cultivated pathogens could be classified into clinically meaningful groups disclosing the major genetic determinants leading to drug-resistant pathogens. In many instances the applied molecular method showed a better performance than traditional methods in identifying the antimicrobial resistance. The present offers clinicians the possibility to have a more rapid and focussed antibiotic therapy in the management of sepsis patients.

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