Molecular panel for detection of sepsis-related microorganisms

Painel molecular para detecção de microrganismos associados à sepse

Leslie Ecker Ferreira[1], Karilene Dalposso[3], Bruna Barbosa Hackbarth[3], Anderson R. Gonçalves[2], Glauco Adrieno Westphal[2], Paulo Henrique Condeixa de França[1], Mauro de Souza Leite Pinho[1]

1. Post-Graduation in Health and Environment Program of Universidade da Região de Joinville – UNIVILLE - Joinville (SC), Brazil.
2. Department of Medicine of Universidade da Região de Joinville – UNIVILLE - Joinville (SC), Brazil.
3. Department of Pharmacy of Universidade da Região de Joinville – UNIVILLE - Joinville (SC), Brazil

This study was developed at the Universidade da Região de Joinville – UNIVILLE - Joinville (SC), Brazil.

Financial support: Universidade da Região de Joinville and Foundation for Support of Scientific and Technological Research of the State of Santa Catarina.

Conflicts of interest: None.

Submitted on December 1st, 2010
Accept on March 10, 2011

Corresponding author:
Leslie Ecker Ferreira
UNIVILLE/ Área de Pesquisa
Rua Paulo Malschitzki, 10 – Campus Universitário – Zona Industrial
Zip Code: 89219-710 – Joinville (SC), Brazil.
Phone: (47) 3461-9197 / Fax (47) 3473-0131
E-mail: leslie.ferreira@univille.br

ABSTRACT

Introduction: Sepsis is a systemic inflammatory response related to high mortality rates in the hospital environment. Delayed etiological diagnosis and inadequate antimicrobial therapy are associated with treatment failures. Molecular tests based on polymerase chain reaction are regarded as faster and more accurate procedures than culture techniques for microbial identification, providing a higher rate of therapeutic success.

Objective: To develop a panel of primers for DNA fragments of sepsis-related microorganisms.

Methods: Primers for amplification of *Enterobacter spp.*, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida spp.* were designed and tested for sensitivity and specificity on the basis of their respective standard strains.

Results: The intended specificity was obtained for *P. aeruginosa, S. aureus* and *Candida spp* primers. Sensitivity tests showed a threshold for detection from 5 ng to 500 fg in blood samples contaminated with microbial DNA.

Conclusions: The molecular panel presented offers the advantage of a flexible 'open' system when compared to other multiplex detection methods.

Keywords: Molecular diagnostic techniques; Polymerase chain reaction; Sepsis; DNA primers/diagnosis; Nucleic acid amplification techniques

INTRODUCTION

In Brazil, there are an estimated 400,000 cases of severe sepsis per year, requiring 17% of the available beds in the intensive care units. [1] Confirmation of the diagnosis depends on microbiological exams based on blood culture techniques, which usually take 24 to 72 h.[2] Thus, in most cases, antibiotic therapy is initiated on the basis of clinical criteria. Inadequate antimicrobial treatment regimens have been associated with the emergence of drug-resistant strains, increasing treatment costs and mortality rates, especially among patients in critical condition.[3] Nevertheless, it is recommended that adequate antimicrobial therapy should be initiated as early as possible because each hour delay results in a 7.6% increase in death rate.[4]

Molecular assays employing polymerase chain reaction (PCR) are important tools for the detection of microorganisms and may contribute to early diagnosis with high sensitivity, even when targets are present at extremely low titers (10 to 100 DNA copies).[5] Microbial investigations by PCR have been

explored with a detection system known as Multiplex-PCR. This technique is based on simultaneous amplification of distinct segments of target DNA by employment of two or more primer pairs in a single reaction vessel, which, in turn, usually means reduced costs and time requirement. The thermodynamics of Multiplex-PCR requires a complex balance among parameters such as levels of salts, primers, and DNA polymerase, as well as compatible primer melting temperatures, resulting in a laborious experimental design.[6,7] In addition, Multiplex-PCR systems have also shown lower sensitivity when compared to individual reactions with the corresponding single-primer pairs.[8]

On the other hand, molecular panels are considered a less complex alternative to Multiplex-PCR. They are performed employing two or more primers pairs designed for distinct loci and are conducted in separate vessels under equal thermocycling conditions. Additional primers pairs targeting other *loci* may be eventually included with no need to redefine the reaction parameters.[9]

The aim of the present study was to develop and standardize a panel of primers for PCR amplification of specific DNA fragments of microorganisms related to sepsis according to a unique protocol for a faster confirmatory diagnosis.

