New Microbiologica, **38**, 251-257, 2015

Development of a real-time PCR assay for the rapid detection of *Acinetobacter baumannii* from whole blood samples

Eliana De Gregorio[1], Emanuela Roscetto[1], Vita Dora Iula[1], Marianna Martinucci[1], Raffaele Zarrilli[2], Pier Paolo Di Nocera[1], Maria Rosaria Catania[1]

[1]Department of Molecular Medicine and Medical Biotechnologies, Federico II University, Naples, Italy;
[2]Department of Public Health, Federico II University, Naples, Italy

SUMMARY

Acinetobacter baumannii is a multidrug-resistant pathogen associated with severe infections in hospitalized patients, including pneumonia, urinary and bloodstream infections. Rapid detection of *A. baumannii* infection is crucial for timely treatment of septicemic patients. The aim of the present study was to develop a specific marker for a quantitative polymerase chain reaction (PCR) assay for the detection of *A. baumannii*. The target gene chosen is the biofilm-associated protein (*bap*) gene, encoding a cell surface protein involved in biofilm formation. The assay is specific for *A. baumannii*, allowing its discrimination from different species of Acinetobacter and other clinically relevant bacterial pathogens. The assay is able to detect one genomic copy of *A. baumannii*, corresponding to 4 fg of purified DNA, and 20 colony-forming units/ml using DNA extracted from spiked whole blood samples.

KEY WORDS: Bacterial bloodstream infection, Molecular diagnostics, TaqMan real-time PCR, Blood culture, Sepsis.

Received June 3, 2014 *Accepted* January 28, 2015

INTRODUCTION

Acinetobacter baumannii has become an increasingly important human pathogen because of the increase in the number of infections caused by this organism and the emergence of multidrug- resistant (MDR), extensively-drug resistant (XDR) and pandrug-resistant (PDR) strains (Durante-Mangoni and Zarrilli, 2011). *A. baumannii* can produce different types of infections, most typically nosocomial pneumonia and bloodstream infections in critically ill patients (Peleg *et al.,* 2008). *A. baumannii* bloodstream infection has a high mortality rate of 34-43.4% for critical patients treated in intensive care units (Wisplinghoff *et al.,* 2004).

Corresponding author
Eliana De Gregorio
Dipartimento di Medicina Molecolare
e Biotecnologie Mediche
Federico II University
Via S. Pansini, 5 - 80131 Napoli, Italy
E-mail: edegrego@unina.it

Additional species in the Acinetobacter genus, such as *Acinetobacter pittii* (Acinetobacter genospecies 3), *Acinetobacter nosocomialis* (Acinetobacter genospecies 13TU) and *Acinetobacter lwoffii*, have been recognized as important nosocomial pathogens more recently (Dijkshoorn *et al.*, 2007; Higgins *et al.,* 2007; Turton *et al.,* 2010). Identification of *non-baumannii Acinetobacter* species is complicated because they are closely related genetically to *A. baumannii* and difficult to differentiate phenotypically using routine laboratory methods (Gerner-Smidt *et al.,* 1991; Gerner-Smidt, 1992; Higgins *et al.,* 2007; Yamamoto *et al.,* 1999). Therefore, these species tend to be grouped in the *A. calcoaceticus-A. baumannii* complex. These organisms are frequently misidentified in routine diagnostic laboratories and there is a need for simple molecular methods to differentiate *A. baumannii* from the related Acinetobacter species.

Bloodstream infections (BSIs) are leading cause of morbidity and mortality in hospitalized pa-

tients, especially posttransplant patients, patients with leukemia (neutropenic patients), and postoperative patients in the intensive care unit.

