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Identification of the Causative Bacteria in Musculoskeletal Infections Using 16s rDNA - Denaturing Gradient Gel Electrophoresis Analysis

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A

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Supervisory Professor: Heidi B. Kaplan, PhD

ABSTRACT

Musculoskeletal infections are infections of the bone and surrounding tissues. They are currently diagnosed based on culture analysis, which is the gold standard for pathogen identification. However, these clinical laboratory methods are frequently inadequate for the identification of the causative agents, because a large percentage (25-50%) of confirmed musculoskeletal infections are false negatives in which no pathogen is identified in culture. My data supports these results. The goal of this project was to use PCR amplification of a portion of the 16S rRNA gene to test an alternative approach for the identification of these pathogens and to assess the diversity of the bacteria involved. The advantages of this alternative method are that it should increase sample sensitivity and the speed of detection. In addition, bacteria that are non-culturable or in low abundance can be detected using this molecular technique. However, a complication of this approach is that the majority of musculoskeletal infections are polymicrobial, which prohibits direct identification from the infected tissue by DNA sequencing of the initial 16S rDNA amplification

products. One way to solve this problem is to use denaturing gradient gel electrophoresis (DGGE) to separate the PCR products before DNA sequencing.

Denaturing gradient gel electrophoresis (DGGE) separates DNA molecules based on their melting point, which is determined by their DNA sequence. This analytical technique allows a mixture of PCR products of the same length that electrophoreses through agarose gels as one band, to be separated into different bands and then used for DNA sequence analysis. In this way, the DGGE allows for the identification of individual bacterial species in polymicrobial-infected tissue, which is critical for improving clinical outcomes. By combining the 16S rDNA amplification and the DGGE techniques together, an alternative approach for identification has been used.

The 16S rRNA gene PCR-DGGE method includes several critical steps: DNA extraction from tissue biopsies, amplification of the bacterial DNA, PCR product separation by DGGE, amplification of the gel-extracted DNA, and DNA sequencing and analysis. Each step of the method was optimized to increase its sensitivity and for rapid detection of the bacteria present in human tissue samples. The limit of detection for the DNA extraction from tissue was at least 20 *Staphylococcus aureus* cells and the limit of detection for PCR was at least 0.05 pg of template DNA. The conditions for DGGE electrophoreses were optimized by using a double gradient of acrylamide (6 – 10%) and denaturant (30-70%), which increased the separation between distinct PCR products. The use of GelRed (Biotium) improved the DNA visualization in the DGGE gel. To recover the DNA from the DGGE gels the gel slices were excised, shredded in a bead beater, and the DNA was allowed to diffuse

into sterile water overnight. The use of primers containing specific linkers allowed the entire amplified PCR product to be sequenced and then analyzed.

The optimized 16S rRNA gene PCR-DGGE method was used to analyze 50 tissue biopsy samples chosen randomly from our collection. The results were compared to those of the Memorial Hermann Hospital Clinical Microbiology Laboratory for the same samples. The molecular method was congruent for 10 of the 17 (59%) culture negative tissue samples. In 7 of the 17 (41%) culture negative the molecular method identified a bacterium. The molecular method was congruent with the culture identification for 7 of the 33 (21%) positive cultured tissue samples. However, in 8 of the 33 (24%) the molecular method identified more organisms. In 13 of the 15 (87%) polymicrobial cultured tissue samples the molecular method identified at least one organism that was also identified by culture techniques. Overall, the DGGE analysis of 16S rDNA is an effective method to identify bacteria not identified by culture analysis.

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INTRODUCTION

Musculoskeletal Infections

Musculoskeletal infections can be defined as infections of the bone and surrounding tissues (29). These infections include various medical conditions: osteomyelitis (bone infection), septic arthritis (infection of the synovial membrane of joints), and pyomyositis (skeletal muscle infections) (11, 29). Generally, a physician's initial diagnosis of a musculoskeletal infection is based on the presence of typical clinical symptoms, which may include high fever, malaise, localized pain, inflammation, pus and loss of function of the involved extremity. In cases of arthritis, swelling of the affected joint may develop (11, 21). A rapid diagnosis of musculoskeletal infections is important to prevent complications and aid in the recovery. The physician's diagnosis should be confirmed by culture analysis, which is the gold standard for pathogen identification (46, 47). A bone or tissue biopsy of the infected area or aspiration of the joint fluid are the samples most likely to give a positive culture (17). However, the current clinical laboratory methods are frequently inadequate for the identification of the causative agents. Recent case reviews and my own data, identify a large percentage (25-50%) of confirmed musculoskeletal infections for which no pathogen is identified in culture (17, 47). It has been stated that for osteomyelitis cases, "the most important step is to isolate the offending organisms so that the appropriate therapy can be chosen" (27). A possible solution to decreasing the number of false negative cultures is to use a method that does not require bacterial growth. This is important because other published studies have

demonstrated that bacterial diversity is severely underestimated when based on culture techniques. It has been estimated that less than 1% of the organisms in nature are cultivable (32). The application of molecular techniques in medical diagnostics of infection diseases should provide increased sensitivity in pathogen identification (6, 14, 43, 48).

Molecular techniques for detection and identification of bacteria

Several molecular techniques have been used for identification of bacterial pathogens. The polymerase chain reaction (PCR) amplification, followed by sequence analysis of the product is one molecular technique that can definitively identify a known organism. PCR amplification allows for selective nucleic acid sequences to be copied and amplified through repeating cycles of denaturing, primer annealing, and elongation of DNA (40). In a study by El-Eragi, et al. (14) this particular molecular method was used to identify *Mycobacterium tuberculosis* in patients suspected of having pulmonary tuberculosis. This study consisted of 135 DNA isolates that were cultured from patients. The isolated DNA was PCR amplified by primers designed for the specific amplification of the *rpoB* gene. PCR amplification of the DNA samples confirmed the identification of *M. tuberculosis* and provided evidence that their PCR amplification and sequence analysis was 100% accurate in identification of the pathogen (14). However, this method of identification required that the bacteria were cultured before the samples were used as template in the PCR.

Antibody recognition is another molecular technique that has been shown to aid in pathogen identification. In one study by Wang, et al. (43) anti-*Salmonella*-antibody-conjugated to oval-shaped gold nanoparticles easily and specifically identified *Salmonella typhimurium* in cultured samples where *Escherichia coli* was also present. This identification method for *S. typhimurium* was based on the observation of the colorimetric change when aggregation of the nanoparticles occurred due to binding to the bacteria present (43). This study also required that the bacteria were first cultured and then used for the molecular detection technique.

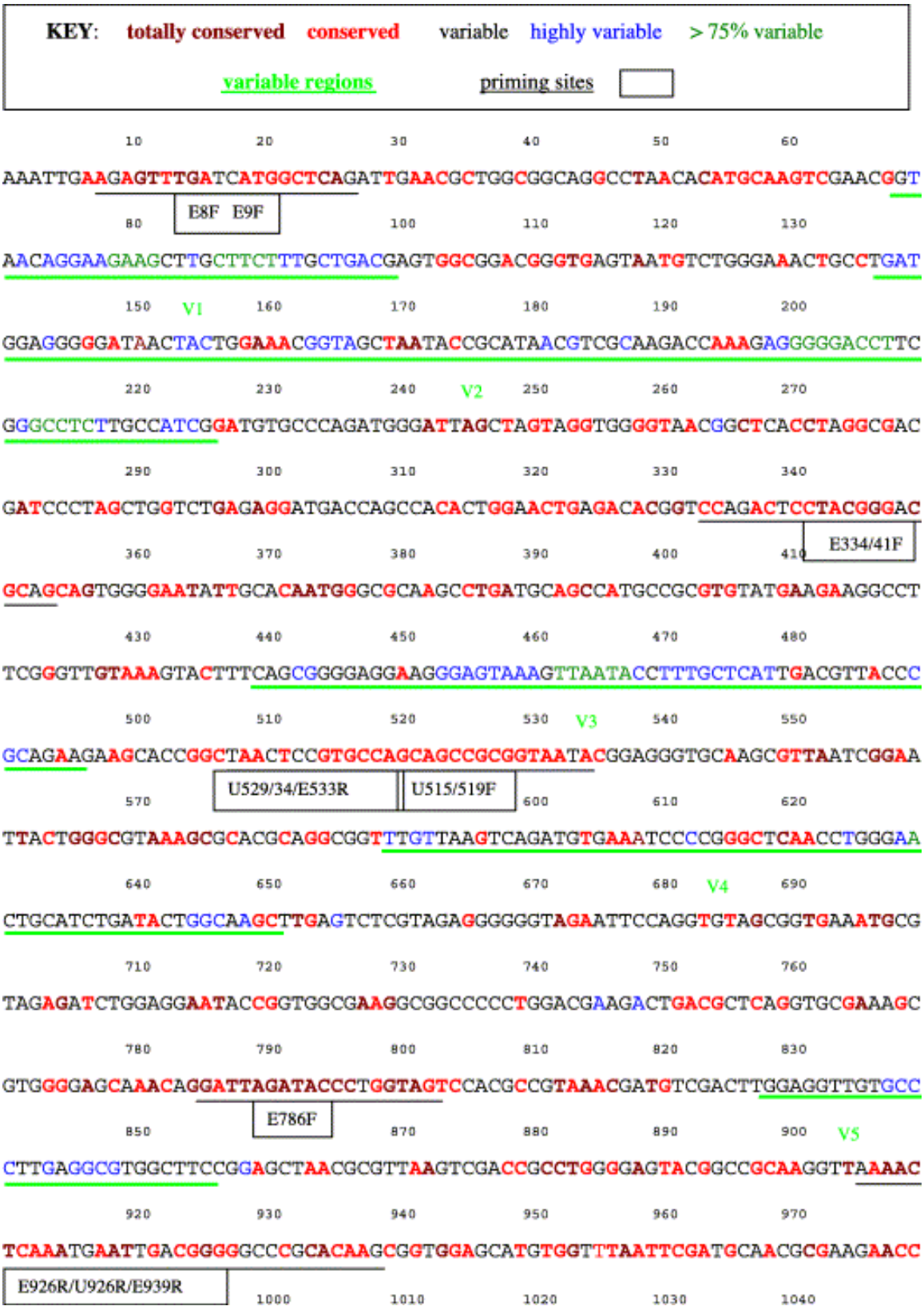
A third molecular technique is the restriction digest of DNA to determine the identity of an organism. Zhan, et al. (48) used *Hpy*CH4 III endonuclease to differentiate *Legionella pneumophila* from non- *L. pneumophila* stains. In this study, 42 ATCC strains of *L. pneumophila* and of non-*L. pneumophila* were cultured and DNA isolation was performed. The enzyme restricted the DNA from *L. pneumophila* differently than non-*L. pneumophila* strains giving different size fragments when the samples were electrophoresed through an agarose gel (48). Identification was based on the pattern of the restriction fragments.

All three of these molecular techniques required the bacteria to be cultured and were designed to detect a specific organism. One molecular technique, a microarray, also detects specific organisms, but can at one time detect several hundred specific organisms. This characteristic of a microarray is an advantage when the organism of interest is thought to be among a group of organisms. In one study, Harrington, et al. (20) designed a microarray with 16S rDNA probes that would identify 162 gastrointestinal bacteria. Fecal samples were obtained from

healthy individuals or patients suffering from ulcerative colitis and the genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). In this study, the microarray results were sensitive and provided new evidence of the complex population of gastrointestinal tract bacteria. However, it must be stressed that all of these molecular techniques require some knowledge about the suspected bacteria to be identified.

Some molecular techniques allow for unbiased screening of bacteria. These methods usually include using PCR amplification of the 16S rRNA gene, followed by sequence analysis for identification. The 16S rRNA gene has been used to examine bacterial phylogeny and taxonomy, since it was first studied by Carl Woese in 1970s and the collection of 16S rRNA gene sequences has grown each year (36). The 16S rRNA gene is unique in that it is present in essentially all bacteria. The function of the gene has not changed over time and it is of sufficient size (1,500 bp) for DNA-based bioinformatics (22). The 16S rRNA gene is composed of alternating regions of DNA that are conserved among all bacterial species and DNA that is variable or unique at the species level (Figure 1). This characteristic allows for primers designed to hybridize to the conserved regions of the gene, to amplify the desired variable sections of the DNA, so that the latter regions can be used to speciate among the thousands of known bacteria (3, 9, 45).

