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UNIVERSAL PRIMERS FOR DETECTION OF COMMON BACTERIAL PATHOGENS CAUSING PROSTHETIC JOINT INFECTION

Pavel Sauer[a], Jiří Gallo[b], Michaela Kesselová[a], Milan Kolář[a], Dagmar Koukalová[a]

[a] *Institute of Microbiology, Faculty of Medicine, Palacký University, Teaching Hospital, Olomouc, Czech Republic*
[b] *Department of Orthopaedics, Faculty of Medicine, Palacký University, Teaching Hospital, Olomouc, Czech Republic*

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The diagnosis of low grade prosthetic joint infection is difficult and time consuming. Nested-PCR for universal bacterial DNA segments detection of "orthopaedic" bacteria was tested in a laboratory setting. This method is based on amplification of the 16S bacterial ribosomal RNA coding sequences. 11 species of the most frequent bacterial pathogens (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus agalactiae, Enterococcus faecium, Enterococcus faecalis, Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Serratia marcescens*) involved in prosthetic joint infections were studied. All could be detected rapidly and sensitively by this method.

INTRODUCTION

Prosthetic joint infection (PJI) is a serious complication of total joint arthroplasty causing a high degree of morbidity in affected patients. In addition, it is a significant economic burden to any health care system. Currently, the frequency of infection after knee or hip arthroplasty ranges from below 1% to 5% with slightly higher figures in revision procedures.[1]

The bacteria most commonly isolated from PJIs include coagulase-negative staphylococci and *Staphylococcus aureus*, followed by streptococci, gram-negative bacilli and others.[2] In general, early manifestation of PJI (within the first 1–3 postoperative months) seems to be associated with more virulent pathogens such as *Staphylococcus aureus*, and often produces a clearer clinical picture of infection. On the other hand, the indolent and shifty nature of late occurring infections is more typical of less virulent bacteria such as coagulase-negative staphylococci.[3] Various algorithms have been proposed to improve joint sepsis diagnostics, including clinical, serological, microbiological, and imaging techniques.[4]

It has been suggested that one useful method for low-grade prosthetic sepsis bacteria identification should be sensitive and specific enough to allow early and adequate therapy. Recent studies suggest that rapid detection systems can decrease costs associated with hospitalization and refine application of antibiotic treatment.[5]

Traditional cultivation methods are time consuming and are not able to detect very small amounts of bacteria occurring in some PJIs or due to antibiotic treatment.[6] This is an argument for the utilization of molecular diagnostics relying on the presence of bacterial DNA. PCR methods are able to detect small amounts of pathogen (even deadones). There are different strategies to PCR amplification of bacterial DNA in clinical samples. The first based on usage of species-specific primers. This method lacks the ability to determine bacterial infection definitely.[7] The second approach involves amplification of sequences found in all bacteria based on universal sequences common in bacteria.[8] A number of primer systems for 16S bacterial rRNA detection have been published but they differ in focus to examinant clinical material and pathogens.[9, 10, 11, 12] The first study in periprosthetic infection diagnostics with the help of PCR methods was published by Levine et al.[13] In the Czech Republic, diagnostic based on PCR detection was published in 1999 by Rozkydal et al.[14]

In this report, the primer system is demonstrated, optimized and tested for the most common orthopaedic pathogens. It is sensitive enough, has broad specifity among bacteria and can be used for a vast range of materials from joint fluid to tissue specimens.

MATERIALS AND METHODS

Bacterial isolates and extraction of DNA

The pure bacterial isolates were collected from cultures of common clinical specimens obtained at The Institute of Microbiology, Teaching Hospital Olomouc and Faculty of Medicine, Palacký University and were identified using the diagnostic test kits ENTEROtest16, STAFYtest and NEFERMtest (Pliva-Lachema, Czech Republic). The investigated species of bacteria were *Staphylococcus aureus* (10 isolates), *Staphylococcus epidermidis* (7), *Streptococcus pyogenes* (4), *Streptococcus agalactiae* (4), *Enterococcus faecium* (2), *Enterococcus faecalis* (2), *Klebsiella pneumoniae* (2), *Escherichia coli* (2), *Proteus mirabilis* (1), *Pseudomonas aeruginosa* (1) and *Serratia marcescens*

(1). For the isolation of DNA, the pure isolates were inoculated onto agar and incubated overnight at 37 °C. One colony of the studied bacterial species was taken for isolation of DNA and resuspended in lysis buffer (10 mM Tris; 0,3 M sucrose; 5 mM $MgCl_2$; 1 % Triton X-100) and harvested by centrifugation (3000 rpm, 15 min). DNA was recovered from the resulting sample using the Lego DNA Isolation kit (Top-Bio, Czech Republic) according to the manufacturers instruction. Briefly, a pellet of the bacterial colony was resuspended in binding buffer, silica was added and mixed 10 min by vortexing. Then the sample was centrifuged (14 000 rpm, 2 min), washed twice with washing buffer, acetone and then dried. DNA was purchased by elution with buffer (10 mM Tris-HCl, 1mM EDTA) and frozen at –20 °C for later usage.