The automated continuous monitoring conventional blood culture (BC) system is a gold standard for diagnosis of bloodstream infection. Unfortunately, this technique is slow, usually requiring 24-72 h to produce a positive result. Early detection and adequate treatment of causative pathogens within the first 6-12 h is critical for a favorable outcome in patients with BSI. In recent years, sensitive molecular methods have been developed for a rapid detection and the specific identification of pathogens involved in BSI. Broad-range real-time PCR methods have been evaluated for the detection of bacteria in blood to improve sensitivity and reduce the time delay before commencing adequate antimicrobial therapy (Hansen *et al.,* 2010; Klaschik *et al.,* 2004; Lehmann *et al.,* 2008; Wang *et al.,* 2014). PCR assays have been developed for the detection of *A. baumannii* from clinical specimens, environmental samples and pure culture by targeting a variety of genes (Clifford *et al.,* 2012; McConnell *et al.,* 2012; Nomanpour *et al.,* 2011; Rice *et al.,* 2013; Wang *et al.,* 2013).

The aim of this study was to develop a polymerase chain reaction (PCR) for identifying *A. baumannii* from whole blood samples. Using as target the gene encoding the bap (biofilm-associated protein) protein, we have established a reliable and efficient procedure for quantitative detection of *A. baumannii*, and effective discrimination from clinically relevant bacteria. This method is simple, rapid and sensitive and could distinguish *A. baumannii* from *A. lwoffii*, *A. junii*, *A. pittii* and *A. nosocomialis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two hundred clinical isolates of *A. baumannii* were included in the study. Strains were isolated from blood (46), respiratory samples (102) and other sources (52) collected at the Laboratory of Clinical Microbiology, University of Naples. *A. baumannii* reference strains (700, 3990, 2735, LUH 5875), assigned to international clonal lineages I, II and III (Giannouli *et al.,* 2013), were used to validate the specificity of the assay. The wholly-sequenced *A. baumannii* AYE (Vallenet *et al.,* 2008), and ATCC17978 (Smith *et al.,* 2007) strains were used as a positive control.

Multiple clinical isolates of four pathogenic species of the Acinetobacter genus (*A. nosocomialis*, *A. pittii*, *A. junii*, *A. lwoffii*) and ten pathogenic species belonging to other genera (*E. coli*, *P. aeruginosa*, *K. oxytoca*, *K. pneumoniae*, *S. maltophilia*, S. *marcescens*, *P. mirabilis*, *E. cloacae*, *S. aureus*, *E. faecalis*) recovered from blood were included as controls.

All tested isolates were identified by the BD Phoenix Automated Microbiology System (Becton Dickinson, Franklin Lakes, NJ, USA). *Acinetobacter* species identification was confirmed by MALDI-TOF MS (Espinal *et al.,* 2012) or PCR amplification and sequencing of *rpoB* (Gundi *et al.,* 2009). Isolates were stored in tryptic soy broth with 20% glycerol at -80°C. All strains was grown on blood agar plates (TSA) and incubated at 37°C for 24 h.

DNA extraction from bacterial specimens

The bacterial harvest was centrifuged for one min at 12,000 rpm, and genomic DNA was extracted using the QIAamp DNA Mini Kit (QiagenTM, Germany) according to the manufacturer's protocol.

The concentration of genomic DNA was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific). The efficiency and sensitivity of primers was evaluated using serial dilutions of genomic DNA from 400 ng (approximately 10^8 genome copies) to 4 fg (approximately 1 genome copy) obtained from the two reference *A. baumannii* strains AYE and ATCC17978. Genomic DNA copy number was estimated on the basis of molecular weight of the *A. baumannii* genome using the copy number calculator at the URI Genomics and Sequencing Center website (http://www.uri.edu/ research/gsc/resources/cndna.html). The specificity of PCR assays was evaluated using DNA lysates prepared from the *A. baumannii* clinical isolates. Bacterial DNA from the non-*baumannii Acinetobacter* species and the non-Acinetobacter genera were used as negative controls in real-time PCR.

DNA extraction from whole blood spiked with bacteria and clinical specimens

Two *A. baumannii* strains AYE and ATCC17978 were used for spiking. One isolate of each of non-*baumannii Acinetobacter* species and the non-Acinetobacter genera above mentioned were used as negative controls in blood spiking experiments. All microorganisms were grown to mid log phase in brain heart infusion broth and the density of the bacterial suspension was adjusted to 0.5 McFarland (approximately $1.5x10^8$ colony-forming units/ml). For *A. baumannii* tenfold serial dilutions in PBS of the bacterial suspensions were spiked into EDTA whole blood, and 100 microliter from each dilution were plated onto TSA plates for counting colony-forming units (CFU). The final bacterial concentrations in spiked whole blood samples ranged from 10 to 10^5 CFU/ml. For non-*A. baumannii* isolates, samples of whole blood were spiked with bacterial suspensions at a concentration of 10^8 CFU/ml. Each dilution series included as negative control PBS or blood without bacteria. Duplicate samples were prepared from each concentration.