The 16S rRNA gene amplification and sequencing method has been used in various studies to detect and identify bacteria (40). Al Masalma, et al. (1) identified bacteria in brain abscesses using PCR amplification followed by cloning of the PCR amplified 16S rDNA and then DNA sequence analysis of the inserted PCR


KEY: totally conserved conserved variable highly variable > 75% variable
variable regions
priming sites
E8F E9F
V1
V2
E334/41F
V3
U529/34/E533R
U515/519F
V4
E786F
V5
E926R/U926R/E939R

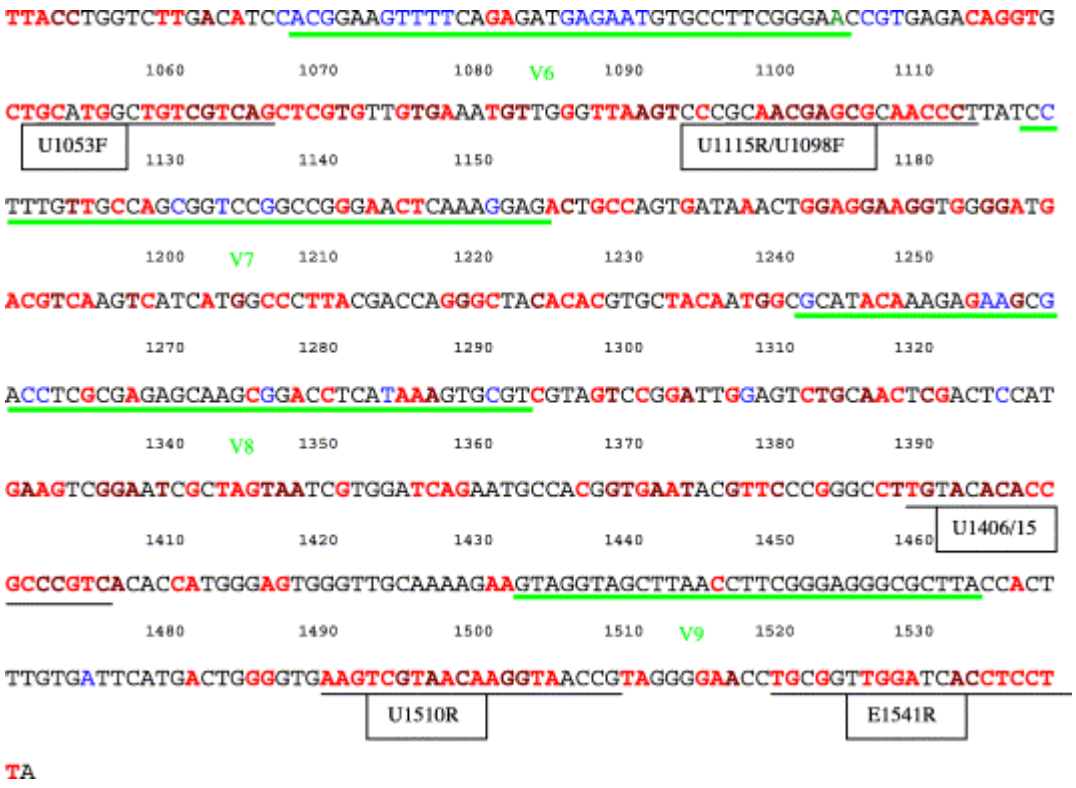


Figure 1: *E. coli* 16S rRNA gene sequence annotated with bacteria and "universal" priming sites and variable regions V1–V9. The sequence is color coded to indicate bacterial sequence variability. Dark red = totally conserved. Red = conserved. Black = variable. Blue = highly variable. Green = > 75% variable. Green = variable regions. Black = priming sites. From reference # 3.

fragment. Cloning was used in this study to separate mixed species PCR products in order to obtain a readable DNA sequence for analysis. The separation of PCR products is required if more than one species of bacteria is present in a sample. Cloning allows for a single PCR product to be incorporated into a vector, and then transformed into one *E. coli* cell. Colonies arising from the individual *E. coli* cells carrying the vector and insert are isolated. In this study in which 125 colonies per specimen were analyzed, it was determined that the molecular technique identified more bacterial species than culture analysis. Some of these species included the anaerobes, *Fusobacterium nucleatum*, *Porphyromonas endodontalis*, and *Prevotella oris*. *M. faucium* was another bacterial species identified that had never been previously associated with brain abscesses. Bittar, et al. (6) used a similar molecular technique to identify pathogens in cystic fibrosis patients. However, in this study only 40 colonies per specimen were analyzed. Since cloning randomly incorporates the PCR products into the vector, the number of colonies needed for analysis is large to provide a high probability that all the unique PCR product sequences amplified are identified. This study also identified bacterial species that had not previously been observed in cystic fibrosis patients.

A more recent molecular technique, termed “pyrosequencing” has been used to bypass the cloning step in the analysis of the 16S rRNA gene. In this approach the DNA sequencing procedure examines many single strands of DNA. This is accomplished using a method termed “sequencing by synthesis” (Figure 2). “Sequencing by synthesis” occurs using a DNA polymerase that generates inorganic pyrophosphate each time it adds a nucleotide. The pyrophosphate forms

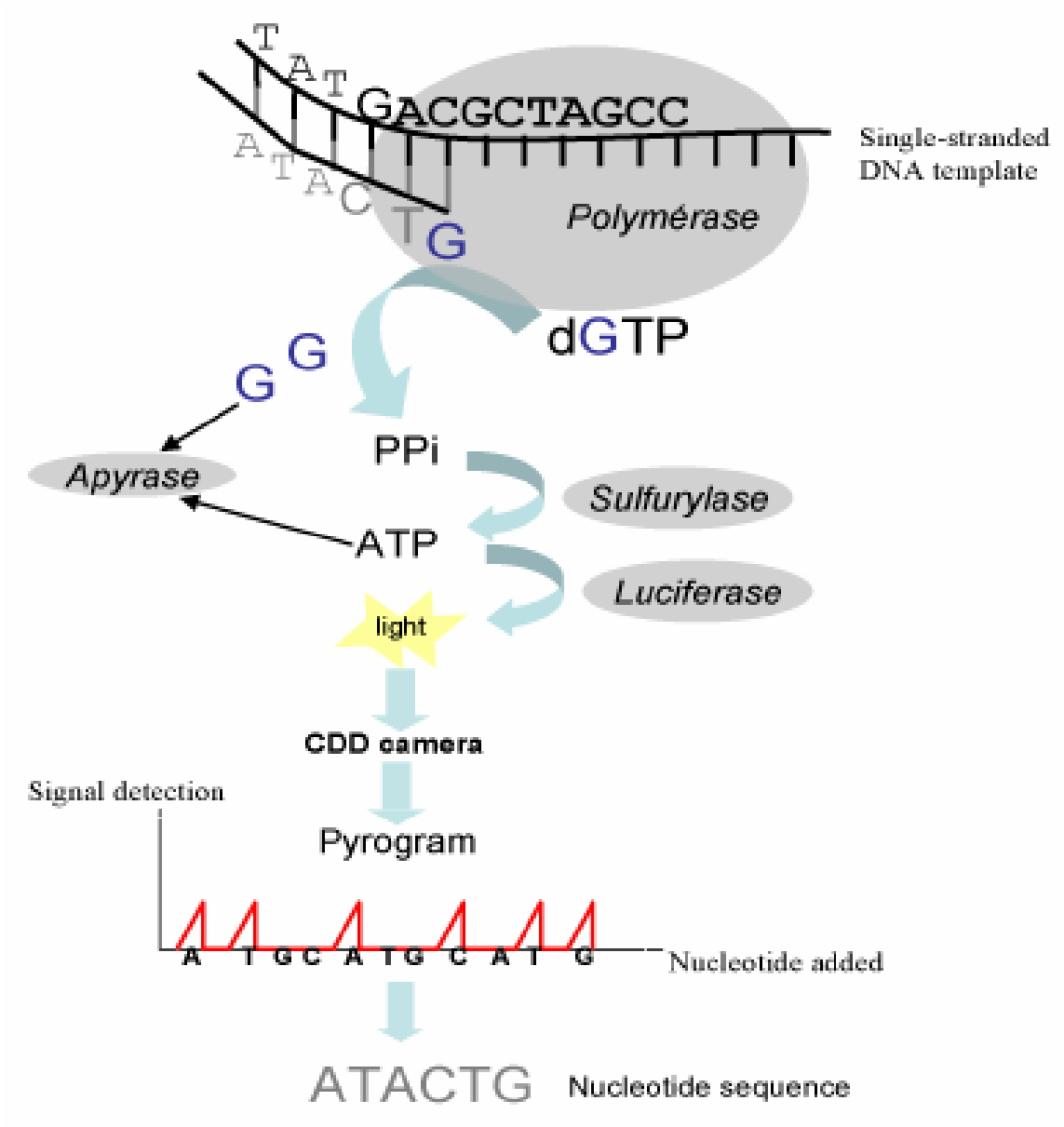


Figure 2: Theory of pyrosequencing. Each peak in the pyrograms represents a pulse of light detected in the instrument. From reference # 15.

ATP and there is an ATP-dependent conversion of luciferin to oxyluciferin. When oxyluciferin is formed it causes the emission of light pulses. The amplitude of each light pulse is directly related to the presence of nucleosides and each type of nucleotide is released separately. This means that for a single time period only one type of nucleotide is released and if that nucleotide is incorporated into the DNA sequence a light pulse will be detected. If the light pulse is doubled then two nucleotides of the same type were incorporated in the DNA sequence. Pyrosequencing allows thousands of sequences to be generated from one PCR reaction. However, one significant limitation of pyrosequencing is that the DNA sequence retrieved is usually no longer than 400 bases (15). In one clinical study by Dowd, et al. (13) 193,890 sequences were generated and analyzed from 4 samples. Using the pyrosequencing technique the authors found greater diversity in chronic wounds then had previously been identified by culture techniques. Specially, the pyrosequencing method detected more strict anaerobes in the chronic wound samples than did the culture method. This new sequencing technique is rapid and sensitive, but at this time it is still too costly for routine use to identify pathogens. For general identification of bacterial pathogens, a molecular method that is unlimited in the number of detectable species and is not prohibitively costly would be useful.

16S rRNA gene PCR-DGGE molecular method

The first goal of this project was to test a rapid, sensitive, and cost-effective molecular technique that could identify all possible bacterial pathogens in musculoskeletal infections. The second goal was to apply this method in a pilot study of 50 human tissue samples. The molecular method of choice used PCR amplification of the 16S rRNA gene to identify all possible bacterial species. If all of the specimens were monomicrobial, then direct sequence analysis of the initial PCR product could be used for identification of the bacterial pathogens. However, a complication to this approach was that the majority of musculoskeletal infections are polymicrobial, which prohibited direct DNA sequencing of the initial 16S rDNA PCR product. One way to solve this problem was to use denaturing gradient gel electrophoresis (DGGE) to separate the PCR products into individual bands based on their melting point. The separation of the PCR products is based on the principle that each double-stranded DNA (dsDNA) fragment has a unique denaturation point, which is based on the order of the specific nucleotides of which it is composed. When DNA is in a denaturing environment, it will begin to denature in specific regions. The temperature at which the DNA begins to denature is called the melting point temperature. Chemical reagents, such as urea and formamide, can also be used to denature dsDNA. The differences in denaturation points of dsDNA can be detected by electrophoresis through an acrylamide gel containing a gradient of urea and formamide. To create greater separation between distinct dsDNA, the concentrations of urea and formamide can be modified. By increasing the gradient concentrations a larger range of denaturing conditions can be provided. A

denaturant gradient is formed by using gradient maker. A constant temperature of 60℃ also contributes to the denaturation of the dsDNA. Once the dsDNA starts to denature, its movement through the acrylamide gel will be arrested. The dsDNA with a lower denaturation point will migrate and stop earlier (or higher) in the acrylamide gel whereas, the dsDNA with a higher denaturation point will migrate longer and stop lower in the acrylamide gel. The differences in migration will result in DNA bands at different positions in the DGGE gel.

The 16S rRNA gene PCR-DGGE molecular method should allow the identification of all causative bacteria present in the tissue samples. This analytical technique allows a mixture of PCR products of the same length that electrophorese through agarose gels as one band, to be separated into different bands that can then be used as template for DNA sequence analysis. In this case, the DGGE allows for the detection of individual bacterial species in polymicrobial infected tissue, which is critical for identification and assessing diversity (10). Combining the 16S rDNA amplification and the DGGE techniques together should provide more complete information to the medical staff treating these infections than is currently provided by the clinical microbiology laboratory. The advantages of this alternative method are that it should increase sample sensitivity and the speed of detection. Furthermore, bacteria that are non-culturable or in low abundance can be detected using this molecular method. Infections, particularly those resulting from polymicrobial biofilms, are difficult to eradicate, making proper identification of the causative agents critical for treatment.

METHODS

Origin of bacterial control strains and experimental tissue samples

The standard control strain used for this study was a clinical isolate of *Staphylococcus aureus*, from an osteomyelitis infection, designated UAMS-1, which was purchased from the American Type Culture Collection as ATCC 94250. During all steps of the analyses, positive and negative controls were included. The tissue samples were obtained from the Memorial Hermann Hospital Clinical Microbiology Laboratory after culture analysis was performed. The collection of specimen occurred throughout the study; some were collected as early as 2007. All the tissues used in this study were obtained as part of our ongoing trial study, which has been approved by the IRB - the Committee for the Protection of Human Subjects at UTHSCH. All patient identification was removed and each tissue sample was assigned a study number. All tissue samples were stored at -20℃ until DNA isolation from tissue was performed. The amount of time spent in storage varies for each specimen.

