Development of a Novel, Rapid Processing Protocol for Polymerase Chain Reaction-Based Detection of Bacterial Infections in Synovial Fluids

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Abstract

We describe the development of a molecular detection system designed for use with synovial fluid (SF)-based infections. The methodology employs a lysis/extraction procedure that effectively disrupts microorganisms allowing for release of the microbial DNA and its amplification by polymerase chain reaction (PCR). We tested the effectiveness of adding a mixed-bed, ion-exchange resin to the extract to remove PCR inhibitory components present in the SF. After centrifugation to separate the resin, DNA contained in the supernatant is subjected to PCR using oligonucleotide primers designed for broad-spectrum microorganism detection. Amplification products are analyzed by agarose gel electrophoresis and/or DNA hybridization methodology. We report here the detection sensitivity and specificity of the protocol using SF inoculated with *Escherichia coli* and *Staphyloccocus aureus*. We have applied this new methodology to clinical SF specimens with results superior to standard laboratory culturing assays.

Index Entries: Synovial fluid; bacterial infection; polymerase chain reaction; joint arthroplasty; clinical diagnostics.

1. Introduction

Current methodologies for the diagnosis of infection associated with orthopedic implants and joint disease are relatively inaccurate, owing to the difficulty of retrieval and detection of infectious microorganisms. The accepted standard for microorganism identification has been arthrocentesis with Gram stain testing and laboratory culturing, techniques that suffer because of unacceptable frequencies of false negative results. Thus, a relatively large number of orthopedic infections remain undiagnosed.

We have applied molecular biological techniques to develop a protocol that greatly improves the sensitivity, speed, accuracy, and reproducibility of identification of bacterial contamination or infection in orthopedic specimens *(1)*. Our protocol involves processing a small volume of synovial fluid (SF) for bacterial lysis and DNA extraction that allows rapid detection using the polymerase chain reaction (PCR) *(2)*. The PCR technology allows the specific targeting of bacterial DNA in a complex mixture of bacterial and host DNA types. We have made use of the fact that many currently known orthopedically relevant bacterial species possess highly conserved, multicopy 16S ribosomal RNA genes, which are amenable to hybridization with a single set of oligonucleotide primers for PCR-based amplification *(3)*.

For rapid, PCR-based testing of orthopedically relevant specimens, the SF sample must be prepared in conditions that will allow its direct addition to the reaction mixture without adverse effects on the activity of the polymerase enzyme. Ideally, a DNA purification step prior to PCR analysis would ensure optimal polymerase activity; however, with the extremely low bacterial

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titers present in some SF aspirates, any purification step would be impractical, if not impossible. Therefore, we have designed a procedure that couples a simple bacterial lysis and DNA extraction system directly with PCR for use with SF samples. This procedure allows the rapid and reproducible detection of bacterial infection/contamination from SF samples.

2. Materials

The described methodology for SF-based bacterial infection detection can be divided into three steps:

1. The lysis/extraction step disrupts the bacterial cell wall to release bacterial DNA into solution.
2. The amplification step uses aliquots of extract directly for PCR using bacteria specific primers.
3. The detection step uses gel electrophoresis to size fractionate DNA amplification products, which are visualized by ethidium bromide (EtBr) staining.

For increased detection sensitivity, a Southern blot hybridization step *(4)* is included using cloned PCR product as probe. As an alternative, amplification products are slot-blotted directly onto nylon membrane for hybridization analysis.

2.1. SF Lysis/Extraction

1. Extraction buffer (KTET): Stock solution (diluted 2:1 with SF), 75 m*M* KCl, 150 m*M* Tris-HCl, pH 8.0, 3 m*M* ethylenediaminetetra-acetate (EDTA), and 0.75% polyoxyethylene sorbitan monolaurate (Tween-20).
2. Aseptic SF, retrieved prior to total joint arthroplasty, 2–10 mL.
3. Fresh cultures of *Escherichia coli* and *Staphylococcus aureus* grown to saturation and titered by counting colonies of plates (in duplicate) inoculated with a dilution series of the cultures.
4. Mixed-bed, mixed-charge ion-exchange resin (MBR), e.g., Bio-Rad (Hercules, CA) biotechnology grade mixed bed resin AG 501-X8, 20-50 mesh.
5. Chelex-100 cation-exchange resin (Bio-Rad).
6. Sterile, double-distilled water, and sterile 0.9% saline solution.
7. Proteinase K (Promega, Madison, WI).