BCs were performed using the BACTEC FX (Becton Dickinson) blood culture systems. Two pairs of culture bottles for aerobes or anaerobes were incubated for five days after inoculating blood drawn from the patient at the bedside. If no bacterial growth was detected within five days, the blood culture was considered negative.

Whole blood samples from three patients confirmed to be positive for *A. baumannii* and nine patients confirmed to be positive for another microrganism by blood culture, were selected. Written informed consent to participate in this study was obtained from the patients.

DNA was extracted from 150 microliter of each spiked sample or blood culture using the extractor NucliSens EasyMag (BioMerieux, Marcy l'Etoile, France) following the manufacturer's instructions.

Real-time PCR

Two primer sets directed against different regions of the *bap* gene were used for PCR assays. AB792 primers and AB792 probe for real-time PCR were designed using the Primer Express 3.0.1 software (Applied Biosystems, Foster City, CA, USA). The specificity of the designed primers was checked using the basic local alignment search tool (BLAST) against the NCBI database.

All amplification reactions were performed on a ABI 7500 FAST instrument (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 microliters. All analyses were performed in triplicate. Three samples without a DNA were routinely included as a no template control.

SYBR Green real-time PCR was done with AB792-A primers (fw: AATGCACCGGT RCTTGATCC;rv:TRTTGCCTGCAGGGTCAGTT; R is A or G). The SYBR Green PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). PCR mixture included 4 microliters of extracted DNA and 200 nM of primers. PCR protocol included an initial denaturation step at 95°C for 10 min, followed by 38 cycles of denaturation at 95°C for 15 s, and annealing and extension at 63°C for 45 s. The cycle threshold (CT) was determined automatically. The data collection was performed during each annealing step. Following amplification, during melting curve analysis, the temperature increases to obtain product specific melting temperature from 65 to 95°C with an increment of 0.5°C.

TaqMan real-time PCR was done with AB792-B primers (fw: CGCTGCAGCATCAAATCATG; rv: TGGGTCAACCGAGAAAGTTACG) and AB792 probe (FAM-AGCACCTGCTGACACCACTCCAC CA-TAMRA). The TaqMan PCR mixture was prepared with 1X Environmental Master Mix (Applied Biosystems, Foster City, CA, USA), 4 microliters of extracted DNA, 900 nM of primers and 250 nM probes. The PCR protocol included an initial denaturation step at 95°C for 10 min, followed by 42 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 45 s.

RESULTS

Identification and in silico specificity test of A. baumannii target gene used in real-time PCR experiments

Real-time PCR analyses designed to identify *A. baumannii* in clinical samples were based

on the amplification of the *secE* (Clifford *et al.,* 2012), $bla_{\text{OXA-51-like}}$ (Nomanpour *et al.,* 2011), *ompA* (McConnell *et al.,* 2012), *gyrB* (Rice *et al.,* 2013) and *pgaD* (Wang *et al.,* 2013) gene coding sequences.

It has been reported that the use of repetitive coding sequences as amplification targets may improve the level of detection in real-time PCR experiments (Fenollar *et al.,* 2004). For this purpose, we searched all wholly sequenced *A. baumannii* genomes for repetitive coding sequences to be used as targets of real-time PCR experiments. We selected the gene encoding the biofilm-associated protein (*bap*) described by Loehfelm *et al.* (2008). The protein is necessary for mature biofilm formation and is involved in adherence of *A. baumannii* to human epithelial cells (Brossard and Campagnari, 2012).