Nested PCR

16S rRNA target sequences of common bacterial pathogens causing the most frequent orthopaedic problems were selected using internet gene database of The Institute for Genomic Research (TIGR) and compared with sequences obtained from other internet gene databases (GenBank, USA). Conservative segments were chosen using the MACAW programme and suitable universal oligonucleotide primer pairs were designed with the help of the PRIMER2 programme. The sequence of primers used was as follows: outer forward UNI_OL 5'-GTGTAGCGGTGAAATGCG-3', outer reverse UNI_OR 5'-ACGGGCGGTGTGTACAA-3', inner forward UNI_IL 5'-GGTGGAGCATGTGGTTTA-3', inner reverse UNI_IR 5'-CCATTGTAGCACGTGTGT-3'.

A master mix of total volume 45 µl was prepared as follows: 37,6 µl H_2O; 5 µl 10× reaction buffer; 0,4 µl dNTPs (10 mM each); 0,8 µl of each primer (100 µM) and 0,4 µl *Taq* polymerase (5U/µl) (dNTPs was obtained from Promega USA, other components from Top-Bio, Czech Republic). 5 µl of DNA of tested bacterial isolate was added to master mix and overlaid with 50 µl of mineral oil (Top-Bio, Czech Republic). Hot-start PCR on MiniCycler (MJ Research, USA) was performed. The method was composed of the following steps: 94 °C 5 min, 94 °C 1 min, 55 °C 1 min for outer primer pair annealing and 72 °C 1 min for elongation. Cycles were repeated 30×. 1 µl from the first run for the second part of nested-PCR was used. Annealing temperature of inner primer pair was changed to 53,6 °C.

To avoid extraneous DNA contamination or residual DNA in *Taq* polymerase, master mix was preincubated with 50 U of DNase I (Fermentas, Lithuania) for 30 min at 37 °C. DNase I was furher denatured by heating at 95 °C for 10 min. Assumed amplicons with 709 bp for outer primer pair and 287 bp for inner primer pair were visualized with ethidium bromide after gel electrophoresis in 2 % agarose gel.

The sensitivity of the reaction was tested with concentration series of *Staphylococcus aureus* and *Staphylococcus epidermidis* DNA.

RESULTS

Primers UNI_OL, UNI_OR, UNI_IL and UNI_IR corresponding to universal bacterial regions of the 16S rRNA gene of *Staphylococcus aureus* were expected to amplify DNA from chosen pathogenic bacteria. Designed primer pairs for nested-PCR were tested with 11 species (36 bacterial isolates) of the most frequent microbial orthopaedic pathogens and in all tested bacteria the 16S rRNA parts were amplified. Amplicons of expected sizes 709 bp and 287 bp were detected after the first and the second run of nested-PCR (Figure 1). A master mix without template DNA and DNase I nontreated by preincubation, showed in the second run of nested-PCR band corresponding to requested amplicon for detection of universal bacteria (287 bp long). After preincubation with 50 U of DNase I, this amplicon was