2.2. PCR Amplification

1. PCR primers targeting the 16S ribosomal RNA (rRNA) gene of *E. coli*: upstream (5') primer sequence is CGGCAGGCCTAACACATGCAAGTCG, downstream (3') primer sequence is GGTTGCGGCCGTACTCCCCAGG.
2. Thermocycling conditions are as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, 35 cycles. A precycle denaturation step at 94°C can be included. We used the Perkin-Elmer (Norwalk, CT) GeneAmp PCR Reagent Kit.
3. Perkin-Elmer and Coy (Ann Arbor, MI) thermocyclers have been used for these studies.

2.3. Gel Electrophoresis and Blot Hybridization

1. Standard high-melting-temperature electrophoresis grade agarose (Fisher, Pittsburgh, PA).
2. Ethidium bromide (EtBr) stock solution, 10 mg/mL, diluted 1:20,000 in molten agarose prior to pouring gel.
3. Nylon membrane filter, e.g., Nytran from Schleicher and Schuell (S&S, Keene, NH) or positively charged membrane from Boehringer-Mannheim Biochemicals (BMB, Indianapolis, IN), for blot transfer using capillary action or S&S Turboblot Transfer System. Slot-blotting is done using an S&S Minifold II Slot Blot system.
4. Megaprime random prime DNA labeling kit (Amersham, Arlington Heights, IL) for ^{32}P-nucleotide incorporation (DuPont NEN, Boston, MA). DIG DNA Labeling and Detection Kit (BMB) for nonradioactive labeling using digoxygenin incorporation.
5. Rad-Free, Lumi-Phos Chemiluminescent Substrate Sheets (S&S) for detection of alkaline phosphatase conjugates.

3. Methods

3.1. Lysis/Extraction Methods

In developing the detection protocol for SF infection, we first compared existing bacterial extraction methods *(5)*. A key consideration for successful and reproducible infection detection is that the lysis/extraction conditions allow for its direct addition to the PCR amplification mixture.

To test various procedures in this regard, we first used bacterial inoculations in sterile saline solution. *E. coli* or *S. aureus* was added to sterile saline at a concentration of 1×10^4 cells/mL. Each lysis/extraction method listed below was tested using 0.1 mL of bacterial sample (1000 cells).

1. Freeze/thaw method: three to five cycles of quick-freezing in dry ice/ethanol bath, and thawing at 65°C, 1 min at each temperature.
2. Freeze/boil method: three to five cycles of quick-freezing in dry ice/ethanol bath, and boiling, 1 min at each temperature.
3. Chelex-100 method *(6)*: Sample was boiled in the presence of 10% (w/v) Chelex-100 cation-exchange resin for 10 min.
4. Proteinase-K method: Samples were made 20 m*M* Tris-HCl, pH 7.5, and 0.5% (v/v) Tween-20. Proteinase K was added to a final concentration of 0.5 mg/mL and incubated for 1 h at 37°C, followed by heat inactivation at 85°C for 10 min. Additionally, the sample was extracted once with an equal volume of phenol:chloroform (1:1). The aqueous phase was carefully harvested for PCR.
5. Proteinase-K treatment was performed as above, but the phenol:chloroform step was omitted.

These five methods were tested several times and a representative outcome is shown in Fig. 1. We had the most consistent success with the freeze/thaw (protocol 1), Chelex-100 (protocol 3), and one of the proteinase-K methods (protocol 5). However, when tested with SF inoculated with known concentrations of bacteria, all methods failed to give reproducible PCR products detectable by EtBr staining of agarose gels, even with bacterial titers as high as 1×10^4 and 1×10^5 cell/100 µL sample (*see* Table 1 and Note 1).

Despite these preliminary negative results using SF, we felt that modification of these methods could yield a workable protocol, except for proteinase-K treatments, which we eliminated because of the lengthy incubation times required (*see* Note 1). Owing to the viscosity of SF, we added a dilution step using KTET buffer *(7)* or water (*see* Note 2). For the freeze/thaw and freeze/boil methods, we added a posttreatment incubation for 10 min at 90°C to ensure complete lysis. Owing to limited initial success with Chelex-100, a cation-ion-exchange resin, we also tested a mixed-charge, mixed-bed ion-exchange resin (MBR), Bio-Rad 501-X8. To test these different conditions, we performed the following experiment: A 100-µL sample of SF, containing 1×10^5 or 1×10^4 cells, was mixed with 200 µL of water or KTET buffer. Samples were subjected to four cycles of freeze/boiling or freeze/thawing, followed by the addition of Chelex-100, MBR, or no resin, and either heat-treated at 90°C for 10 min, or not heated. All samples were centrifuged briefly and aliquots of the supernatant used for PCR analysis. The results are shown in Fig. 2 and summarized in Table 2 (*see also* Note 3).