DNA isolation from tissue biopsies

DNA isolation from tissue was performed by using a modified version of the Puregene DNA Purification kit (Qiagen) for solid tissue. The tissue (0.5 g) was homogenized in cell lysis solution (500 ul) using a sterile disposable tissue grinder (Kendall). These steps of the protocol were performed under a tissue culture hood and all instrumentation and a few solutions (cell lysis solution and sterile water)

were exposed to UV light for 30 min before the DNA isolation was performed. The homogenized tissue samples were incubated for 100 min at 65℃. Afterward, 6 ul Proteinase K solution (20 mg/ml) was added and the sample was incubated at 55℃ overnight. To ensure efficient lysis of the bacterial cells and complete digestion of cell walls, 10 ul lysotaphin (2 mg/ml) and 3 ul Lytic Enzyme Solution (Qiagen) were added and the samples were incubated at 37℃ for 3 hrs, followed by heating to 80℃ for 5 min. This step of the protocol was optimized for *S. aureus* and it efficiently lyses other bacteria including Gram-negative organisms, such as *E. coli*. Protein precipitation was performed by adding 200 ul of protein precipitation solution (10 M ammonium acetate), which was followed by the placing samples on ice for 20 min. The samples were then centrifuged at 15,000 x g for 6 min to collect the supernatant. The supernatant was transferred to a clean microcentrifuge tube. Again the sample was placed on ice for 20 min and centrifuged to collect the supernatant. The supernatant containing the DNA was then transferred to a clean microcentrifuge tube containing 700 µl 100% isopropanol. The microcentrifuge tube was then mixed and centrifuged at 15,000 x g for 6 min. The supernatant was discarded and 800 µl 70% ethanol was added to wash the DNA pellet. The samples were centrifuged at 15,000 x g for 2 min to position the DNA pellet at bottom of the tube. The ethanol was discarded and the pellet was allowed to air dry for 1 hr. The DNA pellet was hydrated overnight at room temperature with water that had been treated with ethidium monoazide bromide (EMA, 9 ug/ml) (see Results). The DNA isolation protocol duration was two days.

Initial PCR amplification

DNA amplification was performed as follows. A positive control of *S. aureus* DNA and blank were included in all DNA amplification steps. Three different concentrations of the tissue-extracted DNA were used as the PCR template (undiluted, 1:10 dilution, and 1:100 dilution). This is an important step in the PCR amplification protocol because large quantities of DNA can decrease the efficiency of the PCR reaction. The PCR amplification cocktail included 10X buffer, 5X Q buffer, 25 mM $MgCl_2$, 10 mm dDNTP, the forward primer (10 pmol/ µl), the reverse primer (10 pmol/ µl), and HotStar Plus Taq (5 units /µl). The oligonucleotide primers used were designed based on the known conserved regions that will amplify variable regions V3, V4 and V5 of the 16S rRNA gene. The primers used were 380F: 5'CCAGACTCCTACGGGAG GCAG'3 and 907R: 5' CCG TCA ATT CMT TTG AGT TT (3). The thermocycler was programmed for the following steps: an initial denaturing step of 95℃ for 5 min; ten cycles of 94℃, 60℃, 72℃ for 30 sec each; then 35 cycles of 94℃, 54℃, 72℃ for 30 sec each. The final step for elongation of the DNA was 10 min at 72℃. The water used in the PCR contained EMA (9 ug/ml) to eliminate contamination in the PCR reagents and PCR tubes (see the Results section below). A 1.5% agarose gel electrophoresis was performed after every DNA amplification to ensure amplification and no contamination.

Generation of the PCR product for DGGE

A second DNA amplification was performed to generate a PCR product for DGGE analysis. This amplification conditions was identical to the initial PCR reaction, except that the oligonecleotide primers used amplified the variable regions V3 and V4 only, and the reverse primer contained a high GC region on the 5' end. This region was selected because an alignment of the 16S rRNA gene of the most reported bacterial pathogens in musculoskeletal infections revealed that these variable regions provide enough sequence information to speciate almost all of the pathogens (Table 1). The primers used were 380F (see above) and 759R CGV3V4:5'CGCCGCGCGCGGCGGGCGGGGCGGGGCGGGGGGCCGCATT YCACCKCTACAC'3. The template for this PCR reaction was the PCR product from the first PCR amplification, which had been diluted to less than a nanogram of DNA per microliter (usually a 1:100 dilution).

Bacterial species associated with musculoskeletal infections

1. *Achromobacter xylosoxidans* (9)
2. *Acinetobacter baumannii** (9)
3. *Acinetobacter calcoaceticus* (9)
4. *Acinetobacter lwoffii** (16)
5. *Actinomyces israelii** (9)
6. *Actinomyces meyeri* (9)
7. *Aerococcus viridans* (9)
8. *Alcaligenes faecalis* (16)
9. *Alkane-degrading soil bacterium MVAB Hex1* (9)
10. *Anaerococcus vaginalis* (16)
11. *Arcanobacterium pyogenes* (9)
12. *Bacillus cereus* (9)
13. *Bacteriodes fragilis** (9)
14. *Brucella melitensis* (9)
15. *Burkholderia cepacia* (9)
16. *Burkholderia gladioli* (39)
17. *Campylobacter jejuni* (9)
18. *Citrobacter diversus* (9)
19. *Citrobacter freundii* (19)
20. *Clostridium botulinum** (9)
21. *Clostridium septicum* (9)
22. *Comamonas terrigena* (16)
23. *Corynebacterium confusum*(18)
24. *Corynebacterium jeikeium** (9)
25. *Corynebacterium striatum* (16)
26. *Coxiella burnetii* (9)
27. *Eikenella corrodens* (19)
28. *Enterobacter aerogenes** (9)
29. *Enterobacter hormaechei**(9)
30. *Enterococcus gallinarum* (9)
31. *Enterococcus faecium** (9)
32. *Escherichia coli**(9)
33. *Escherichia vulneris* (9)
34. *Faecalibacterium prausnitzii* (9)
35. *Francisella tularensis* (9)
36. *Fusobacterium alocis* (9)
37. *Fusobacterium equorum* (9)
38. *Fusobacterium naviforme* (9)
39. *Fusobacterium necrophorum* (9)
40. *Fusobacterium nucleatum* (9)
41. *Fusobacterium periodonticum* (9)
42. *Fusobacterium sulci* (9)
43. *Granulicatella adiacens* (16)
44. *Haemophilus aphrophilus* (9)

45. *Haemophilus parahaemolyticus* (9)
46. *Haemophilus parainfluenzae* (9)
47. *Haemophilus paraphrophilus* (9)
48. *Haemophilus pittmaniae* (9)
49. *Kingella kingae* (16)
50. *Klebsiella pneumonia** (9)
51. *Leclercia adecarboxylata* (16)
52. *Leptospira interrogans* (9)
53. *Listeria grayi* (9)
54. *Listeria monocytogenes* (9)
55. *Moraxella catarrhalis* (9)
56. *Morganella morganii** (16)
57. *Mycobacterium avium* (9)
58. *Mycobacterium fortuitum* (9)
59. *Mycobacterium goodii* (8)
60. *Mycobacterium gordonae* (9)
61. *Mycobacterium haemophilum* (38
62. *Mycobacterium intracellulare* (9)
63. *Mycobacterium kansasii* (9)
64. *Mycobacterium malmoense* (44)
65. *Mycobacterium marinum* (42)
66. *Mycobacterium tuberculosis* (9)
67. *Mycobacterium wolinsky* (8)
68. *Mycobacterium xenopi* (12)
69. *Mycoplasma pneumoniae* (24)
70. *Neisseria lactamica* (9)
71. *Neisseria meningitis* (28)
72. *Nocardia brasiliensis* (9)
73. *Oligella urethralis* (9)
74. *Pasteurella multocida** (37)
75. *Peptoniphilus lacrimalis** (16)
76. *Peptostreptococcus hareii** (16)
77. *Peptostreptococcus micros** (9)
78. *Porphyromonas asaccharolytica* (16)
79. *Porphyromonas somerae** (16)
80. *Prevotella bivia** (16)
81. *Prevotella buccalis** (16)
82. *Prevotella disiens* (19)
83. *Proteus mirabilis** (9)
84. *Proteus vulgaris* (9)
85. *Providencia stuartii* (19)
86. *Pseudomonas aeruginosa** (9)
87. *Pseudomonas putida* (19)
88. *Pseudomonas stutzeri** (33)
89. *Ralstonia pickettii* (35)
90. *Rhodococcus equi* (9)

91. *Rickettsia rickettsii* (9)
92. *Rochalimaea henselae* (9)
93. *Salmonella paratyphi A* (9)
94. *Salmonella typhimurium* (9)
95. *Serratia marcescens** (9)
96. *Shewanella algae* (7)
97. *Shigella dysenteriae* (9)
98. *Sphingobacterium mizutae* (9)
99. *Staphylococcus aureus** (9)
100. *Staphylococcus capitis* (16)
101. *Staphylococcus caprae* (9)
102. *Staphylococcus epidermidis** (9)
103. *Staphylococcus haemolyticus* (9)
104. *Staphylococcus hominis* (9)
105. *Staphylococcus intermedius* (9)
106. *Staphylococcus lugdunensis* (9)
107. *Staphylococcus saprophyticus* (9)
108. *Staphylococcus schleiferi* (9)
109. *Staphylococcus simulans** (9)
110. *Staphylococcus warneri* (9)
111. *Stenotrophomonas maltophilia* (16)
112. *Streptococcus agalactiae** (28)
113. *Streptococcus gordonii* (9)
114. *Streptococcus sp. 'group G' ** (19)
115. *Streptococcus iniae* (25)
116. *Streptococcus pyogenes* (9)
117. *Streptococcus salivarius* (9)
118. *Streptococcus uberis* (9)
119. *Streptococcus viridans* (19)
120. *Veillonella parvula* (16)
121. *Vibrio cholerae* (9)
122. *Yersinia enterocolitica* (9)

Table 1: Bacterial species in the 16S rRNA gene alignment previously identified as the causative agents in musculoskeletal infections. Names of bacteria species reported to be the causative agents of musculoskeletal infections, which was used for the alignment of the 16S rRNA gene that revealed that variable regions 3 and 4 provide enough sequence information to speciate most of the pathogens. Bacterial species with an asterisk (*) indicate that it was identified by the 16S PCR molecular method. References are listed in the parentheses.

Denaturant gradient gel electrophoresis

After obtaining the second PCR product, this PCR product was separated using DGGE, which used the Bio-Rad DCode system for the casting of the gel and electrophoreses (4). The denaturing gradient gel used was composed of 6-10% acrylamide/bis and a 30-70% denaturing solution of urea and formamide. The 30% denaturing solution contains 12 ml of formamide and 12.6 g of urea. The 70% denaturing solution contains 28 ml of formamide and 29.4 g of urea. The proper gel concentration was determined by comparing band migration and separation in a variety of gel concentrations: 6%, 8%, 6-10%, and 8-10%, acrylamide/bis and 30-70% and 40-70% denaturant solution. The addition of 10% ammonium persulfate and TEMED served to catalyze the gel polymerization. The gradient gel was allowed to polymerize for 2 hrs at room temperature and was stored at 4℃ overnight. The running buffer (0.5 TAE) was heated to 60℃ before the samples were loaded into the wells. Each sample contained PCR products varying in DNA concentration (200 ng to 1ug) and 2x loading dye. The minimum DNA concentration, 200 ng, that can be detected with a UV light source and GelRed Nucleic Acid stain (Biotium) at 3X concentration (5) was determined by loading decreasing amounts DNA of the control stain of *S. aureus*. The duration of electrophoresis was 15 hrs at 100 V. This condition was determine by comparing band migration and separation in gels that had run for various times and voltages. Once electrophoresis was completed the gel was removed from the DGGE

chamber and stained with GelRed stain (3X) for 45 min. The gel was viewed under UV light and all bands were excised using sterile scalpel blades.

DNA extraction of DNA from DGGE bands

Several methods for DNA extraction from the DGGE bands were explored. One method was extraction of DNA using the QIAquick gel extraction kit (Qiagen) modified for acrylamide gels based on the manufacturer's recommendations. Second, a small piece of gel slice was placed directly into the PCR tube to serve as the DNA template in a PCR reaction. Third, the gel slice was cut into pieces and placed in a centrifuge tube with water and left overnight. The DNA would then diffuse into the water, which served as the template for PCR. The fourth method involved shredding the gel slice by bead beating it in a 1.7 ml microcentrifuge tube containing 0.2 g of glass beads in 0.5 ml of water overnight at a setting of 4℃. This solution then served as the DNA template for PCR. The fourth method was the most successful template to produce PCR products, as a result it was the method of choice and included in the optimized protocol.

PCR of DNA extracted from DGGE bands

DNA amplification of the DGGE gel extracted DNA was performed using Fail Safe polymerase (Epicentre Biotechnologies). This polymerase was tested and was more efficient then the Hot Star Plus Taq (Qiagen), used in the initial PCR.

In the development of this method a few other were tested. First, a shorter reverse primer without the GC region was used, however, with this primer the PCR was less efficient. In addition, the GC reverse primer containing the long GC region was tested and showed better amplification of the DNA, but it produced an extensive amount of primer dimers. These primer dimers were a problem in the subsequent sequencing reaction. An agarose gel extraction using the Mini Qiagen kit was used to purify the PCR product in this case.