BLAST searches carried out against the 17 complete genomes and 291 *A. baumannii* draft genomes deposited at GenBank in December 2013 revealed that bap is conserved in most (290) isolates and is therefore a good candidate for identification purposes. The bap protein described by Loehfelm is a 8621 aa protein which contains seven variants of 86-106 bp modules (A to G repeats) all featuring HYR (hyaline repeat) motifs (Loehfelm *et al.,* 2008). This large protein carries 19 B repeats and 28 D repeats. The number of B repeats varies among strains, and is lower (1-4 copies) in many draft genomes. It must however be stressed that variations of repeated sequences denoted by BLAST searches are mostly spurious, because repetitive sequences organized in tandem arrays tend to be underlooked in the assembly of sequence contigs in unfinished genomes.

Specificity and sensitivity of the bap SYBR Green real-time PCR assay

The AB792-A primers pair selected for SYBR real-time PCR experiments amplified a 205 bp segment of the bap B repeat, reiterated 23 times in *A. baumannii* AYE but present in one copy only in the ATCC17978 genome. The species specificity of the assay was determined using genomic DNA from bacterial cultures of 200 clinical isolates of *A. baumannii*, and 14 clinical isolates of non-*baumannii Acinetobacter* species and non-Acinetobacter genera (the list of Gram negative and Gram positive microorganisms tested is in Materials and Methods).

TABLE 1 - *Ct values of dilutions of A. baumannii purified DNA obtained by SYBR Green PCR.*

A. baumannii AYE	
ng/µl	*Ct values*
1x10	11.9±0.11
$1x10^{-1}$	15.2±0.09
$1x10^{-2}$	18.5±0.34
$1x10^{-3}$	21.9±0.21
$1x10^{-4}$	25.6±0.08
$1x10^{-5}$	29.2±0.57
$1x10^{-6}$	31.8±0.61

The primers targeting the *bap* gene amplified products from all *A. baumannii* clinical isolates. Melting curve profiles showed low levels of non specific amplification from *P. aeruginosa*, *E. coli*, *S. aureus* strains. As expected from *in silico* analysis, the AB792-A primers also amplified a product from *A. pittii* and *A. nosocomialis*.

The sensitivity of the assay was evaluated generating a standard curve by use of tenfold serial dilutions of *A. baumannii* AYE DNA. The assay was able to detect one genomic copy (corresponding to 4 fg). Ct values for three replicates of tenfold dilutions of AYE DNA are reported in Table 1. The standard curve showed a good linear response (R^2: 0.994). Standard regression analysis of the linear part of the slope gave a coefficient of -3.316, which yielded a PCR efficiency of 100.24%. The specificity of the assay is supported by the finding that *A. baumannii*, *A. nosocomialis* and *A. pittii* have different melting temperatures of 82°C, 83.5°C and 84°C, respectively, and specific peaks.

Detection limit on DNA extracted from spiked whole blood samples by SYBR Green real-time PCR assay

Next, the performance of the SYBR Green real-time PCR assay was checked on blood samples. DNA extracted from serial dilutions (10^5, 10^4, 10^3, 10^2, 10^1 CFU/ml) of *A. baumannii* AYE and ATCC17978 cells mixed to whole blood samples was amplified in duplicate. The minimum detection level was 20 CFU/ml (AYE, R^2 value: 0.998; slope value: -3.438; PCR efficiency: 95.37%; ATCC17978, R^2 value: 0.99; slope value: -3.41; PCR efficiency: 96.467%; for Ct values see Table 2). Similar experiments have been

TABLE 2 - *Ct of A. baumannii DNA extracted from diluited blood spiked samples obtained by SYBR Green and TaqMan real-time PCR.*

	A. baumannii AYE		*A. baumannii ATCC17978*	
	SYBR Green	*TaqMan*	*SYBR Green*	*TaqMan*
CFU/ml	*Ct values*		*Ct values*	
10^5	17.9±0.06	22.8±0.02	20.7±0.09	22.0±0.11
10^4	21.3±0.11	26.2±0.22	24.2±0.47	25.4±0.09
10^3	24.8±0.35	29.6±0.43	27.5±0.25	28.9±0.37
10^2	28.4±0.19	32.9±0.39	30.9±0.51	32.3±0.28
2x10	30.8±0.55	34.8±0.38	32.9±0.62	34.3±0.59

carried out with DNA extracted from whole blood spiked with 10^8 CFU/ml of the strains of non-*baumannii Acinetobacter* species and the non-Acinetobacter genera. Curve profiles allowed to discriminate specific and non-specific amplifications.