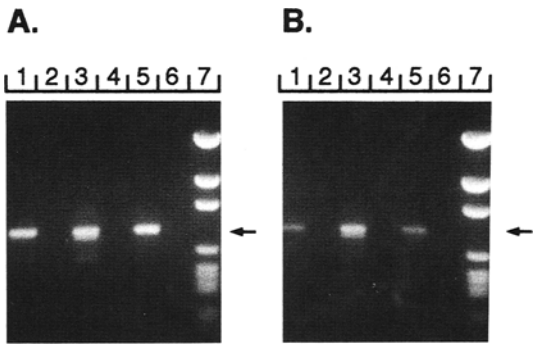

Fig. 1. EtBr-gel electrophoresis detection of PCR amplification products of bacterial DNA prepared by five different lysis/extraction methods using saline solution inoculated with **(A)** *E. coli* or **(B)** *S. aureus*. A 100-µL volume of saline sample containing 1×10^5 bacterial cells was used for each extraction. A 10-µL volume of extract was used for each 100-µL PCR amplification, of which 20 µL were used for electrophoresis on 1.4% agarose gels. Lane 1, freeze/thaw method; lane 2, freeze/boil method; lane 3, Chelex-100 method with 10 min 100°C heat treatment; lane 4, proteinase-K digestion with organic extraction; lane 5, proteinase-K digestion without organic extraction; lane 6, negative control, saline solution extracted without added bacteria; lane 7, DNA molecular size markers (pGEM, Promega). Arrows indicate position of the 881-bp PCR product generated from bacterial 16S ribosomal RNA gene. Note that methods 1, 3, and 5 were the most successful in yielding PCR products for both bacterial species, although for *E. coli* PCR yields are higher with methods 1 and 5.

Table 1
Efficiency of Different Lysis/Extraction Methods for PCR Detection of Bacteria[a]

Method	Sample composition	
	Saline	SF
Freeze/thaw (three cycles)	++	–
Freeze/boil (three cycles)	+	–
Chelex-100	++	–
Proteinase K		
With organic extraction	+	–
Without organic extraction	++	–

[a]The methods are as described in Section 3.1. Samples contained 1×10^5 *E. coli* cells inoculated in 100 µL of sample volume (saline or SF). A 10-µL volume of each extract was used for a 100-µL PCR test. Amplification products were subjected to agarose gel electrophoresis and detected using EtBr staining of the gels (*see* Fig. 1A). Results from three separate tests were scored as follows: ++, consistent, reproducible PCR products detected by EtBr staining of agarose gels; +, PCR products detectable, but with reduced consistency and/or lower yields; –, no PCR products detected.

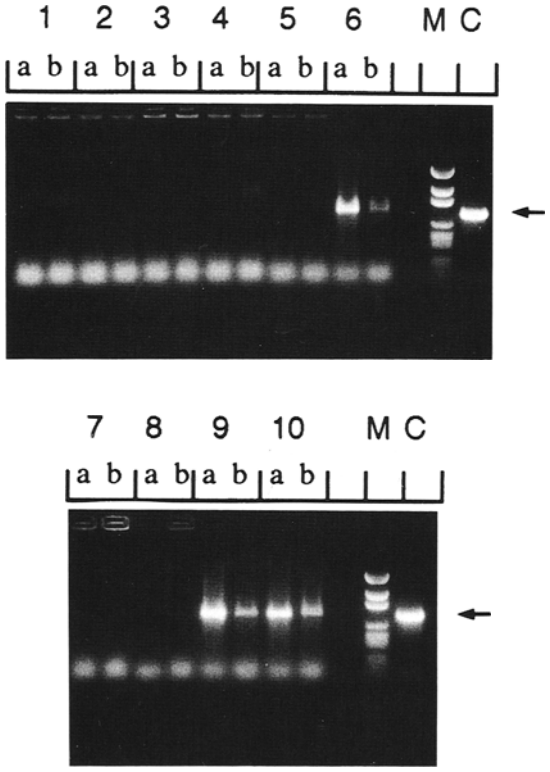

Fig. 2. EtBr-gel electrophoresis detection of PCR products generated from SF samples using the modified lysis/extraction protocols described in Table 2. A 100-µL volume of SF containing either 1×10^5 (lanes a) or 1×10^4 (lanes b) *E. coli* cells was used for each extraction method. PCR was performed as described in Fig. 1. Amplification products for all 10 methods are shown in sets of lanes 1–10 (refer to Table 2 and text for details of each method tested). Lane M, DNA molecular size markers (pGEM DNA Markers, Promega); lane C, positive control PCR using 8 ng of purified *E. coli* genomic DNA as template; 881-bp PCR product is indicated by arrows.