The primers used were forward and reverse primers that contained linker regions with either AT or GC in content (31). 380F-AT-M13: 5'- GTAAAACGA CGGCCAGTAATTAAAATAAAAATGAAAAAAACCAGACTCCTACGGGAGGC -3'. 759R-CG-M13: 5'- CAGGAAACAGCTATGACGGGCGGGGCGGGGGCACGG GGGGCGCATTYCACCKCTACAC -3'. These primers were chosen to create a buffer region for the sequencing reaction. This resulted in a DNA sequence that begins 40 base pairs upstream of the actual bacterial DNA sequence. This ensured that the entire 16S rDNA sequence was available for analysis. Also, these primers do not form primer dimers and as a result an agarose gel extraction step is not required. These primers were shown to be the most efficient in production of PCR products and allowed complete bacterial DNA sequencing.

DNA sequencing and analysis of the PCR products extracted from the DGGE gel slices

To perform DNA sequencing, the purified PCR products were first incubated with the Exo SAP-IT enzyme to digest the single-stranded DNA in the reaction,

unconsumed dNTPS and primers. ExoSAP-IT contains two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase. The Exonuclease I enzyme removes the unused primers in the PCR reaction. The Shrimp Alkaline Phosphatase removes the residual dNTPs from the PCR reaction (41). The DNA sequencing reaction was performed as followed: 20 ng of the PCR product was added to Big Dye, 5X sequencing buffer, and forward primer (10 pmol/ µl). The Big Dye and 5X were reagents supplied by the Center for Clinical and Translational Sciences Genetics Core Lab of the University of Texas Medical School at Houston. The DNA sequencing reaction was performed on a thermocycler with the following program for 35 cycles: 96℃ for 15 sec, 55℃ for 15 sec, 65℃ for 2 min. The sequencing reaction products were purified using Sam solution and Big Dye Xterminator, which were supplied by the Genetics Core Lab. The sequencing products were analyzed using the ABI 3137 XL in the Genetics Core Lab. DNA sequencing was performed on the control strains and the DGGE PCR products. Even though the DGGE PCR products appeared to not have primer dimers in the agarose gel, the sequence obtained from some of these PCR products was not always reliable. Several modifications of the sequencing reaction were tested, including increasing the amount of nucleotide dyes used per sequencing reaction, decreasing the DNA concentration used in sequencing reaction to 20 ng, and altering the temperatures of the thermocycler steps. None of the modifications tested significantly improved the DNA sequence quality. However, the addition of linker primers (see above) (31) to the 3' end and the 5' end of the PCR product used for sequence analysis did improve the percentage of identify to the known

sequences. The linker primers allowed for the full PCR product sequence to be analyzed. DNA sequences that did not have significant background signal were analyzed by searching the bacterial database (30) with the complete sequence using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) nucleotide-nucleotide server (2). Identification was based on the comparison that provided the highest degree of identity. All identifications including speciation were defined as having a > 90% identity to a known 16S rDNA sequence in the nucleotide database when compared with the BLAST sequence results.

Overall the 16S rRNA gene PCR-DGGE method includes several critical steps: DNA extraction from tissue biopsies, amplification of the bacterial DNA, PCR product separation by DGGE, amplification of the gel-extracted DNA, and DNA sequencing and analysis (Figure 3). This method can detect the presence of bacteria in 3 days by PCR amplification of the 16S rRNA gene and can identify the organisms in 5 days by DNA sequence analysis.

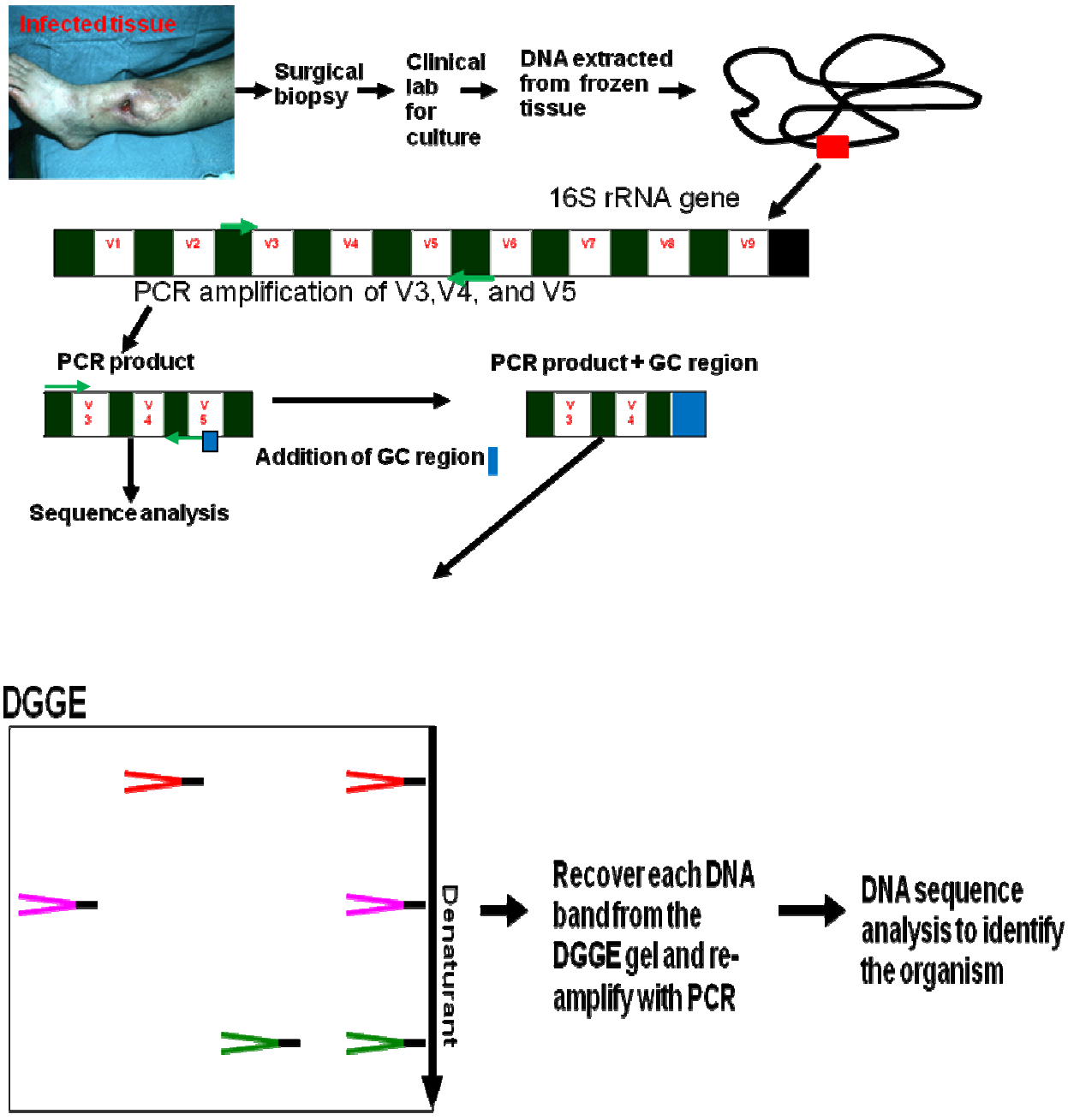


Figure 3: Overview of 16S rRNA gene PCR-DGGE method. This method includes DNA extraction from tissue biopsies, amplification of the bacterial DNA, PCR product separation by DGGE, amplification of the gel-extracted DNA, and DNA sequencing and analysis.

RESULTS

Section 1: Optimization and validation of the method

Isolation of DNA

Limit of detection for DNA extraction

Efficient lysis of the bacterial cells is important to achieving high efficiency and sensitivity for the 16S rRNA gene PCR-DGGE molecular detection technique. The isolation of DNA protocol used in this study was modeled after the Puregene DNA Purification kit (Qiagen). Several modifications were made to the Puregene kit (Qiagen) that increased the efficiency of bacterial cell lysis. *S. aureus* was used as a model bacterial cell because it is a common pathogen in musculoskeletal infections and requires specific steps for efficient cell lysis. The high degree of crossing-linking of the pentaglycine bridge between the ε-amino group of lysine and the terminal D-alanine of an adjacent tetrapeptide makes the *S. aureus* cell wall extremely strong and difficult to lyse (23). For efficient cell lysis of *S. aureus* the addition of lysostaphin (2 mg/ml) to the DNA isolation protocol was important. To test how efficient the DNA isolation protocol was, an experiment using a range of *S. aureus* cells (2 to 2,000) was used in the DNA isolation protocol. The PCR products of the resulting extracted DNA were electrophoresed through a 1.5% agarose gel and visualized after staining with ethidium bromide. The PCR product resulting from the extracted DNA showed that this DNA isolation protocol was able to detect at least 20 bacterial cells (Figure 4). These data indicate that this method is very sensitive. It is interesting to note that when less DNA that was used as the template,

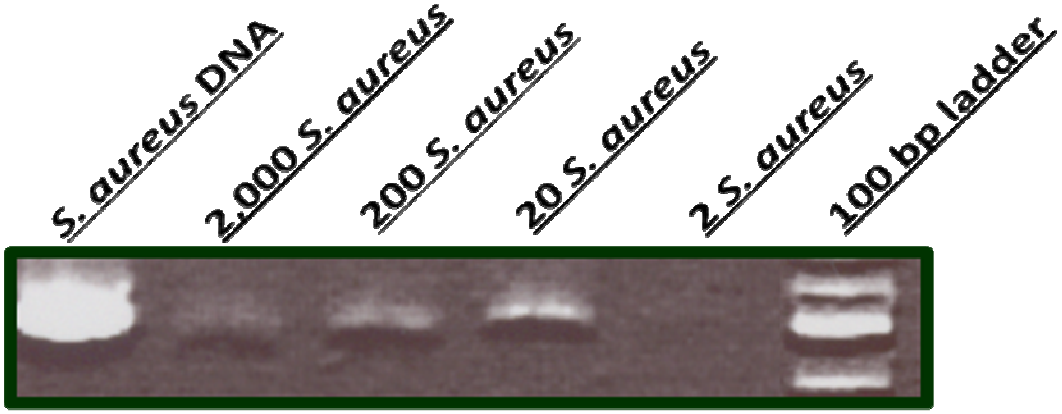


Figure 4: The limit of detection for DNA extraction. The PCR products of extracted DNA from different numbers of *S. aureus* cells ranging from 2 to 2,000 was electrophoresed through a 1.5% agarose gel and visualized after staining with ethidium bromide. The PCR product of the extracted DNA shows that the DNA isolation protocol method is able to detect at least 20 bacterial cells.

there was more PCR product synthesized. These results indicate that the PCR reaction is a more effective reaction with a limited amount of DNA template.

PCR amplification

The optimization of EMA concentration

Ethidium monoazide bromide (EMA) is a photoreactive chemical that is an analogue of ethidium bromide. EMA contains an azide group in the eighth position, whereas ethidium bromide contains an amino group in that position (Figure 5). When EMA is exposed to a long wavelength (> 400 nm), the azide group covalently crosslinks with nucleic acids. Any nucleic acid that becomes crosslinked with EMA will be unable to serve as template for PCR amplification reaction (34). The ability of EMA to bind to nucleic acids can be used to eliminate contaminates from being amplified along with the template. This is important because amplification of contaminates can give misleading results. In this study EMA was only used for the pretreatment of the PCR reaction mix prior to the addition of the template DNA. To determine the appropriate range of EMA necessary for the elimination of contamination in PCR reactions, 0.1 pg of *S. aureus* DNA was added to each PCR reaction to serve as artificial contamination in one of the PCR amplification reagents. Also added were various amounts of EMA: 5 ug/ml, 10 ug/ml, 15 ug/ml, 20 ug/ml; and there was a tube with no added EMA. After the addition of the EMA the PCR reaction was exposed to a halogen light for 5 minutes. All the PCR reactions were amplified as described previously. The PCR products were electrophoresed through 1.5% agarose gel and visualized after staining with ethidium bromide. The PCR reaction

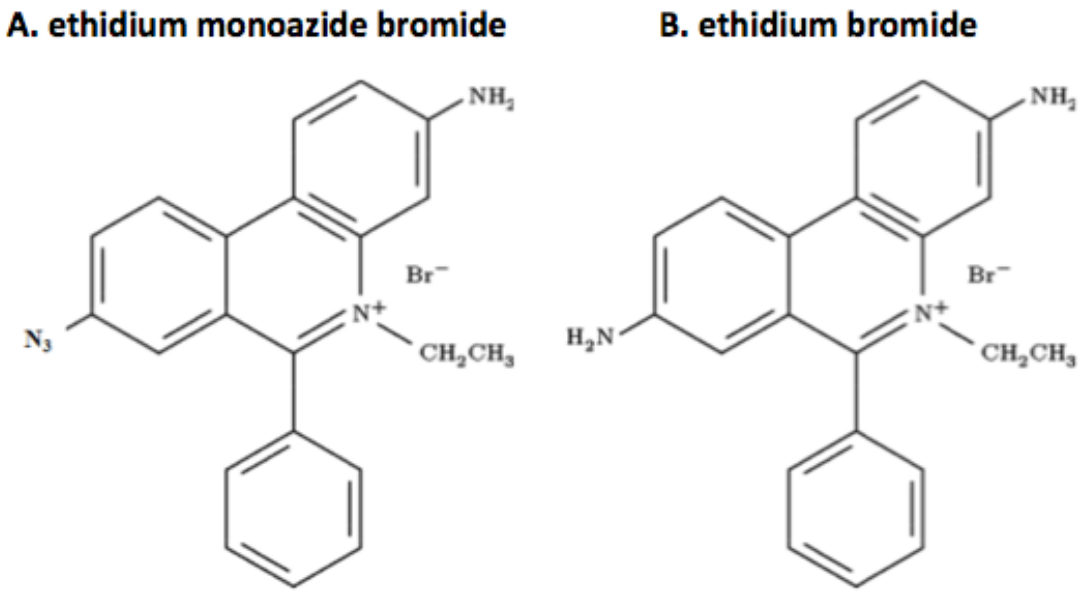


Figure 5: Structural comparison of ethidium monoazide bromide to ethidium bromide. Ethidium monoazide bromide (EMA) (Panel A) is a photoreactive chemical that is an analogue of ethidium bromide (Panel B). EMA contains an azide group in the eighth position where as ethidium bromide contains an amino group. When EMA is exposed to a long wavelength (> 400 nm) the azide group covalently cross links with nucleic acids and prevents their amplification in a PCR reaction.

that contained no EMA and no standard template (blank) showed amplification of the artificial contaminant DNA. However, when EMA at 5 ug/ml or more was added to the PCR reaction before the addition of the standard template DNA, no amplification of the artificial contaminant DNA was observed. This is important to ensure that reagent contamination was not amplified during the PCR reaction. The PCR reactions in which EMA was added at concentrations of 10, 15, and 20 ug/ml seemed to be less efficient in amplification of the template DNA (Figure 6). We chose to use 10 ug/ml of EMA to decontaminate the reagents in the standard protocol. However, 5 ug/ml of EMA would have been a better choice.