METHODS

All procedures adopted in this study were approved by the Institutional Research Ethics Committee of the University of Joinville Region - UNIVILLE.

Primer design

A panel of PCR primers was developed for detection of five microorganisms related to sepsis, namely, *Enterobacter spp.*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida spp.* A series of nucleotide sequences of each microorganism were selected from the GenBank database, retrieved from the 23S rRNA gene for *P. aeruginosa*, *Enterobacter spp.* and *S. aureus* and from 18S rRNA for *Candida spp.* For each respective set, a consensual sequence was generated by ClustalW® software.[10] These were submitted to Visual OMP® software[11] to simulate a set of primers for simultaneous amplification. Multiple oligonucleotides were then tested *in silico* regarding specificity of the BLAST tool against all nucleotide sequences available in GenBank.[12] Similarly, the *LacZ* gene was defined for primers intended for *Escherichia coli* identification using the PRIMER3 ® software.[13]

Microorganisms

Reference strains of *Candida albicans* (ATCC 10231), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019), *Enterobacter aerogenes* (ATCC 13048), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus (*ATCC 6538) were acquired from the Tropical Culture Collection of the André Tosello Foundation.

DNA preparation

Qiamp DNA Mini Kit® (Qiagen, Hilden, Germany) was applied for DNA extraction of strains maintained in solid media or present in human blood samples. DNA extraction of *Candida* strains was preceded by cell disruption with glass beads. Qualitative and quantitative assessments of DNA preparation were performed through spectrophotometric measurements.

Polymerase chain reaction

Reactions were performed at a defined volume of 50 µL and included 50 – 500 ng DNA, 1 U Taq DNA polymerase (LGC Biotecnologia, São Paulo, Brazil), 200 µM dNTP's (GE Healthcare, Little Chalfont, United Kingdom), 1 - 2 mM $MgCl_2$ (LGC Biotecnologia), 10X PCR Buffer (LGC Biotecnologia) and 20 pmol of each primer (Invitrogen, São Paulo, Brazil). To achieve a common optimal annealing temperature for all primer pairs, individual reactions were tested in temperature gradients from 40°C to 60°C, according to vessels position in the thermocycler device (LGC XP thermocycler - BIOER Technology Co., Tokyo, Japan). The initial denaturation was performed at 94°C for 3 minutes, followed by 40 cycles encompassing three consecutive 45-seconds steps at 94°C, 50°C, and 72°C. An extension step at 72°C for 10 minutes ended the procedure. The amplicons obtained were submitted to electrophoresis in 1% agarose gel and stained by ethidium bromide (0.5 µg/mL) for UV light analysis and digitized (MiniBis-Pro photodocumentation system - DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Sensitivity tests

Sensitivity tests were performed by serial dilutions of DNA extracted from human blood samples *in vitro* contaminated by the above-mentioned microbial strains.

Specificity tests

Specificity tests were performed employing DNA extracted from blood samples (human DNA) or pure cultures (DNA of microorganisms included or not in the panel) (see also **DNA preparation**).

RESULTS

Analysis of multiple oligonucleotide sequences suggested by Visual OMP® software resulted in a panel of four pairs of compatible primers for amplification of respective microorganisms in the same thermocycling protocol. As *Enterobacter spp.* primers showed crossed amplification to *E. coli,* an additional pair of primers based on the *E. coli LacZ* gene was developed for differential diagnosis in a further reaction when required. These primers generated amplicons of 526 bp (*Enterobacter spp.)*, 430 bp (*Candida spp.*), 407 bp (*E. coli*), 377 bp (*P. aeruginosa*), and 198 bp (*S. aureus*) (Table 1).