We next tested the effectiveness of KTET buffer and MBR addition with either the freeze/boil step or the 90°C heat treatment. Also, we compared the effectiveness of adding the MBR before or after the heat treatment. As shown in Table 3, we found that the 90°C, 10-min heat treatment, without a freeze/boil step, was adequate for bacterial lysis when done in KTET. The addition of MBR after the heat treatment gave consistently superior PCR results compared to prior addition (*see* Note 4).

The ability of this relatively simple method to yield PCR-compatible extract was tested in a bacterial dilution series. A 100-µL sample of SF containing either 1×10^5, 1×10^4, 1×10^3, 1×10^2, or 1×10^1 cells/sample was mixed with 200 µL KTET and heated at 90°C for 10 min. After cooling, MBR was added to 10% (w/v) and samples were vortexed 30–60 s and centrifuged for 10 min at 4°C, at 14,000*g*. Supernatant containing extracted DNA was removed and used for PCR analysis. For samples containing 1×10^5 to 1×10^3 cells/100 µL SF, we analyzed PCR products on EtBr-stained agarose gels (Fig. 3). Southern analysis was performed on samples containing 1×10^3 cells/100 µL of SF or less to increase detection sensitivity (Fig. 4; *see also* Note 5). We found that a bacterial concentration of 1×10^3 cells/100 µL was the limit for accurate visualization on gels.

We extended our bacterial detection methodology to analyze clinical SF specimens obtained from knee joints of patients who had undergone total knee arthroplasty (TKA, *see* ref. *1*) and subsequently returned to their orthopedic surgeons with a variety of symptoms including swelling, pain, and warmth about the affected

Table 2
Efficiency of Various Combined Dilution/Extraction/Lysis Procedures for PCR Detection of Bacteria in SF Samples[a]

Procedure	Components/steps							
	Water, 200 µL	KTET, 200 µL	Freeze/thaw	Freeze/boil	Chelex-100	MBR	90°C, 10 min	PCR[b]
1a	yes	–	yes	–	–	–	yes	–
b	yes	–	yes	–	–	–	yes	–
2a	yes	–	–	yes	–	–	yes	–
b	yes	–	–	yes	–	–	yes	–
3a	yes	–	yes	–	yes	–	–	–
b	yes	–	yes	–	yes	–	–	–
4a	yes	–	–	yes	yes	–	yes	–
b	yes	–	–	yes	yes	–	yes	–
5a	yes	–	yes	–	–	yes	–	–
b	yes	–	yes	–	–	yes	–	–
6a	yes	–	–	yes	–	yes	yes	++
b	yes	–	–	yes	–	yes	yes	++
7a	–	yes	yes	–	–	–	–	–
b	–	yes	yes	–	–	–	–	–
8a	–	yes	yes	–	–	yes	–	–
b	–	yes	yes	–	–	yes	–	–
9a	–	yes	–	yes	–	–	yes	++
b	–	yes	–	yes	–	–	yes	++
10a	–	yes	–	yes	–	yes	yes	++
b	–	yes	–	yes	–	yes	yes	++

[a]Two different synovial fluid dilution strategies were tested in combination with various lysis/extraction methods, ion exchange resins, and heat treatments as outlined in Section 3.1. Each of the 10 protocol combinations were tested on 100-µL synovial fluid samples containing: 1×10^5 *E. coli*/sample (a) or 1×10^4 *E. coli*/sample (b).

[b]A 10-µL volume of extract was used for PCR and analyzed by EtBr-stained agarose gels as described for Table 1 (*see* Fig. 2): ++, consistent, reproducible PCR products detected by EtBr staining of agarose gels; +, PCR products detectable, but with reduced consistency and/or lower yields; –, no PCR products detected.

joint. For this study, 15 specimens collected from patients with symptomatic TKA knees, asymptomatic TKA joints (usually from the "good" knee from a bilateral TKA patient), and from previously unoperated, unaffected knees were processed in a random, "blinded" manner in three separate experiments. For each test, a 100-µL volume of synovial specimen was extracted and subjected to PCR analysis as described above, and the results from one test are shown in Fig. 5. As described in Section 3.3., Southern blot hybridization was performed on electrophoretically fractionated PCR products using both radioactive and nonradioactive probing strategies (Fig. 5A,C). PCR products were also analyzed by slot-blot techniques using the same hybridization conditions as for Southerns (Fig. 4B,D). As with all clinical specimens, each patient sample was subjected to standard microbiological culturing assays routinely used for detection of infectious organisms. Our PCR analysis was performed without knowledge of culturing results, and clinical decisions made by the orthopedic surgeon concerning treatment regimens were made without knowledge of the PCR data (*see* Note 7).

3.2. PCR Amplification

1. For PCR amplification, a 10–20-µL aliquot of extract was used for a 100-µL reaction volume. Reaction conditions were as follows: 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 200 m*M* each of dATP, dCTP, dGTP, dTTP, 0.5 µ*M* of each primer, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer).