The limit of detection for PCR amplification

The PCR amplification of the chromosomal DNA is an important step for the 16S rRNA gene PCR-DGGE method. It is essential that this step be efficient and sensitive in amplifying the variable regions V3, V4, and V5 of the 16S rRNA gene. To determine the efficiency and sensitivity of the PCR amplification, various amounts of *S. aureus* DNA were used as template for the PCR reaction (0.01 pg to 1 pg). This range of *S. aureus* DNA is approximated to be equal to the DNA in 3 - 325 cells base on a chromosome of 3 Mb per cell (30). The PCR amplification was performed using the protocol described previously. The PCR products were electrophoresed through a 1.5% agarose gel and visualized after staining with ethidium bromide. The agarose gel showed that the limit of detection was between 0.01 pg – 0.05 pg of *S. aureus* DNA, which is equivalent to 3-15 cells.

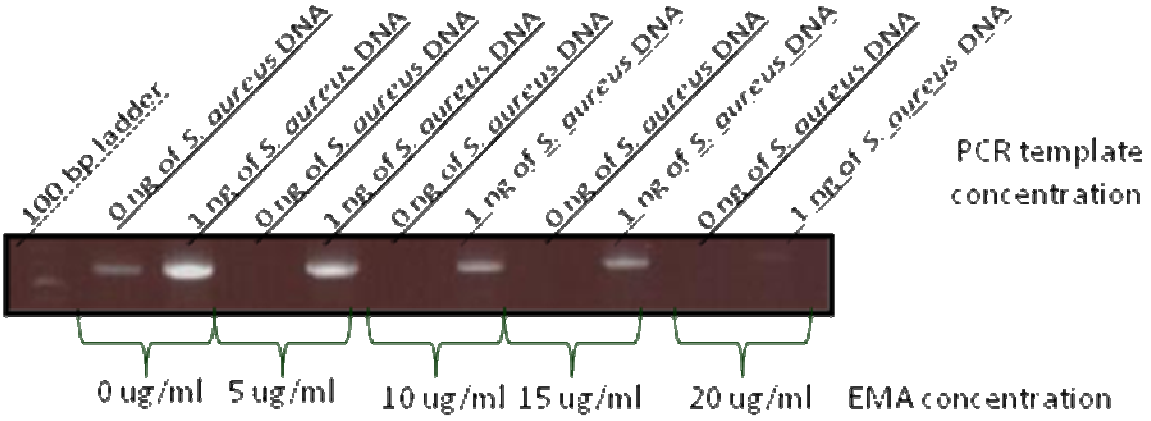


Figure 6: The range of EMA necessary to eliminate PCR contamination for PCR. *S. aureus* DNA (0.1 pg) was added to each PCR reaction to serve as an artificial contamination of the PCR amplification reaction. Also added were various amounts of EMA, 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml or no EMA. After the addition of the EMA the PCR reaction was exposed to a halogen light for 5 min. All the PCR reactions were subjected to PCR amplification cycle described previously. The PCR products were electrophoresed through a 1.5% agarose gel and visualized after with ethidium bromide. The PCR reaction that contained no EMA and no standard template showed amplification of the artificial contaminates. However, when 5 ug/ml of EMA or above was added to the PCR reaction before the addition of the template DNA, no amplification of the artificial DNA contamination was observed.

A PowerWave microplate spectrophotometer (BioTek) was used with a Gen5 data analysis software to determine the *S. aureus* DNA concentration used (Figure 7).

A second PCR reaction was performed to determine if the PCR amplification would be altered in its efficiency in the presence of human DNA. Two different concentrations of human DNA were added to the PCR reactions: 19 ng and 190 ng. These concentrations reflect the concentration of the human DNA that was typically present in 1:10 and 1:100 dilutions of tissue samples, respectively. Each concentration of human DNA was added to PCR reactions with various amounts *S. aureus* DNA as template, 0.01 pg, 0.5 pg, 1 pg, and 125 pg. As described above, DNA concentrations were determined with a microplate spectrophotometer. The PCR amplification was performed according to the PCR amplification protocol described previously. The electrophoresis of the PCR products on a 1.5% agarose gel indicated that in the presence of human DNA less than 100 ng the efficiency of PCR amplification is greater and the sensitivity is increased from 0.05 pg to 0.01 pg of *S. aureus* DNA (Figure 8). This is somewhat surprising and interesting in the light that all of the samples that were analyzed contained human tissue. The results from both PCR amplification experiments showed that the presence of human DNA in the PCR reaction seemed to increase the sensitivity of the reaction. In the presence of human DNA the limit of detection was decreased to 0.01 pg of *S. aureus* DNA.

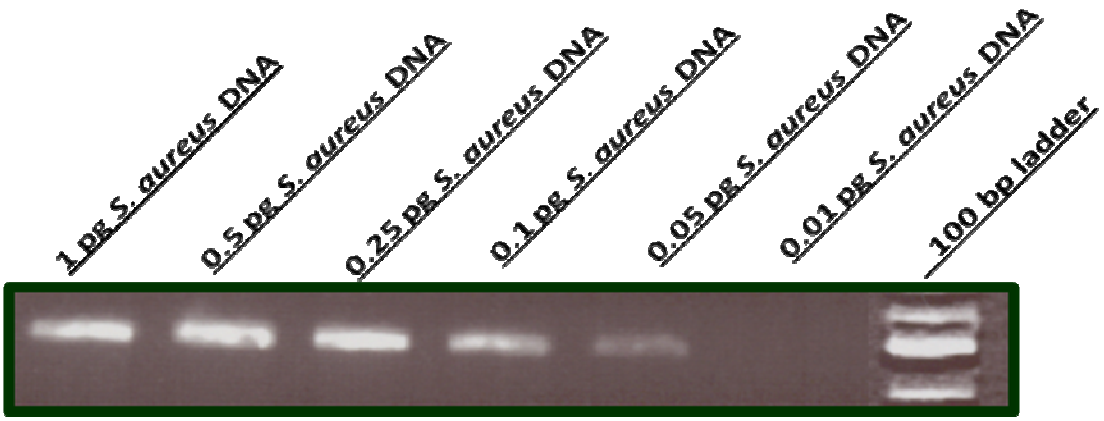


Figure 7: The limit of detection for PCR using *S. aureus* DNA as template. DNA from *S. aureus* cells ranging from 0.01 pg to 1 pg was used as template in the PCR amplification reaction using PCR conditions describe previously. The limit of detection for PCR amplification using *S. aureus* DNA is at least 0.05 pg.

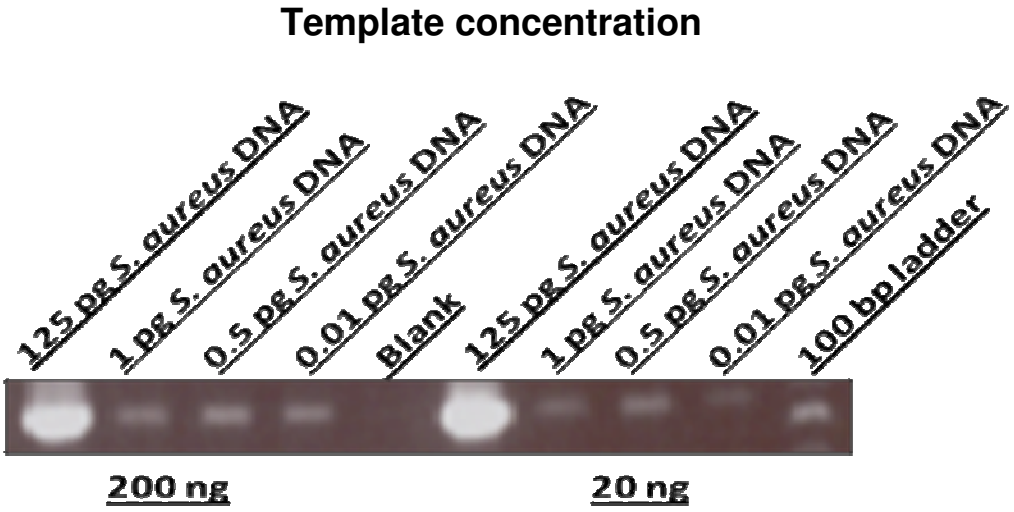


Figure 8: The limit of detection for PCR using S. aureus DNA as template in the presence of human DNA. Two different concentration of human DNA was added to the PCR reaction, 2 ng and 19 ng. Each concentration of human DNA was added to PCR reactions with various amounts *S. aureus* DNA as template, 0.01 pg, 0.05 pg, 1 pg, and 125 pg. The PCR amplification was performed according to the protocol describe previously. The PCR products were electrophoresed through a 1.5% agarose gel and visualized after with ethidium bromide. The results from the agarose gel show that the limit of detection for PCR using *S. aureus* DNA in the presence of human DNA is at least 0.01 pg.

DGGE gel electrophoresis

Determining the most appropriate preparation of the DGGE gel

Two methods to distribute the unpolymerized acrylamide into the DGGE system were tested: a 475 gradient former that is included in the Bio-Rad DCode system and a gravity gradient maker. The 475 gradient former uses two syringes to hold the different liquid acrylamide concentrations, which are moved by a calibrated wheel to form the gel (Figure 9). The second method uses a gravity gradient maker that is connected to a peristaltic pump. To test the two gradient forming devices the high concentration denaturing solution was stained with red food dye. Once the gels solidified they were scanned and pixel analysis determined that the gravity gradient maker made the gradient gel with the most gradual and consistent gradient.

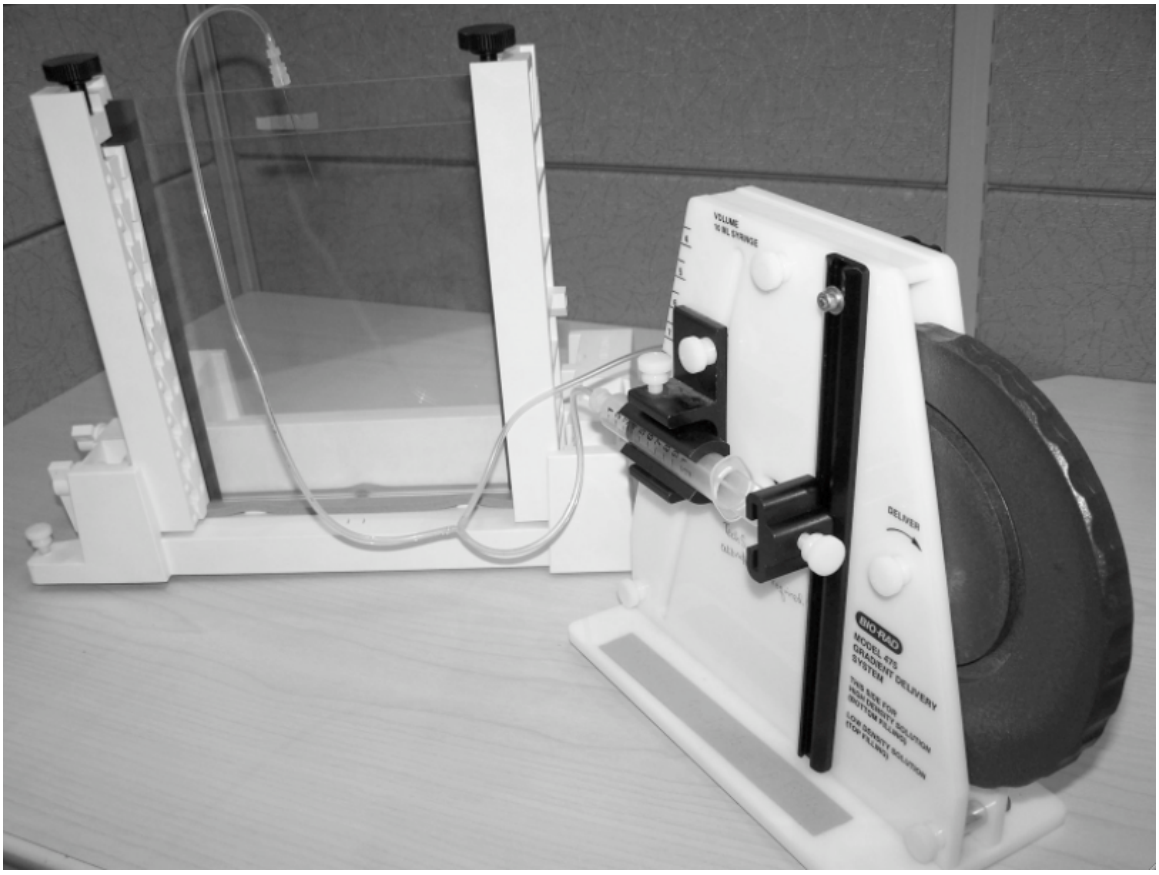


Figure 9: Bio-Rad DCode DGGE apparatus. The DGGE gradient maker uses a calibrated wheel apparatus to dispense the high and low denaturing solution to cast a gradient gel. The wheel applies selective pressure to two syringes containing the high and low concentrations of the denaturing solution. The two solutions flow through the plastic tubing where they become mixed and poured in between two glass plates.