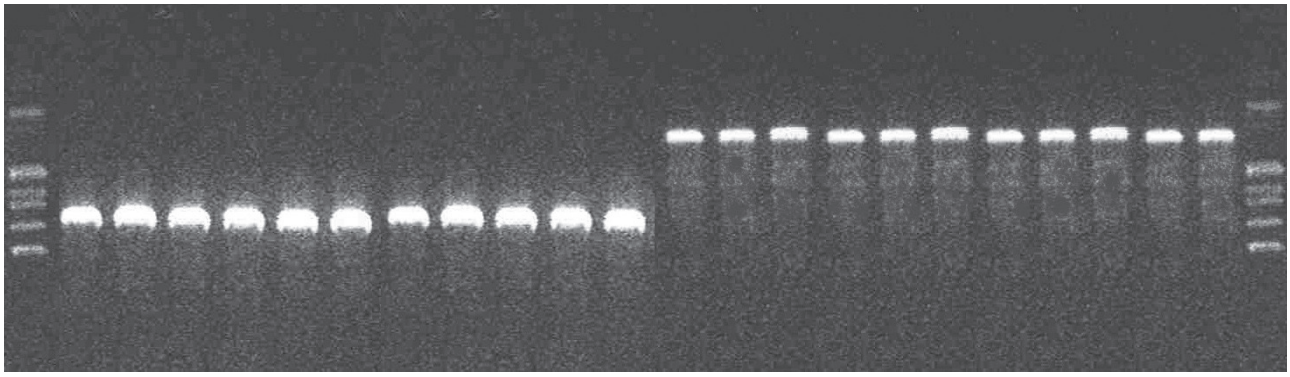

Fig. 1. PCR detection of DNA from different bacterial cultures. Lanes 1 and 24, molecular weight marker (155, 447, 544, 595, 750, 970 bp). Lanes 2-12 show a 709 bp band, lanes 13-23 a 287 bp band corresponding to *Staphylococcus aureus* (2, 13); *Staphylococcus epidermidis* (3, 14), *Streptococcus pyogenes* (4, 15), *Streptococcus agalactiae* (5, 16), *Enterococcus faecium* (6, 17), *Enterococcus faecalis* (7, 18), *Klebsiella pneumoniae* (8, 19), *Escherichia coli* (9, 20), *Proteus mirabilis* (10, 21), *Pseudomonas aeruginosa* (11, 22), *Serratia marcescens* (12, 23).

not visible. Detectable concentration of DNA isolated from *Staphylococcus aureus* and *Staphylococcus epidermidis* was 19 fg per µl, 6 fg per µl respectively.

DISCUSSION

It is well known that bacterial DNA or RNA can serve as an effective target for amplification and detection of bacterial DNA.[15, 16] Nevertheless the PCR-based assay may be so sensitive that is very easy to contaminate it regardless when during the pre-amplification or the post-amplification phase. The sources of false positive amplification may be at the site of specimen retrieval (commensal skin organisms) or during laboratory processing (reagents, pipettes, laboratory environment). Traces of DNA in *Taq* polymerase may be the source of false positivity too.[17] Hence false positivity has frustrated researchers and clinicians due to unreliable interpretation of positive molecular results. To eliminate these problems, a number of recommendations (UV irradiation, DNase I or restriction endonucleases pretreatment, detection threshhold) and laboratory protocol advices have been published.[18, 19] Simultaneously, specific PCR configurations and complicated structure of inner controls were developed and tested. Mariani et al tested a protocol with preamplification DNase treatment and standard radioactive or non-radioactive labeling combined with *Escherichia coli* 16S rRNA gene as a probe.[20] Later they published excellent clinical results for synovial fluid molecular analysis of PJI(ref.[21]). Hoeffel et al stressed the role of residual *Escherichia coli* DNA in commercially available products.[22] They attempted to eliminate this false-positive amplification by developing so-called genus focused PCR primers designed not to hybridize and amplify *Escherichia coli* 16S rRNA DNA. However, the results of this strategy were rather disillusioning (sensitivity 71 %, specificity 49 %). The results of our study strongly support the tactic using DNase I preamplification treatment as a more valid way to prevent false positive results. On the other hand, Meier et al treated their PCR master mix with ultraviolet radiation for 1 hour and obtained similar results.[23] Based on these statements, there is evident that many ways achieve the same status, so the problem seems to be successfully solved.

PCR-based techniques have also been referred to the context of false negative results. This may occur due to the very high detection threshold of the method, failure in extraction of bacterial DNA or due to the presence of PCR inhibitors in clinical samples. Currently, clear recommendations for detection threshold do not exist. This ranges from 10 to more than 1000 colony forming units per cubic centimetre in published studies.[24] However, this was beyond the scope of our study.

Our configuration of broad range nested-PCR is able to reliably detect the most common bacterial pathogens involved in prosthetic joint infection. According to clinical experiences the most relevant PJI pathogens are *Staphylococcus aureus*, *Staphylococcus epidermidis* and other coagulase-negative staphylococci. Other bacteria tested in the present study have been captured at a very low rate. Nevertheless, all eleven tested bacteria were identifiable by this approach and after PCR amplification two expected products 709 bp and 287 bp long were detected. The detection limit was 6 fg per µl and 19 fg per µl for *Staphylococcus epidermidis* and *Staphylococcus aureus*, respectively. No other main products were detected if the master mix was pretreated with DNase I. The source of false positivity was probably DNA contamination from *Taq* polymerase occurring under our laboratory settings. Non-treated master mix showed byproducts. To remove them, at least 50 U of DNase I for one PCR tube was needed.

Based on this result it may be concluded that this method seems to be applicable for the detection of bacteria from joint fluid and tissue specimens. Simultaneously, there is a need to continue to test this procedure as part of well designed clinical studies.

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