Table 3
Efficiency of MBR-Based Procedures for PCR Detection of Bacteria in SF Samples[a]

Procedures[a]	Samples[b]		
	1×10^5 cells/100 µL	1×10^4 cells/100 µL	1×10^3 cells/100 µL
Freeze/boil, three cycles, then add MBR	++	+	–
90°C, 10 min MBR added before heating	+	–	–
90°C, 10 min MBR added after heating	++	++	++

[a]Three MBR-based lysis/extraction procedures based on and modified from the results shown in Table 2, and described in Section 3.1., were each tested with three *E. coli* concentrations as indicated.

[b]Each 100-µL synovial fluid sample inoculated with *E. coli* as indicated was diluted with two volumes of KTET buffer prior to extraction. PCR was performed as described in Tables 1 and 2, and amplification products were analyzed by EtBr staining of agarose gels: ++, consistent, reproducible PCR products detected by EtBr staining of agarose gels; +, PCR products detectable, but with reduced consistency and/or lower yields; –, no PCR products detected.

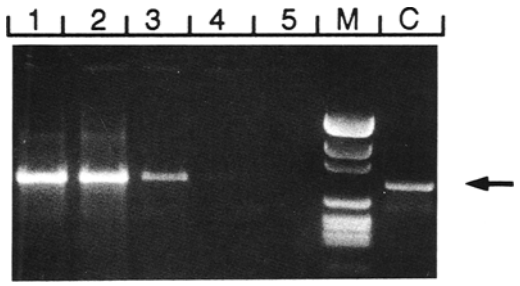

Fig. 3. Sensitivity of EtBr-gel electrophoresis detection of PCR products generated from SF samples processed by the MBR-based lysis/extraction protocol. SF samples containing a dilution series of *E. coli* concentrations were used for each extraction. Samples were processed as described in Section 3.1. Unless otherwise indicated, 10-µL aliquots of supernatant were used for PCR, and agarose gels were run as for Figs. 1 and 2. Lane 1, 1×10^5 cells/100 µL SF sample; lane 2, same extract as used in lane 1, except that 20 µL were used for PCR; lane 3, 1×10^4 cells/sample; lane 4, 1×10^3 cells/sample; lane 5, negative control, SF sample containing no bacteria processed in parallel; lane M, DNA molecular size markers (pGEM); lane C, positive control reaction using 8 ng of purified *E. coli* DNA as template in PCR; 881-bp PCR product is indicated by arrow. In comparing lanes 1 and 2, we have found that increasing the volume of extract added to the PCR does not increase PCR yields. In fact, PCR efficiency decreases with increasing extract input in some cases, possibly owing to inhibitory components present in the SF. Using the PCR conditions described here, the sensitivity limit for product detection is 1×10^3 bacterial cells/sample when using EtBr-stained gels.

2. The primers used for all PCR herein were derived from the *E. coli* sequence for the 16S rRNA gene *(8)*, a highly conserved sequence among many bacterial species, which targets all orthopedically relevant species we have tested *(9)*. These primers yielded an 881-bp product from *E. coli* genomic DNA and an identical product from *S. aureus*. As positive control, PCR was done using 1–10 ng of purified *E. coli* DNA as template. For negative controls, reactions were performed using all PCR reagents on extracts from saline solution, sterile water, or aseptic SF samples containing no added bacteria.

3.3. Gel Electrophoresis, Slot-Blotting, and Hybridization Analysis

1. Amplification products were size fractionated on 1.4% high-melting-temperature agarose (Fisher) in 1X TAE buffer (40 m*M* Tris-acetate, pH 8.0, 2 m*M* EDTA). EtBr was included in the gel during electrophoresis to stain DNA for detection with UV transillumination. Electrophoresis was performed at 5 V/cm under constant voltage. After electrophoresis, gels were photographed prior to Southern blot transfer. Calibration of the electrophoretogram was based on pGEM DNA size markers (Promega).
2. For Southern analysis, DNA products were blot-transferred to nylon membranes using alkaline transfer conditions. Gels were soaked in 0.4*N* NaOH for 30 min prior to transfer.