Determining the appropriate amount of acrylamide and denaturing gradient

It is necessary for the DGGE gradient be large enough to separate closely related PCR products. To test which concentration gradient would be the most appropriate, several concentrations of acrylamide and denaturing gradient were tested. These concentrations included: 8%, 8-10%, and a 6-10% acrylamide. These gels were loaded with 500 ng of PCR products amplified from the 16S rDNA of *Staphylococcus aureus, Enterobacter hormaechei, and Acinetobacter baumannii. A. baumannii* and *E. hormaechei* were chosen because the two bacterial DNA PCR products migrate and stop at the top and bottom of the DGGE gel, respectively. The denaturing solutions tested were 40-70% and 30-70%. From these trials, the gel concentration of 6-10% acrylamide and 40-70% denaturing solution was determined to have the best separation for the bacterial DNA tested (Figure 10).

Determination of the most appropriate duration of denaturing gradient gel electrophoresis

It was important to determine the amount of time necessary for the PCR products to migrate to their melting point positions, otherwise PCR product separation may not occur. To determine the correct running time for the DGGE gel, 340 ng of *S. aureus* DNA was loaded into the denaturing gradient gel wells every two hrs for 10 hrs. The DNA bands at 8 and 10 hr migrated the same distance in the denaturing gradient gel. This shows that the DGGE needs at least 8 hrs for maximal migration (Figure 11).

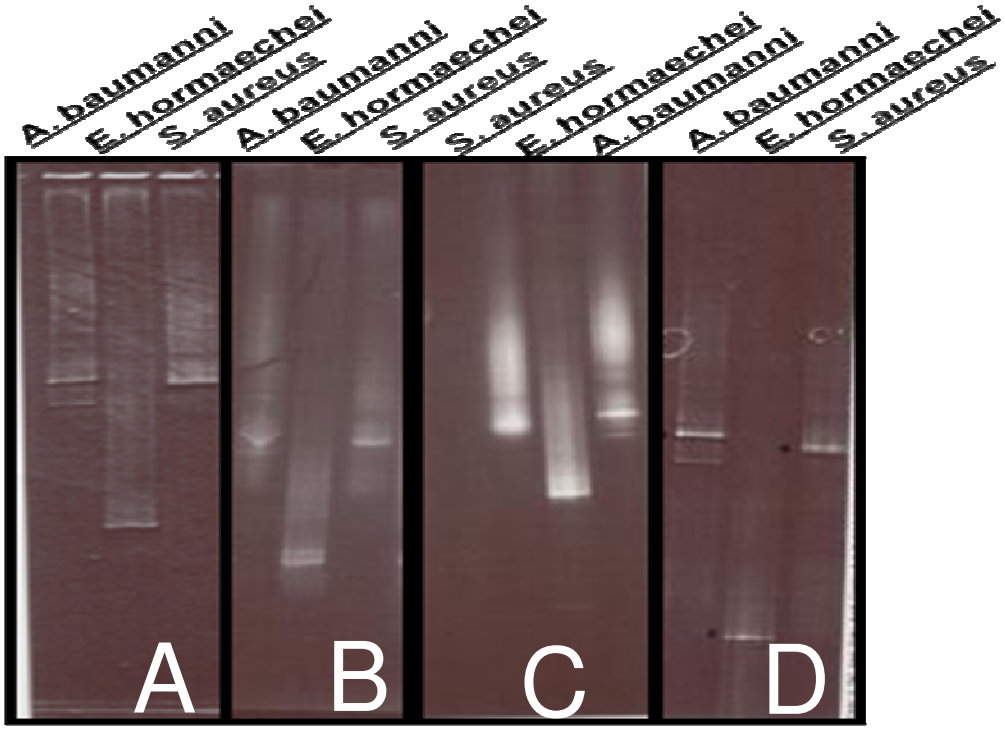


Figure 10: Determining the most appropriate amount of acrylamide and denaturing gradient. Panel A: This DGGE gel is comprised of 8% acrylamide/Bis with 40-70% denaturing solution. Panel B: This DGGE gel is comprised of DGGE 8-10% acrylamide/Bis with 30-70% denaturing solution. Panel C : This DGGE gel is comprised of 6-10% acrylamide/Bis with 40-70% denaturing solution. Panel D: This DGGE gel is comprised of 6-10% acrylamide/Bis with 30-70% denaturing solution. Several concentrations of acrylamide and denaturing gradient were tested. The 8% acrylamide/bis and 8-10% acrylamide/bis were run with 500 ng of *S. aureus, E. hormaechei, and A. baumannii.* The denaturing solutions tested were 40-70% and 30-70%. From these trials, the gel concentration of 6-10% acrylamide and 40-70% maximal migration denaturing solution was determined to have the best separation for the bacterial DNA tested.

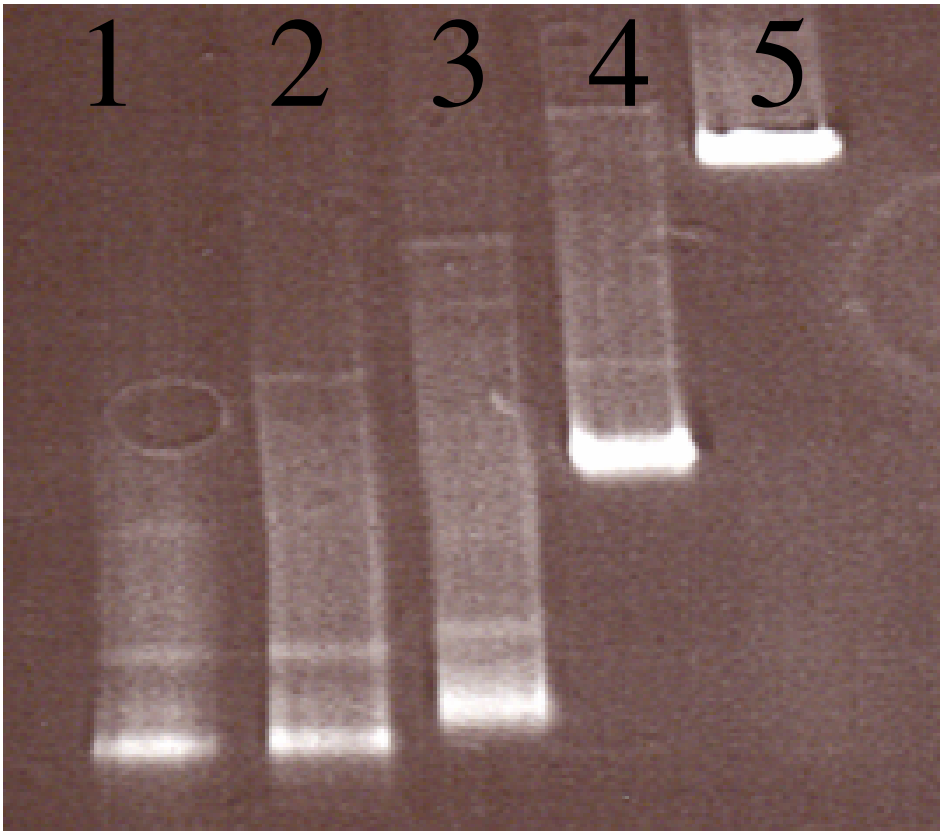


Figure 11: Determining the most appropriate duration of denaturing gradient gel electrophoresis. Electrophoresis of *S. aureus* DNA (340 ng), which was applied to the gel every two hours for ten hours. The DGGE gel was composed of 6-10% acrylamide/bis with 30-70% denaturing solution and electrophoresed at 100 V. The DNA bands (lanes 1 and 2) at 8 and 10 hrs migrated the same distance in the DGGE gel. Lane 1 = 10 hrs, lane 2 = 8 hrs, lane 3 = 6hrs, lane 4 = 4 hrs, lane 5 = 3 hrs.

Determination of the most appropriate amount of DNA to load into the DGGE

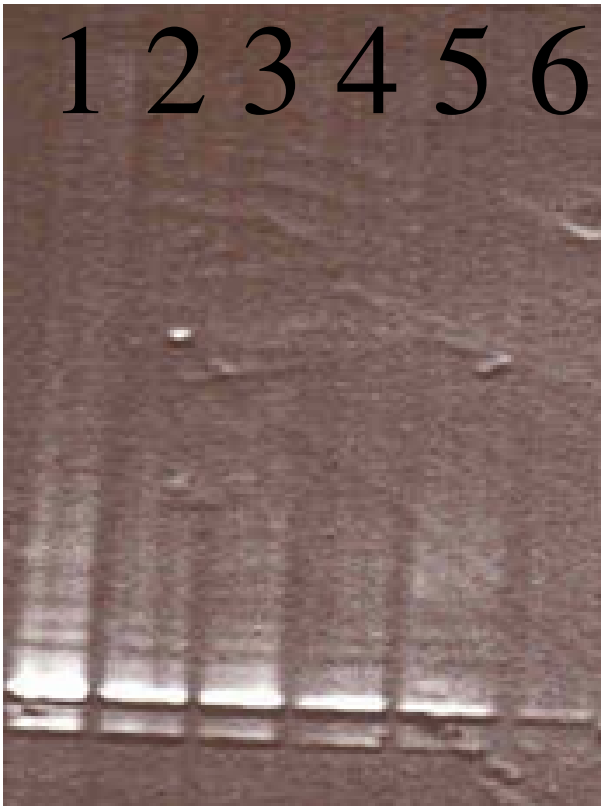
Visualization of the DNA bands in the DGGE gel is important for the recovery and re-amplification of the DNA for sequencing analysis. To determine the amount of DNA needed for visualization with Gel Red stain under UV light, a range of 50 ng -500 ng of *S. aureus* and *A. baumannii* DNA was loaded into the DGGE gel and electrophoresed using our standard conditions, for 15 hrs at 100 V. The 50 ng loading of *S. aureus* and *A. baumannii* were still visible under UV light (Figure 12).

Mixed template PCR amplification and DGGE analysis of bacterial strains

DNA isolation from polymicrobial tissue samples would have multiple DNA templates for PCR amplification. To test the efficiency of PCR amplification of multiple templates in one reaction, DNA of three bacteria strains*, S. aureus, Enterococcus faecalis, and Enterobacter aerogenes,* were PCR amplified together and separately. 50 ng of each bacterial stain, *S. aureus, E. faecalis, and E. aerogenes* served as template separately for PCR amplification. Mixed template PCR was also performed using all three bacterial DNA as template. One PCR reaction contained an equal low concentration of DNA from each organism as template (0.5 ng). A second PCR reaction contained *S. aureus* (5 ng*), E. faecalis* (0.5 ng), and *E. aerogenes* (50 ng). A third PCR reaction contained an equal high concentration of DNA from each organism (50 ng). All PCR amplification products were electrophoresed through a DGGE gel overnight at 100V. The results from this experiment indicate that the efficiency of mixed template PCR amplification

Figure 12: Determining the appropriate amount of DNA to load onto the DGGE.

A: Electrophoresis of various amounts of *S. aureus* DNA ranging from 500 ng to 50 ng.



B: Electrophoresis of various amounts of *A. baumannii* DNA ranging from 500 ng to 50 ng.

1 = 500 ng, 2 = 250 ng, 3 = 200 ng, 4 =150 ng, 5 =100 ng, 6 = 50 ng

decreased when multiple templates were present. However, higher magnification analysis of the bands indicates that DNA for all three bacteria can be observed when equal concentrations of the templates were present (Figure 13 and 14).