In parallel, SYBR Green real-time PCR assays were performed on dilutions of whole blood samples spiked with *A. baumannii* AYE, using primers that amplify single copy $bla_{\text{OXA-51-like}}$ and *secE* genes previously described (Clifford *et al.,* 2012; Nomanpour *et al.,* 2011). The $bla_{\text{OXA-51-like}}$ and *secE* assay had a limit of detection of 100 CFU/ml and 1000 CFU/ml, respectively.

Specificity and sensitivity of real-time PCR TaqMan assay

To increase the specificity of the assay, a real-time PCR TaqMan assay was developed and tested on DNA extracted from bacterial cultures of 200 clinical isolates of *A. baumannii*, and 14 clinical isolates of non-*baumannii Acinetobacter* species and non-Acinetobacter genera. The real-time PCR yielded positive results for all tested isolates of the *A. baumannii*, and no amplification was detected for the strains of non-*baumannii Acinetobacter* species and the non-Acinetobacter genera. The sensitivity of the real-time PCR TaqMan assay was evaluated on DNA extracted from whole blood spiked with 10^8 CFU/ml of strains of non-*baumannii Acinetobacter* species and the non-Acinetobacter genera and from serial dilutions of blood spiked with *A. baumannii* AYE and ATCC17978. Fluorescence signal was observed only for the *A. baumannii* strains, and the limit of detection was 20 CFU/ml in both cases (AYE, R^2 value: 0.969; slope value: -3.191; PCR efficiency: 105.774%; ATCC17978, R^2 value: 0.989; slope value: -3.213; PCR efficiency: 104.742%). It is of interest to note that the assay was negative when genomic DNA extracted from blood samples spiked with 10^8 CFU/ml of *A. pittii*, *A. nosocomialis* and *A. junii*, indicating that TaqMan assay was specific for *A. baumannii*. Ct values for three replicates of tenfold dilutions of the blood spiked with *A. baumannii* AYE and ATCC17978 are reported in Table 2.

In clinical samples, mixtures of pathogens can either reflect a polymicrobial infection or contamination during sample acquisition. In both instances, the detection of the species of interest is hampered. We checked the ability of TaqMan real-time PCR to detect low copy levels of *A. baumannii* in samples containing high levels of another pathogen. Amplification was carried out on genomic DNA extracted from whole blood spiked with 10^3 CFU/ml of *A. baumannii* AYE and 10^6 CFU/ml of *E. coli* or *S. aureus*. In both cases, *A. baumannii* could be detected.

Based on the performance with spiked blood, we chose to test TaqMan real-time PCR using 12 BC-positive samples. BC identified eight Gram-negative bacteria (two *P. aeruginosa*, three *A. baumannii*, one *K. pneumoniae*, two *E. coli*) and four Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *S. hominis* and *E. faecalis*). As expected, the amplification was observed only for *A. baumannii* positive BC.

DISCUSSION

A. baumannii is identified in approximately 9% of bloodstream infections, and has a high mortality rate for critical patients treated in intensive care units. Blood culture is the current gold standard method for detecting BSI micro-

bial pathogens, although in many cases it is too slow or insufficiently sensitive. The turnaround time of BC ranges from 24 to 72 hours, which implies that results might become available too late to be of clinical utility. Early and accurate identification of the responsible pathogens is of critical importance for patient survival. Development of molecular diagnostic tools is essential to improve the clinical benefit of detection of pathogens in blood. The real-time PCR assay is a promising tool for detecting bacterial genomic DNA from biological fluids such as blood, urine and sputum. In this study, we designed a reliable and efficient TaqMan real-time PCR assay for detection and quantification of *A. baumannii* from spiked whole blood.