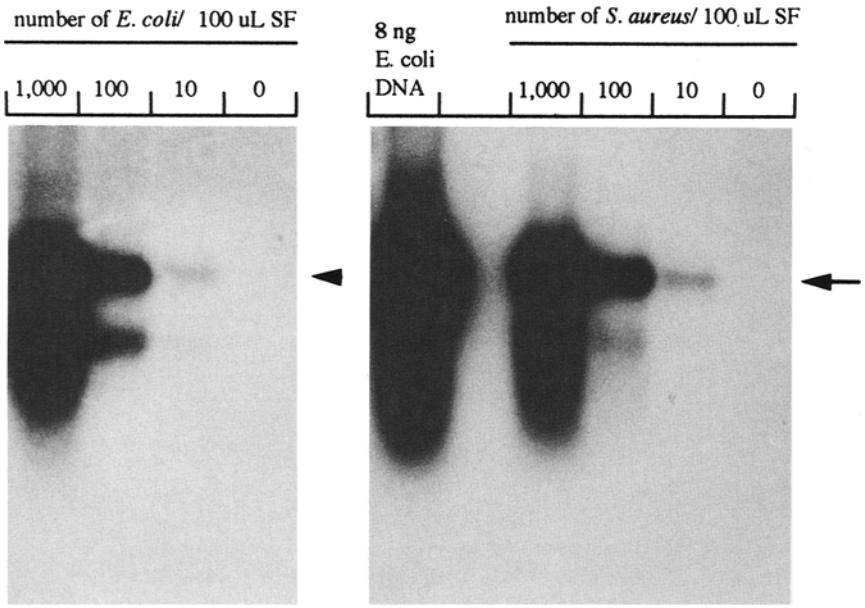

Fig. 4. Sensitivity of Southern blot hybridization detection of PCR products generated from SF samples processed by the MBR-based lysis/extraction protocol. SF samples containing known concentrations of *E. coli* as indicated were used for each extraction as described in Fig. 3 and Section 3.1. After gel electrophoresis, the DNA was blot-transferred to nylon filter membranes and hybridized with a cloned, ^{32}P-labeled *E. coli* 16S rRNA gene PCR product as probe and hybridization product detected by autoradiography (exposure time, 4 h). Arrows indicate position of 881-bp PCR product. For bacterial concentrations in the 1×10^3 to 1×10^2cells/sample range, autoradiographic exposures of 5–60 min are adequate for detection. For concentrations lower than 100 cells, exposures of several hours to overnight are adequate.

Transfers usually proceeded for 4–16 h (overnight) to ensure complete and reproducible transfer of low DNA concentrations. We also used the Turboblot transfer apparatus (S&S) under alkaline conditions and achieved transfer efficiency in 2 h similar to 16 h transfers under standard conditions. Filters were neutralized in 200 m*M* Tris-HCl, pH 7.0, 2X SSC (1X = 0.15*M* sodium chloride, 0.015*M* sodium citrate, pH 7.0) prior to air drying and UV-crosslinking the nucleic acids to the membrane, or baking at 120°C for 15 min, or at 80°C for 90 min.

3. For hybridization analysis, the cloned 16S rRNA gene PCR product generated from purified *E. coli* DNA was used as probe. Probes were labeled with either ^{32}P (NEN, Dupont) or digoxygenin (BMB) in random-primed labeling reactions using the Klenow fragment of *E. coli* DNA polymerase I. Routinely, we performed hybridizations under high stringency conditions at 68°C in 0.5*M* sodium phosphate buffer, pH 7.5, 5% sodium dodecyl sulfate, 1% (w/v) bovine serum albumin. Filters were washed at 68°C in 40 m*M* sodium phosphate buffer, pH 7.5, and 1% (w/v) sodium dodecyl sulfate for ^{32}P probe, or as described in BMB manual for digoxygenin-labeled probes. The digoxygenin-labeling protocol employs an antidigoxygenin alkaline phosphatase-conjugated antibody. After antibody binding and washing, hybridization products were detected based on chemiluminescence using Rad Free substrate sheets (S&S). For both chemiluminescence (Fig. 5A) and ^{32}P (Fig. 5C) detection, autoradiography was done using Kodak X-OMAT X-ray film. For ^{32}P detection, film exposure was done at –70°C in the presence of intensifying screens (Kodak, X-Omatic).
4. Alternatively, the gel electrophoresis step could be omitted and PCR products blotted directly onto nylon membranes using a standard slot- or