Analysis of the temperature gradients for all primer pairs yielded a consensual annealing temperature of 50ºC (Figure 1). Additional PCR differentiation between *Enterobacter spp.* and *E. coli* was performed employing distinct annealing temperatures (60ºC).

Sensitivity analysis showed a lower threshold for detection of microbial DNA at 5 ng (*Enterobacter spp.)*, 5 pg (*Candida spp.*, *E. coli, P. aeruginosa*) and 500 fg (*S. aureus)* (Figure 2). Optimal specificity results were obtained with all primer pairs when compared to divergent microbial and human DNA (Figure 3), except by the above-mentioned *Enterobacter spp. – E. coli* crossed reaction.

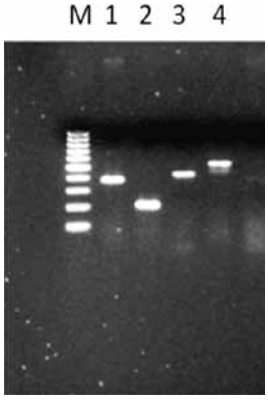

Figure 1 - Simultaneous amplification in separate vessels of (1) *P. aeruginosa,* (2) *S. aureus* (3) *C. albicans,* and (4) *E. aerogenes* with optimized common annealing temperature (50°C). (M) Molecular Standard (100 bp ladder, Fermentas, Ontario, Canada).

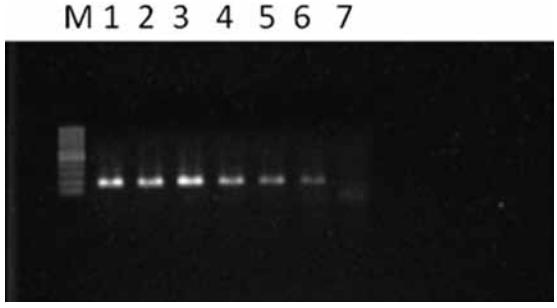

Figure 2 - Sensitivity test of the PCR system designed for *S. aureus*. (1) 50 ng, (2) 5 ng, (3) 500 pg, (4) 50 pg, (5) 5 pg, (6) 500 fg, and (7) 50 fg. (M) Molecular Standard (100 bp ladder, Fermentas).

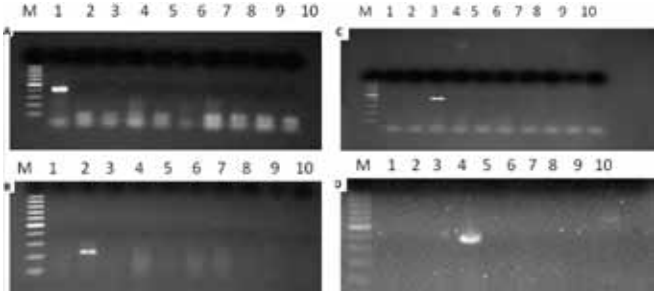

Figure 3 - Specificity test of the PCR system designed for: (A) *E. coli,* (B) *S. aureus,* (C) *P. aeruginosa,* (D) *Candida spp.* (M) Molecular Standard (100 bp ladder, Fermentas, Ontario, Canada). (1) *E. coli*, (2) *S. aureus,* (3) *P. aeruginosa,* (4) *Candida albicans* (5) *A. baumannii,* (6) *E. aerogenes,* (7) *Streptococcus salivarius,* (8) *Streptococcus mutans,* (9) *H. sapiens,* and (10) negative control.

Table 1- Primer database

Primer sequence	Target microorganism	Frame	Amplicon (bp)
CACCTATTTTCTATCTA	*S. aureus*	Sense	198
CCTATAATCGTTTTAAT		Antisense	
GTCAGTGTTACCTAA	*P. aeruginosa*	Sense	377
GAAAGGATCTTTGAA		Antisense	
GTTAAGGTATTTACATT	*Candida spp.*	Sense	430
TCAGTTATCGTTTATT		Antisense	
GAACATCAAACATTAAA	*E. coli*	Sense	454
AATCAGTCGAAGATA		Antisense	
GTACGATTTGTTGTTA	*Enterobacter spp.*	Sense	526
AAAGAAAGCGTAATA	*E. coli*	Antisense	