Rapid detection of *A. baumannii* is valuable due to tendency to spread and innate and acquired antimicrobial resistance of the pathogen (Higgins *et al.,* 2007). The limit of detection of genomic DNA target is critical for the clinical relevance of the assay. In septicemia the blood concentration of pathogens varies from on less 10 CFU/ml (>50%) to >100 CFU/ml (~25% of cases), thus necessitating a detection sensitivity of at least 100 CFU/ml. For all molecular pathogen identification methods, an efficient isolation from the clinical sample that results in amicrobial DNA template of high purity is important to achieve accurate results. High levels of human DNA, heme and anticoagulants (e.g. EDTA) in the blood collection tube are known to interfere with pathogen detection. Nucleic acid-based amplification assays considered highly-sensitive have a limit of detection of less than 50 targets per ml. Our assay was very sensitive being able to detect 20 CFU/ml. Several PCR assays have been reported for the detection of *A. baumannii* by targeting a variety of genes (Clifford *et al.,* 2012; McConnell *et al.,* 2012; Nomanpour *et al.,* 2011; Rice *et al.,* 2013; Wang *et al.,* 2013). Real-time PCR assays based on targeting the *bap* repeat were approximately 50 and fivefold more sensitive than the PCR using $bla_{\text{OXA-51-like}}$ or *secE*, respectively.

Our results also showed that the primers and/or probes designed for *A. baumannii* targeting *bap* gene are highly specific. This provides the opportunity to discriminate *A. baumannii* strains which more frequently spread into the hospital setting from other *Acinetobacter* species. Rapid detection of *A. baumannii* by real-time PCR appears to be a valuable tool, allowing earlier pathogen-adopted antimicrobial therapy in critically ill patients.

Development of primers and probes designed to target additional Acinetobacter species may be relevant for clinical diagnostics; however, this issue warrants further investigation.

ACKNOWLEDGEMENTS

This work was supported by PON Ricerca e Competitività 2007-2013 of the Agency of the Italian Ministry of University and Scientific Research (PON01_02589).

REFERENCES

Brossard K.A., Campagnari A.A. (2012). The *Acinetobacter baumannii* biofilm-associated protein plays a role in adherence to human epithelial cells. *Infect. Immun.* **80**, 228-233.

Clifford R.J., Milillo M., Prestwood J., Quintero R., Zurawski D.V., Kwak Y.I., Waterman P.E., Lesho E.P., Mc Gann P. (2012). Detection of bacterial 16S rRNA and identification of four clinically important bacteria by real-time PCR. PLoS One. **7**, e48558.

Dijkshoorn L., Nemec A., Seifert H. (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat. Rev. Microbiol.* **5**, 939-951.

Durante-Mangoni E., Zarrilli R. (2011). Global spread of drug-resistant Acinetobacter baumannii: molecular epidemiology and management of antimicrobial resistance. *Future Microbiol.* **6**, 407-422.

Espinal P., Seifert H., Dijkshoorn L., Vila J., Roca I. (2012). Rapid and accurate identification of genomic species from the Acinetobacter baumannii (Ab) group by MALDI-TOF MS. *Clin. Microbiol. Infect.* **18**, 1097-1103.

Fenollar F., Fournier P.E., Robert C., Raoult D. (2004). Use of Genome Selected Repeated Sequences Increases the Sensitivity of PCR Detection of Tropheryma whipplei. *J. Clin. Microbiol.* **42**, 401-403.

Gerner-Smidt P., Tjernberg I., Ursing J. (1991). Reliability of phenotypic tests for identification of Acinetobacter species. *J. Clin. Microbiol.* **29**, 277-282.

Gerner-Smidt P. (1992). Ribotyping of the Acinetobacter calcoaceticus-*Acinetobacter baumannii* complex. *J. Clin. Microbiol.* **30**, 2680-2685.

Giannouli M., Antunes L.C., Marchetti V., Triassi

M., Visca P., Zarrilli R. (2013). Virulence-related traits of epidemic Acinetobacter baumannii strains belonging to the international clonal lineages I-III and to the emerging genotypes ST25 and ST78. *BMC Infect. Dis.* **13**, 282.

Gundi V.A., Dijkshoorn L., Burignat S., Raoult D., La Scola B. (2009). Validation of partial rpoB gene sequence analysis for the identification of clinically important and emerging Acinetobacter species. *Microbiology.* **155**, 2333-2341.