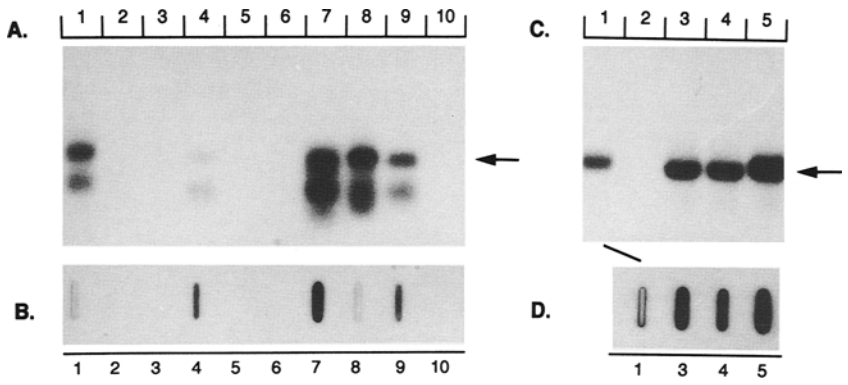

Fig. 5. Southern and slot-blot hybridization analysis of extracts from clinical SF samples. A 100-µL knee joint SF sample, obtained aseptically, was processed for PCR for each patient as described in Section 3.1. using the lysis/extraction protocol described in Fig. 4. A 20-µL volume of PCR mixture was used for Southern analysis **(A,C)**, and 50 µL of the same mixture were used for the slot-blot analysis **(B,D)**. Each lane and accompanying slot corresponds to SF sample from an individual patient as described in Section 3.1. The Southern and corresponding slot-blot filters shown in A and B were analyzed using digoxygenin-incorporated probe and chemiluminescence detection (4-h X-ray film exposure). Filters shown in C and D were analyzed using a ^{32}P-labeled probe (1-h exposure). Arrows indicate position for 881-bp 16S rRNA gene PCR product generated in positive control reaction. Hybridization signal from positive clinical specimens indicates a wide range of PCR product yields, most likely reflecting the different levels of infection among patients. The generation of different sized PCR products as detected in Southern blot (A) can be eliminated with a "hot-start" PCR step as was performed for the Southern blot shown in (C).

dot-blot apparatus. After PCR, 1*M* NaOH and 500 m*M* EDTA, pH 8.0, were added to a portion of or to the entire 100-µL sample to give final concentrations of 0.4*M* and 2 m*M*, respectively. Samples were mixed and incubated at room temperature for 20–30 min (or at 100°C for 10 min) prior to application to the filter as described in detail elsewhere *(10)*. Slot- or dot-blot filters were probed using the same hybridization strategies as described above. Slot-blotting would be considered when direct visualization of the PCR products was not required. Figure 5 shows slot-blot hybridization analysis compared to Southern analysis using chemiluminescence detection (Fig. 5A,B) as well as using a radioactive probe (Fig. 5C,D). For the slot-blot experiment, 50 µL of PCR reaction mixture were applied to the filter for each sample, whereas 15 µL were used for each gel electrophoresis lane.

4. Notes

1. Although the existing protocols we tested gave positive PCR results with saline inoculations, no SF specimens yielded amplification products reproducibly. The viscous, complex macromolecular and ionic mixture found in SF clearly contained components that are inhibitory to PCR when introduced into the reaction mix. We believe that this is the reason that none of the existing protocols gave consistent results. To circumvent this problem, we initially included a brief centrifugation of the specimen to pellet the bacteria, prior to performing the lysis/extraction. This approach was unsuccessful because of the cosedimentation of high-mol-wt synovial components, which interfered with resuspension and extraction efforts. Because of the inability to harvest bacteria from SF by centrifugation, we turned our attention to modifying existing lysis/extraction protocols to allow addition of processed specimen directly to PCR (*see* Note 2). Additionally, boiling of SF under certain conditions leads to the coagulation of synovial proteins making this approach intractable. Also, lysis protocols requiring proteinase-K activity may be less effective owing to inhibition of the enzyme by

SF components, or the overwhelming abundance of synovial proteins.