DISCUSSION

As current confirmatory diagnostic procedures for sepsis require a 24-72 h period for confirmation of an infectious etiology and identification of the pathogen, antibiotic therapy is usually started on an empirical basis.[2]

However, studies comparing blood cultures and molecular tests for detection of microorganisms related to sepsis involve more rapid reporting when performed using PCR-based techniques.[14-16]

The time required for specific microbiological diagnosis using the molecular panel developed in the present study is approximately six hours, except in cases of positivity for *Enterobacter spp.*, when an additional two-hour procedure is required to differentiate samples from *E. coli*. It must also be considered that this panel includes the detection of *Candida spp.*, in view of the difficulty of breaking the fungal cell wall for genomic DNA extraction.[17]

Considering the high sensitivity of PCR-based detection methods, the addition of certain measures must be followed to prevent false-positive results. Such measures include internal controls using non-specific primers based on preserved nucleotide sequences from different species and strictly safe protocols for DNA extraction and amplification.[18]

Other studies have proposed different methods of PCR-based diagnostic procedures.[19-21] For instance, a molecular strategy to detect 62 pathogens via PCR followed by hybridization has been proposed;[22] however, this approach does not involve microorganism differentiation. In this context, large-scale detection systems using highly sensitive microarray and PCR methods have been used to identify 800 sepsis-related microorganism genes;[23] however, the technique's clinical value is impaired by the intrinsic complexity and high costs.

In a recent study,[9] a *set* of primers was reported for detecting the five most prevalent microorganisms in blood cultures and suggested that molecular panels show higher sensitivity and are faster than blood culture. Furthermore, fast and sensitive molecular panel-based methods for identification of bacterial and fungal pathogens causing sepsis directly from blood specimens appears promising for a rapid fine-tuning of empirical antibiotic therapy.[15]

CONCLUSION

Compared to conventional multiplex detection systems, where all primers are mixed in a single 'closed' reaction kit, the molecular panel offers the additional advantage of an 'open' system, where primers targeting other microorganisms may be added to the unique PCR protocol reaction with no delay in diagnostic time.

We conclude that the molecular panel developed is a reliable method for detecting sepsis-related microorganisms, and further studies must be undertaken to assess its role in a clinical environment.

ACKNOWLEDGEMENTS

This study was supported by grants from FAPESC (Santa Catarina State Research Foundation) and FAP-UNIVILLE (Research Fund from University of Region of Joinville). We also thank Carmen Diamantina Teixeira Heyder and Roseneide Campos Deglmann for their technical support.

RESUMO

Introdução: A sepse é uma resposta inflamatória sistêmica relacionada com altas taxas de mortalidade no meio hospitalar. O diagnóstico etiológico tardio e terapia antimicrobiana inadequada se associam a falhas do tratamento. Exames moleculares baseados na reação em cadeia da polimerase são considerados métodos mais rápidos e precisos do que técnicas de hemocultura para identificação microbiana, proporcionando uma taxa mais elevada de sucesso terapêutico.

Objetivo: Desenvolver um painel de seqüências iniciadoras (*primers*) para fragmentos de DNA de microrganismos associados à sepse.

Métodos: Seqüências iniciadoras para amplificação de *Enterobacter spp.*, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* e *Candida spp.* foram desenvolvidos e testados quanto a sensibilidade e especificidade com base em suas respectivas cepas padrão.

Resultados: A especificidade pretendida foi obtida para os *primers* de *P. aeruginosa, S. aureus* e *Candida spp*. O teste de sensibilidade mostrou um limite de detecção de 5 ng a 500 fg em amostras de sangue contaminado com DNA microbiano.

Conclusões: O painel molecular apresentado oferece a vantagem de constituir um sistema flexível "aberto" em comparação a outros métodos de detecção múltipla.

Descritores: Técnicas de diagnóstico molecular; Reação em cadeia da polimerase; Sepse; Primers de DNA/diagnóstico; Técnicas de amplificação de ácido nucleico

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