Hansen W.L., Beuving J., Bruggeman C.A., Wolffs P.F. (2010). Molecular probes for diagnosis of clinically relevant bacterial infections in blood cultures. *J. Clin. Microbiol.* **48**, 4432-4438.

Higgins P.G., Wisplinghoff H., Krut O., Seifert H. (2007). A PCR-based method to differentiate between *Acinetobacter baumannii* and Acinetobacter genomic species 13TU. *Clin. Microbiol. Infect.* **13**, 1199-1201.

Klaschik S., Lehmann L.E., Raadts A., Book M., Gebel J., Hoeft A., Stuber F. (2004). Detection and differentiation of in vitro-spiked bacteria by real-time PCR and melting-curve analysis. *J. Clin. Microbiol.* **42**, 512-517.

Lehmann L.E., Hunfeld K.P., Emrich T., Haberhausen G., Wissing H., Hoeft A., Stüber F. (2008). A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples. *Med. Microbiol. Immunol.* **197**, 313-324.

Loehfelm T.W., Luke N.R., Campagnari A.A. (2008). Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *J. Bacteriol.* **190**, 1036-1044.

McConnell M.J., Pérez-Ordóñez A., Pérez-Romero P., Valencia R., Lepe J.A., Vázquez-Barba I., Pachón J. (2012). Quantitative real-time PCR for detection of *Acinetobacter baumannii* colonization in the hospital environment. *J. Clin. Microbiol.* **50**, 1412-1414.

Nomanpour B., Ghodousi A., Babaei A., Abtahi H., Tabrizi M., Feizabadi M. (2011). Rapid, cost-effective, sensitive and quantitative detection of *Acinetobacter baumannii* from pneumonia patients. *Iran J. Microbiol.* **3**, 162-169.

Peleg A.Y., Seifert H., Paterson D.L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* **21**, 538-582.

Rice L.M., Reis A.H. Jr, Ronish B., Carver-Brown R.K., Czajka J.W., Gentile N., Kost G., Wangh L.J. (2013). Design of a single-tube, endpoint, linear-after-the-exponential-PCR assay for 17 pathogens associated with sepsis. *J. Appl. Microbiol.* **114**, 457-469.

Smith M.G., Gianoulis T.A., Pukatzki S., Mekalanos J.J., Ornston L.N., Gerstein M., Snyder M. (2007). New insights into Acinetobacter baumannii pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev.* **21**, 601-614.

Turton J.F., Shah J., Ozongwu C, Pike R. (2010). Incidence of Acinetobacter species other than *A. baumannii* among clinical isolates of Acinetobacter: evidence for emerging species. *J. Clin. Microbiol.* **48**, 1445-1449.

Vallenet D., Nordmann P., Barbe V., Poirel L., Mangenot S., Bataille E., Dossat C., Gas S., Kreimeyer A., Lenoble P., Oztas S., Poulain J., Segurens B., Robert C., Abergel C., Claverie J.M., Raoult D., Médigue C., Weissenbach J., Cruveiller S. (2008). Comparative analysis of Acinetobacters: three genomes for three lifestyles. PLoS One. **3**, e1805.

Wang H.Y., Kim S., Kim H., Kim J., Kim Y., Park S.D., Jin H., Choi Y., Uh Y., Lee H. (2014). Real-time PCR TaqMan assay for rapid screening of bloodstream infection. *Ann. Clin. Microbiol. Antimicrob.* **13**, 3.

Wang Q., Zhou Y., Li S., Zhuo C., Xu S., Huang L., Yang L., Liao K. (2013). Real-time fluorescence loop mediated isothermal amplification for the detection of *Acinetobacter baumannii*. PLoS One. **8**, e66406.

Wisplinghoff H., Bischoff T., Tallent S.M., Seifert H., Wenzel R.P., Edmond M.B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39**, 309-317.

Yamamoto S., Bouvet P.J., Harayama S. (1999). Phylogenetic structures of the genus Acinetobacter based on gyrB sequences: comparison with the grouping by DNA-DNA hybridization. *Int. J. Syst. Bacteriol.* **49**, 87-95.