2. The inability to harvest bacteria from SF required processing of the sample to allow its direct addition to the amplification reaction. Our goals were to reduce the viscosity of the fluid, achieve complete bacterial lysis and DNA extraction, and eliminate PCR inhibitors. As mentioned above, lysis by heat treatment often led to coagulation, especially if a clinical sample contained blood, but diluting the sample in KTET buffer effectively reduced viscosity, greatly improved lysis/extraction efficiency, and prevented coagulation during heating. The inclusion of Chelex-100 or MBR gave dramatically improved PCR results, most plausibly by binding and sequestering charged ionic and/or macromolecular synovial components. Brief centrifugation of the extract allowed easy separation of resin-bound compounds from bacterial DNA that remained in the supernatant. Typically, one-half the starting volume (150 µL) can be recovered as supernatant, 10 µL of which is used for PCR, and the rest can be stored indefinitely at –20°C for further analysis.
3. As shown in Table 2, the freeze/boil method was more successful than freeze/thawing, as a result of more efficient bacterial lysis and/or destruction of heat-labile PCR inhibitors. The addition of a 10-min incubation at 90°C also enhanced PCR amplification, especially when the lysis/extraction was done in KTET buffer. Two of the three most successful tests also included addition of MBR, whereas Chelex-100 addition was less successful. In comparing many identically processed samples, we found that Chelex-100 gave inconsistent PCR results. It is plausible that the resin may physically break down during vortexing, heating, and/or centrifugation and small quantities of resin contaminating the supernatant could affect PCR. Chelex-100 is a divalent cation chelator and its inclusion in PCR could inhibit *Taq* polymerase activity by reducing magnesium ion concentration. The use of MBR, however, gave consistent and reproducible PCR under the same assay conditions. Bio-Rad AG 501-X8 MBR, which chelates both cations and anions, was overall more effective in neutralizing, binding, and/or eliminating the synovial-based PCR inhibitors.
4. In comparing freeze/boil and 90°C heat-treatment methods, we were surprised to find that, in the presence of KTET, the 10-min, 90°C heat treatment alone was sufficient to give reproducible PCR results. The KTET buffer containing EDTA and nonionic detergent, Tween-20, is very efficient in bacterial lysis, especially under hypotonic conditions. Based on this result, we felt that a single heat-treatment step was superior to multiple transfers between ethanol/dry ice and boiling water. On several occasions, placement of 100°C tubes into ethanol/dry ice led to the leakage of ethanol through the cap as tubes quick-cooled, and trace ethanol subsequently inhibited PCR. In testing the effectiveness of adding MBR to the sample after, instead of prior to, heat treatment, we found markedly improved PCR results. According to the manufacturer, AG 501-X8 resin is not recommended for use at temperatures above 50°C; thus, some physical or chemical breakdown may occur with its inclusion in the heat treatments. Such breakdown would impair its binding activity or capacity, and trace amounts of MBR in the PCR mix could be inhibitory.
5. Southern hybridization using cloned 16S rRNA gene PCR product as probe has been included in experiments involving low bacterial concentrations and for all clinical samples. Hybridization analysis allows detection of PCR products not visualized by fluorescent dye staining. Probe specificity allows discrimination between bacterial PCR products and any background products resulting from nonspecific annealing of the oligonucleotide primers to co-extracted human DNA. The selective use of DNA hybridization thus provides high specificity as well as enhanced sensitivity. In some Southern analyses, we detected two bands from bacterial DNA after PCR, the larger fragment of correct length and a smaller, minor fragment. The lower band may be generated from 16S rRNA gene templates that may have internal secondary structure, leading to a skipping of the polymerase over this region and producing a shorter product that is amplified in subsequent cycles. We can eliminate this secondary product by em-

ploying a "hot start" step prior to PCR, which may prevent aberrant template conformations or primer annealings (compare the two Southern blots shown in Fig. 5). Eventually, unequivocal identification of bacterial genotypes can be achieved by means of restriction endonuclease digestion of PCR products to detect sequence polymorphisms as analyzed by standard Southern blotting.

6. Slot-blot procedures allow direct hybridization analysis of the sample without additional manipulation of the DNA, and circumvent its potential loss during electrophoresis and/or the nonuniformity of transfer from the gel. Also, larger sample aliquots, such as the entire 100-µL PCR sample, can be applied to the filter by slot blotting, as opposed to only 15–20 µL used for gel electrophoresis. We have found that direct application of undiluted PCR mix to the filter results in uneven flow through the manifold, often yielding a nonuniform hybridization image (*see* Fig. 5B, slots 1 and 8, and 5D, slot 1). A two- to fourfold dilution of the sample prior to alkaline treatment circumvents this problem. Additionally, hybridization signals from Southern gel samples occasionally differ from corresponding signals from slot-blotted samples. It is likely that, in agarose gels, the lower DNA concentrations result in reduced blot transfer efficiency and corresponding lower signals (Fig. 5A, lane 4), whereas slot-blotting is more effective as long as the sample is properly diluted.
7. The clinical SF specimens processed in this study varied with respect to contamination with blood or solid tissue. Several samples contained a significant volume of blood, which did not interfere with our extraction or PCR efforts, although we have not yet determined if PCR yields were below expected yields for the given levels of infection. We routinely avoid transferring any solid tissue into our extraction mixture. The processing of solid tissue samples is being tested in a separate study. Samples that contained at least 50% blood may coagulate during heating, but vigorous vortexing during the MBR step will break down the colloid and allow normal processing of sample. For PCR, it may be necessary to use a smaller volume of extract in the reaction mixture because of the potential inhibitory nature of heme group products. Also, PCR mixtures from bloody samples may contain excess proteins or peptide fragments that occasionally interfere with the electrophoretic mobility of DNA amplification products in the agarose gel. A short centrifugation of the PCR mixture to pellet contaminants prior to removing the gel electrophoresis aliquot can improve the quality of the gel mobility. In this study we detected the presence of bacterial infection in 9 of 15 patient samples. The clinical correlation of our PCR data with microbiological culturing, Gram stain testing, and other standard clinical assays is discussed elsewhere (Mariani et al., in preparation).

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