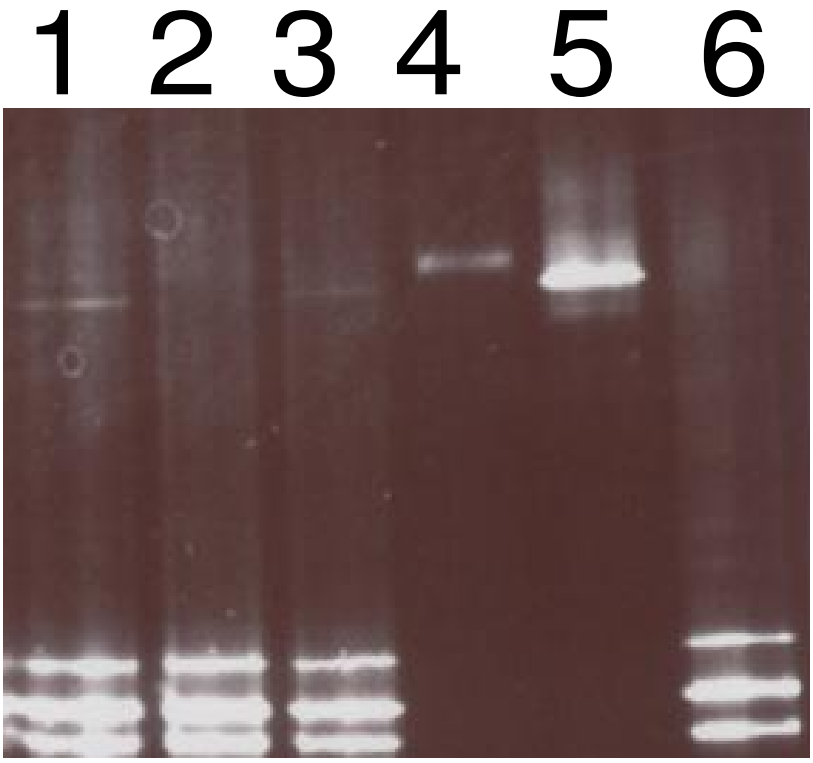


Figure 13: DGGE gel electrophoresis of mixed template PCR products.

Different DNA concentrations of three bacteria strains were PCR amplified together and separately. The PCR products of each PCR amplification reaction was then electrophoresed through a DGGE gel overnight at 100 V. Lane 1 = *Staphylococcus aureus* (0.5 ng*), Enterococcus faecalis* (0.5 ng), *and Enterobacter aerogenes* (0.5 ng). Lane 2 = *S. aureus* (5 ng*), E. faecalis* (0.5 ng), and *E. aerogenes* (50 ng). Lane 3 = *S. aureus* (50 ng), *E. faecalis* (50 ng), and *E. aerogenes* (50 ng). Lane 4 = *S. aureus* (50 ng). Lane 5 = *E. faecalis* (50 ng). Lane 6 = *E. aerogenes* (50 ng).

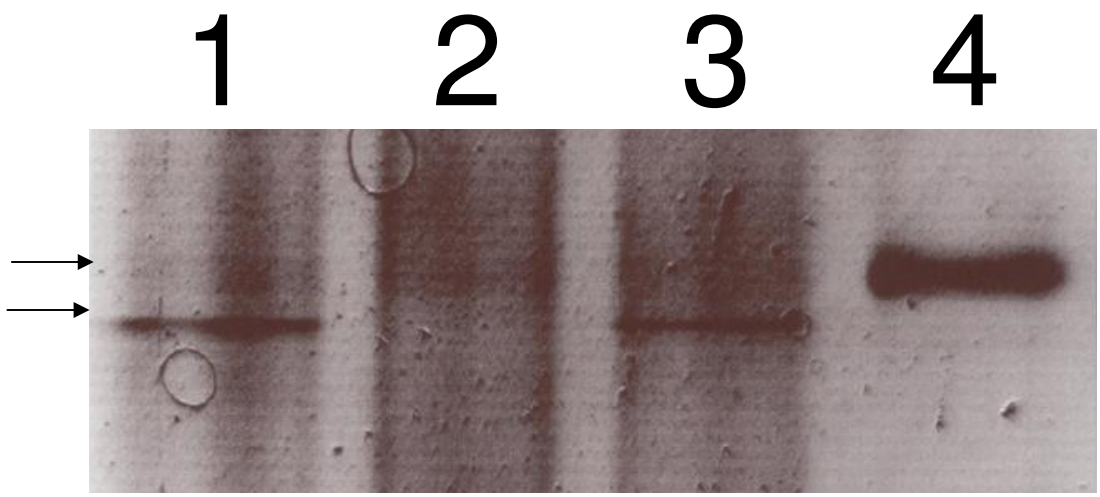


Figure 14: Higher magnification of the DGGE gel electrophoresis of mixed template PCR products. A higher magnification view of lanes 1-4. Lane 1 = *S. aureus* (50 pg*), Enterococcus faecailis*, *and Enterobacter aerogenes* (50 pg). Lane 2 = *S. aureus* (5 ng*), E. faecalis* (50 pg), and *E. aerogenes* (50 ng). Lane 3 = *S. aureus* (50 ng), *E. faecalis* (50 ng), and *E. aerogenes* (50 ng). Lane 4 = *S. aureus* (50 ng). Looking closely the DGGE gel reveals bands in lanes 1-3 that are in a similar position to the *S. aureus,* pictured in lane 4.

Section 2: Results of the pilot study of fifty tissue samples

Molecular analysis of 50 tissue samples

The optimized 16S rRNA gene PCR-DGGE method was used to analyze 50 tissue biopsy samples chosen randomly from our collection. Each tissue sample was subjected to the following steps of the protocol: DNA isolation, PCR amplification with 16S rDNA and DGGE primers, DGGE electrophoresis, extraction of DNA gel slices, DNA sequencing and analysis. The DNA sequencing results were compared to the sequences in the NCBI non-redundant nucleotide database (30) with the BLAST program (2). Identification for each sample was based on comparison with the organism whose 16S rDNA sequence had the highest degree of identity. All identifications were made with > 90% identity with reference sequence giving by BLAST tool. Each result was compared to the culture results of the Memorial Hermann Hospital Clinical Microbiology Laboratory listed in the Memorial Hermann Hospital Electronic Record Resource.

In this study, 17 of the 50 (34%) tissue samples were culture negative. The molecular method was congruent for ten of the 17 (59%) culture negative tissue samples. Six of the 10 (60%) congruent tissue samples were obtained from patients who were not clinically diagnosed with an infection. With this high percentage of clinically uninfected tissue samples, it is not surprising that the molecular method was congruent with ten negative culture samples. In seven of the 17 (41%) culture negative samples the molecular method identified an organism (Table 2).

Tissue Number	Culture Results	16S PCR-DGGE
23	Negative	Negative
110	Negative	Negative
96	Negative	Negative
1506	Negative	Negative
125A	Negative	Negative
0647	Negative	Negative
111*	Negative	Negative
111B*	Negative	Negative
111E*	Negative	Negative
2217*	Negative	Negative
2764	Negative	*Streptococcus agalactiea* (99%)
1921*	Negative	*Staphylococcus aureus* (98%)
2057	Negative	*Abiotrophia defectiva* (98%)
111C	Negative	*Staphylococcus aureus* (90%)
3460*	Negative	*Gemmatimonadetes* (93%)
93*	Negative	*Pseudomonas stutzer* (93%)
3514	Negative	*Acinetobacter sp.* (90%)

Table 2: Culture negative tissue samples. Seventeen tissue samples of 50 were identified as negative by culture. 16S rRNA gene PCR-DGGE method identified 11 out of 50 as negative. Red = congruency. Tissue numbers with an asterisk (*) indicate that *Geobacillus sp.* were identified in the sample by the molecular method.

The culture positive table can be divided into two sections, the mono-microbial and the polymicrobial tissue samples. Eighteen of the 50 (36%) tissue samples were identified to be mono-microbial by culture techniques. The molecular method was congruent with the culture identification for seven of the 33 (21%) positive culture tissue samples. For three of the 18 (17%) samples identified as mono-microbial by the culture results, the molecular method identified more organisms. Fifteen of the 50 (30%) tissue samples were identified as polymicrobial by culture techniques. In 13 of these 15 (87%) the molecular method identified at least one organism that was also identified by culture techniques. In five of these 15 (33%) samples the molecular method identified addition organisms (Table 3).

In tissue samples 67 and 864 *Enterobacter sp.* was identified by the molecular method. However, *Enterobacter* has an identical DNA sequence to *Klebsiella* in the variable 3 and 4 regions which were used for the identification. This means that for these two tissue samples the identification of an *Enterobacter* could also mean an identification of *Klebsiella.* In order to differentiate between *Enterobacter* and *Klebsiella* specific primers to each bacterium could be used for PCR amplification. This is the only problem for identification that has arisen.

Throughout tables 2 and 3, some tissue samples have asterisks, this indicates that *Geobacillus sp*. was identified in that sample by the molecular method. Specifically, *Geobacillus stearothermophilus* spores are widely used for the validation of moist heat sterilization in autoclaves. Autoclaves are commonly used in

Tissue Number	Culture Results	16S PCR-DGGE
103	*Clostridium sp.*	*Clostridium sp.* (99%)
0689	*Staphylococcus aureus*	*Staphylococcus aureus* (99%)
2543	MRSA	*Staphylococcus aureus* (99%)
3368	MRSA	*Staphylococcus aureus* (99%)
2945	*Staphylococcus aureus*	*Staphylococcus aureus* (100%)
2362	*MRSA*	*Staphylococcus aureus* (94%)
3059*	MRSA	*Staphylococcus aureus* (92%)
3361	MRSA	*Staphylococcus aureus (99%), Corynebacterium sp,* (95%) *Streptococcus agalactiae* (99%)
67*	MRSA	*Staphylococcus aureus* (98%), *Enterobacter sp.* (96%)
2920	*Staphylococcus sp.*	*Staphylococcus simulans* (96%), *Corynebacterium sp.* (97%)
65	*Staphylococcus sp.*	*Corynebacterium sp.* (96%)
127B	*Pseudomonas aeruginosa*	*Clostridium sp.* (100%)
117B*	*Staphylococcus aureus*	Negative
131C*	*Staphylococcus sp.*	Negative
107	MRSA	Negative
127A	*Pseudomonas aeruginosa*	Negative
131D*	*Staphylococcus sp.*	Negative
1216*	MRSA	Negative
112	*MRSA, Serratia marscesens, Enterococcus sp.*	*Staphylococcus aureus* (95%), *Serratia sp.* (99%)
97	*Proteus mirabilis, Corynebacterium, Staphylococcus sp.*	*Proteus mirabilis* (94%), *Clostridium sp.* (98%), *Porphyromonas somerae* (94%)
797*	*Streptococcus sp., Staphylococcus aureus, Staphylococcus sp., Corynebacterium*	*Streptococcus pneumonia* (98%), *Candidatus peptoniphilus massiliensis* (96%)

Tissue Number	Culture Results	16S PCR-DGGE Results
864	Group B *Streptococcus*, Gamma *Streptococcus Corynebacterium, Staphylococcus sp.*, *Prevotella sp.*	*Streptococcus agalactiae (97%), Enterococcus faecalis* (97%), *Enterobacter* (93%)
101	Beta Hemolytic *Streptococcus , Pseudomonas aeruginosa , Acinetobacter baumanii*, MRSA	*Streptococcus dysagalactiae* (99%), *Corynebacterium* (97%)
108 *	*Corynebacterium, Staphylococcus aureus,* Group B *Streptococcus*	*Corynebacterium sp.* (99%), *Actinobacterium sp.* (92%)
113*	*Pseudomonas aeruginosa, Enterobacter cloacae, Staphylococcus sp.*	*Pseudomonas aeruginosa* (96%)
1044	*Pseudomonas aeruginosa, Enterococcus, Citrobacter freundii*	*Pseudomonas aeruginosa* (100%)
2669	*Streptococcus sp., Pseudomonas aeruginosa, Staphylococcus* sp.	*Streptococcus anginosus* (99%)
1025	*Enterococcus sp., Enterobacter aerogenes, E. coli, Prevotella, Pseudomonas aeruginosa*, Gamma Hemolytic	*Enterococcus faecalis* (99%)
2085	*Enterococcus sp., Prevotella sp.*	*Enterococcus faecalis* (99%)
2986	*Corynebacterium sp., Pseudomonas aeruginosa,*	*Corynebacterium sp.* (90%)
94B*	*Gram neg. rods, lactose fermenters, Achromobacter*	*Pseudomonas aeruginosa* (96%)
104*	MRSA, *Staphylococcus sp.*, *Corynebacterium*	Negative
104B*	MRSA, Staphylococcus sp. Corynebacterium sp.	Negative

Table 3: Culture positive tissue samples. Eighteen out of the 50 tissue samples were monomicrobial. Fifteen out of the 50 tissue samples were polymicrobial. Red = congruency. Anaerobes are underlined. Tissue numbers with an asterisk (*) indicate that *Geobacillus sp.* were identified in the sample by the molecular method.

medical settings for sterilization of medical instrumentations and medical waste (26). *Geobacillus* has not been found to cause human infection, however, it was present in 12 of the 50 tissue samples. It is possible that the molecular method is detecting DNA from the *Geobacillus* spores left in the autoclaves after the validation of sterilization testing that have contaminated the autoclaved medical instruments used for the biopsies in this study.

DISCUSSION

The overall goal of this project was to use PCR amplification of a portion of the 16S rRNA gene to test an alternative approach for the identification of the causative agents in musculoskeletal infections and to assess the diversity of the bacteria involved. The advantages of this alternative method are that it should increase sample sensitivity and the speed of detection. In addition, bacteria that are non-culturable or in low abundance can be detected using a molecular method. The 16S rDNA PCR-DGGE method, applied many molecular techniques, which included several critical steps: DNA extraction from biopsied tissue, amplification of the bacterial DNA by PCR, PCR product separation by DGGE, extraction and re-amplification of the DNA from the gel slices, and DNA sequencing and analysis.

The first goal of this project was to optimize each step of the protocol so that it was sensitive and rapid as possible. The first step in the protocol to be optimized was the DNA isolation. The DNA isolation protocol required two days to complete. Several steps were modified to increase sensitivity. These steps included the addition of lysostaphin for the efficient lysis of *S. aureus* cells. Without the addition of lysostaphin the detection of *S. aureus* cells was greatly diminished. This is important because *S. aureus* is one of the most common pathogens in musculoskeletal infections. Our results confirmed this to be the case in our 50 samples, as *S. aureus* was identified in ten samples by the molecular approach and in 11 by culture.

Another factor that was important in the DNA isolation step was the prevention of contamination. Contamination was a problem in the beginning of this study. The use of disposable tissue grinders and UV light treatment of cell lysis

solution, sterile water and instruments before DNA isolation was crucial to prevent contamination. The use of EMA to eliminate DNA contamination of the reagents was also important.

The second step of the molecular method was PCR amplification of the 16S rRNA gene. The choice of primers for the PCR amplification was important. The goal of the molecular method was to identify all possible bacterial causative agents in musculoskeletal infections. To accomplish this goal, the primers must be able to hybridize to the conserved regions of the 16S rRNA gene. It was also important that the variable regions amplified contained enough unique information content to ensure that an identification of the species could be made. The choice of which variable region to amplify was based on the comparison of 123 16S rRNA gene sequences of the most common pathogens in musculoskeletal infections. This sequence comparison determined that variable region V3 and V4 would be the best choice for this study because it would speciate almost all of the possible bacterial organisms.

The third step in this method involves the use of DGGE to separate the 16S rDNA PCR products present in polymicrobial samples. This technique has not been widely used for medical diagnostic purposes. This molecular technique was essential for the overall success of the identification of organisms in the molecular method. Without this technique 15 out of the 50 tissue samples would not have provided readable DNA sequences for identification. The DGGE protocol has several components that are important for DNA electrophoresis and visualization. The method of pouring of the acrylamide solution was critical to ensure uniform

movement and arrest of the PCR products. The Bio-Rad DCode system included a calibrated wheel that was used. This was not an ideal method to use to prepare generate the gradient gels. It was time consuming to setup and the gradient was less consistent then the one obtained with a gravity gradient maker. The latter was used for the analysis of the 50 tissue samples in this study.

The acrylamide and denaturing solution concentrations were critical to the optimized migration of the PCR products. Several concentrations of both acrylamide and denaturants were tested, but the best separation observed between the PCR products was seen with a double gradient gel of acrylamide and denaturant. The visualization of the DGGE gel bands also was a problem in the beginning of this project. However, after performing experiments in which various amounts of DNA were electrophoresed through the DGGE gel, it was determined that there was a balance between over loading the gel, which produced smears, and loading too little, which could hardly be seen. In addition, the use of GelRed to stain the DGGE gel proved to be very useful. GelRed does not require a destaining step and is non-toxic, which is helpful when staining a large gel.

Several methods for DNA extraction from DGGE bands were explored. One method was extraction of DNA using the QIAquick gel extraction kit (Qiagen) modified for acrylamide gels based on the manufacturer's recommendations. This method gave very poor recovery of the DNA. About half of the DNA loaded into the gel was lost. Secondly, a small piece of gel slice was placed directly into the PCR tube to serve as the DNA template in a PCR reaction. This method only worked for a few DGGE slices. It was not very consistent in producing a PCR product .Thirdly,

the gel slice was cut into pieces and placed in a centrifuge tube with water and left overnight. The DNA would then diffuse into the water, which served as the template for PCR. This method was consistent and most DGGE slices gave a strong PCR product. However, the question of whether all of the DNA was being released into the water remained. The fourth method involved shredding the gel slice by bead beating and leaving it overnight at 4℃. This method produced the most successful template and all of the DNA present was most likely being released into the solution to be used as a template for PCR amplification.

The sequencing and analysis of the DNA from the DGGE slices was the final step in the molecular method. To obtain an optimal DNA sequence with no background signal, it was important that primer dimers were not present in the sequencing template. The use of linker primers was critical to the success of this project because it allowed a DNA sequence of the entire PCR product to be obtained (31). This was important because the PCR product size was not very large (360 bp) and all the PCR product DNA sequence was needed to identify the species with confidence.

The second goal of this study was to use the optimized 16S rDNA PCR-DGGE method to analyze 50 tissue biopsy samples chosen randomly from our collection. The molecular method was congruent for ten of the 17 (59%) culture negative tissue samples. In seven of the 17 (41%) culture negative samples the molecular method identified a bacterium. The molecular method was congruent with the culture identification for seven of the 33 (21%) positive cultured tissue samples. However, in eight of the 33 (24%) the molecular method identified more organisms.

In 13 of the 15 (87%) polymicrobial cultured tissue samples the molecular method identified at least one organism that was also identified by culture techniques.

Forty-seven out of the 50 (94%) tissue samples had anaerobic cultures performed. Within those 47 tissue samples the molecular method found two tissue samples to contain strict bacterial anaerobes that were not detect by culture analysis. In one of the three tissue samples that did not have an anaerobic culture performed, the molecular method found two strict anaerobe bacterial species. Even though for the majority of the cases anaerobic cultures were taken the molecular method was still able to identified additional strict anaerobes.

Eight tissue samples (1921, 2764, 93, 3514, 797, 65, 103, 864) in the 50 were collected when the patient was already on antibiotics. Specifically, for tissue #1921 which was negative culture, samples were taken from a patient with a positive culture of MRSA four days earlier, which may indicate that the antibiotic was eradicating the infection but the molecular method was still able to detect *Staphylococcus aureus* cells.

Another interesting case is tissue # 2764 which was found to be culture negative, but had two cultures performed 12 and 14 weeks later. At 12 weeks the culture analysis identified MRSA and *Acinetobacter*. At 14 weeks later the culture analysis identified *Acinetobacter*, Group B *Streptococcus*, and *Corynebacteri*um. The molecular method was able to detect the *Streptococcus sp*. much earlier than the culture method. Tissues #93 and #3514 were culture negative and had no further evidence of infection after the course of antibiotics. Tissues #797, #65,

#103, and #864 were found culture positive even though the patient was on antibiotics at the time of tissue biopsy.

The differences in identification between the two methods could be due to many reasons. The first and simplest reason is that each tissue sample tested by the molecular method was not exactly the same as the sample that was used for the culture method. All of the tissue samples used for the 16S rDNA PCR method were the discarded tissue from the Medical Microbiology Lab and may not be a true representative of the original tissue sample that was tested in their laboratory. This potential problem could be resolved if the IRB protocol was modified to allow that the biopsied tissue be mixed and split between the clinical laboratory and the research laboratory immediately after the collection.

The second reason for a discrepancy between the culture and the molecular results could be that the DNA extraction, PCR, or DGGE do not represent all bacterial species in the tissue samples. Some kind of bias could be occurring in the PCR amplification step of the molecular method. The experiment in which the PCR amplification of three different templates in one reaction was performed seemed to indicate that some PCR amplification bias may be occurring during this step. When a low concentration of *E. faecalis* was added into a PCR amplification reaction with templates that were 10 x and 100x more concentrated, it was difficult to observe the *E. faecalis* PCR product in the DGGE gel. This PCR reaction represents the worst case scenario that could possibly occur, which is having a DNA species that represents only 1% of the DNA template. A bias in amplification of rare DNA species is also supported by the fact that most species missed by the molecular

method were usually noted as being rare or few in the culture results. This is the case in tissue # 97; the molecular method detected *Proteus mirabilis* as the most predominant bacterial species, but did not detect *Corynebacterium* and *Staphylococcus* sp. which were described as 'rare' and 'few' respectively. A possible solution for this problem could be using different conserved primers that may be more efficient in amplification of the low percentage DNA species in a mixed template PCR reaction. A second possible solution is performing a multiplex PCR reaction which uses multiple primers in one PCR reaction. This type of PCR amplification could be better in amplifying low percentage DNA species. A third possible solution is to change the molecular method protocol of the second PCR amplification step which adds the high GC region at the 5' end, to use the chromosomal DNA as template for the PCR reaction instead of the initial PCR product as template. It is possible that a nested PCR reaction may not be very efficient in amplifying rare DNA species and that returning to the chromosomal DNA would be more efficient. The mixed template PCR amplification experiment may indicate that an amplification bias against low percentage DNA species may exist. However, additional experiment s should be done to understand this amplification bias thoroughly.

A less likely reason for differences in identification between the two methods could be insufficient separation between the PCR products of similar DNA species. Some DNA species such as *Pseudomonas* and *Staphylococcus aureus* seem to arrest their migration through the DGGE gel in very similar positions. *Pseudomonas* and *S. aureus* were not found together by the molecular method, but they were

identified together in one tissue sample by culture analysis. In cases where very similar DNA PCR products or PCR products that migrate to a similar location in the gel are present, a smaller DGGE gradient can be used to improve the separation. Specifically to create greater separation between *Pseudomonas* and *S. aureus* a 45-60% denaturing solution gradient should be used. However, for most cases the DGGE of the PCR products were found consistently in the same location. Almost all bacterial species were consistently found is similar positions in the DGGE gel. Some tissue samples were done multiple times at different time and consistently showed the same pattern of DGGE DNA banding.

Overall, the DGGE analysis of 16S rDNA seems to be an alternative method to identify bacteria not identified by culture analysis. This method does have limitations for the identification of pathogens. The method requires specific instrumentation and a person trained in these molecular techniques in order to be successful. It relies on efficient amplification of all DNA species present and correct separation of the PCR products in the DGGE gel, which has not been definitely proven by this method. As a result this method may not be ideal for use in the clinical microbiology laboratory for the general identification of pathogens. However, in cases were no pathogen is cultured and the patient is not recovering with the prescribed antibiotics, this method may provide some information that can aid in the patient's recovery.

PERSPECTIVES

Several molecular techniques for bacterial identification have been investigated by others in the field. These molecular techniques include antigen recognition (43), PCR amplification of genes that are specific to the suspected organism (14), microarrays (20), and 16S PCR amplification (40). However, these investigations usually examine a small number of samples and do not compare the molecular results to that of patient culture results. The goal of this project was to develop a rapid, sensitive and unbiased molecular method to identify all possible pathogens directly from infected tissue samples, without the need for culture. PCR amplification of the 16S rRNA gene has been widely used because of the ability to amplify thousands of possible bacteria present in the sample. After the PCR amplification, sequence analysis is usually performed in order to identify the organism. If several distinct PCR sequences are present in the PCR product direct sequencing is not reliable. New sequencing technology may solve this problem. Pyrosequencing is able to sequence multiple templates. However, currently this technology is still expensive for mass clinical use as a method of pathogen identification. In addition, pyrosequencing is limited to sequencing DNAs about 400 bp. If improvements are made in pyrosequencing or other types of single strand sequencing methods and the cost of analysis is reduced this may be a good option for pathogen identification.

Another molecular technique that may be a good option for rapid identification of pathogens is mass spectrometry. This molecular technique is very rapid and is becoming more commonly used. However, accurate identification of all

species in polymicrobial infections using mass spectrometry is still under discussion. Also a reference spectrum is required to obtain a definite identification of the pathogen. At this point the database of reference spectrums is limited, but it is growing rapidly. In the future this molecular technique may also be a suitable option for molecular identification of all pathogens not just bacterial.

In the present time however a microarray may be the best option for a molecular method to identify pathogens. The price of a microarray has decreased and has become widely used and easier to construct. A tiled microarray of portions of the entire 16S rRNA genes of hundreds of bacterial pathogens could be a rapid and more standardized method for the identification of pathogens. Practically, several different microarrays could be constructed, for the purposes of this study a microarray with the most common and hard to culture musculoskeletal bacterial pathogens would be used. However, if needed a microarray could be constructed with pathogens associated with meningitis, cardiovascular infections or other types of infections.

This investigation chose to develop a 16S rRNA gene PCR amplification method for the identification of bacteria. To solve the problem of resolving mixed DNA sequences in a PCR product, we chose to use a denaturing gradient gel electrophoresis. This molecular technique separates DNA molecules into individual species. DGGE has been used for monitoring bacterial populations in environmental samples and has not been used extensively for pathogen identification. The use of DGGE seemed promising as a rapid method for separating bacterial 16S rRNA gene PCR products for sequence analysis. However, much like other 16S rRNA

gene methods, the 16S rRNA gene PCR-DGGE method had problems. The results of this study indicate that this method as currently optimized is not able to identify all possible pathogens in a tissue sample. This molecular method may serve best to identify pathogens in tissue samples that are culture negative.

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