The Use of the 16S rRNA Gene and the Internal Transcribed Spacer (ITS2) Region for Rapid and Specific Identification of Bacteria and Fungi from Clinical Specimens

By

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THE USE OF THE 16S rRNA GENE AND THE INTERNAL TRANSCRIBED SPACER (ITS2) REGION FOR RAPID AND SPECIFIC IDENTIFICATION OF BACTERIA AND FUNGI FROM CLINICAL SPECIMENS

BY

CHRISTINE YVETTE TURENNE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

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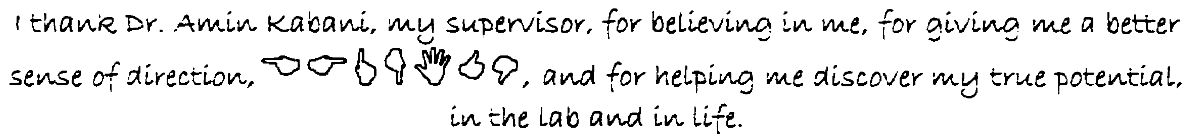
I thank Dr. Amin Kabani, my supervisor, for believing in me, for giving me a better sense of direction, , and for helping me discover my true potential, in the lab and in life.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSI	bloodstream infection
bp	base pair
CFU	colony forming unit
CoNS	coagulase-negative *Staphylococcus*
dNTP	deoxynucleotide triphosphate
dUTP	deoxyuracil triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
H_20	water
HCl	hydrogen chloride
ITS2	internal transcribed spacer 2
kb	kilobase
NaCl	sodium chloride
NaOH	sodium hydroxyde
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
rDNA	ribosomal DNA
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid

SD	standard deviation
SDS	sodium dodecyl sulfate
SPS	sodium polyanetholesulfonate
SSCP	single-stranded conformation polymorphism
$T_{10}E_1$	aqueous solution containing 10 mM Tris and 1 mM EDTA, pH 8.0
UDG	uracil DNA glycosylase
UV	ultraviolet

ABSTRACT

Bacteremia and fungemia contribute significantly to the mortality and morbidity of patients. The use of blood cultures is not rapid, sensitive or specific enough to identify or exclude all causes of a febrile episode, resulting in empiric administration of broad-spectrum antibiotics based on clinical grounds. To overcome these limitations, we have made use of the sequence variability of the 16S ribosomal DNA (rDNA) of bacteria and the intergenic transcribed spacer region (ITS2) of fungi for rapid DNA amplification, detection and identification of pathogens from positive blood cultures using an automated fluorescent capillary electrophoresis sequencer. Bacterial 16S rDNA amplification using universal primers resulted in conformational differences between species detectable by single-stranded conformation polymorphism (SSCP). Fungal ITS2 amplification using universal primers resulted in variability of PCR product length among species detected upon fragment analysis. We determined the sensitivity of the PCR reaction using both the bacterial and fungal universal primers to be 10 and 1 CFU/ml whole blood, respectively. Amplification was tested for cross-reactivity with human DNA, effect of prolonged sample storage and presence of various antibiotics. The SSCP patterns of the most commonly isolated bacteria from blood cultures and the ITS2 length of 47 various fungal species were determined using control strains. The molecular identification of 304 blood culture positive clinical specimens and 101 seeded yeast blood cultures, using the determined SSCP patterns or ITS2 length as reference, was compared to conventional identification in a double-blinded fashion. All species demonstrated

consistent and reliable patterns most being unique to the species. Time to identification from blood culture positivity ranged on average from 2 to 5 days using conventional testing whereas identification by fluorescent capillary electrophoresis was obtained within 7 hours. Cost savings were significant. The limitations encountered included preferential amplification in mixed specimens, unreliable 16S rDNA internet sequence databases and identical SSCP/fragment analysis results obtained between some species. The resolution of these issues will render this methodology a promising tool for the rapid and specific diagnosis of bloodstream infections.

The Use of the 16S rRNA Gene and the Internal Transcribed Spacer (ITS2) Region for Rapid and Specific Identification of Bacteria and Fungi in Blood Specimens

A. INTRODUCTION

1. Bloodstream Infections and Their Treatment

a. Bacterial and Fungal Bloodstream Infections

In the US, there are approximately 250,000 episodes of nosocomial bloodstream infections (BSI) annually (CDC, 1992) and the number of BSI is doubled with the addition of community acquired infections (Weinstein *et al.*, 1997). It is estimated that 3-5% of patients who are discharged from hospital have acquired a nosocomial infection during their stay (Haley *et al.*, 1992). Of these, approximately 8% constitute primary BSI (Horan *et al.*, 1984). The rate of bloodstream infections now ranges from 1.3/1000 discharges for small non-teaching hospitals to 6.5/1000 discharges for large teaching hospitals (Banerjee *et al.*, 1991).

Blood pathogens are most commonly acquired from intravascular catheter sites, genitourinary and respiratory tracts (Weinstein *et al.*, 1997). The first indication of bacteremia include fever, chills, hyperventilation, rash and diarrhea, and often progresses further to more serious disease states such as shock, multiple organ failure and disseminated intravascular coagulation, which carry a high mortality rate. These infections also add to the economic burden of health care as a result of treatment and the attendent morbidity (Pittet *et al.*, 1994).

The crude mortality rate of bacteremia ranges from 25-50%, with close to one third of deaths being directly attributable to these infections (Wenzel, 1988; Pittet *et al.*,

1997; Nucci *et al.*, 1997). This varies greatly depending on the underlying condition, infecting pathogen and treatment administered.

The spectrum of infecting organisms has evolved over the last two decades from a predominance of Gram-negative organisms to Gram-positive organisms. Gram-positive organisms account for 46-84%; Gram-negative organisms 16-42%; and fungi 4-7% while polymicrobial infections constitute 12-14% of infections (Pittet *et al.*, 1997; Weinstein *et al.*, 1997; Elting *et al.*, 1997; Bochud *et al.*, 1994). Most community-acquired infections are due to *Streptococcus* species and *Escherichia coli*. Other *Enterobacteriaceae* and non-fermenting Gram-negative bacteria (ie: *Pseudomonas* and *Acinetobacter spp.*), which persist in the hospital environment, colonize and infect hospital patients, resulting in nosocomial infections (Weinstein *et al.*, 1997). Infections caused by organisms such as *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CoNS), *Enterococcus* and *Candida* species (Weinstein *et al.*, 1997) have increased as a result of a higher number of immunocompromised patients and a more frequent use of prosthetic and indwelling devices (Banerjee *et al.*, 1991).

The incidence of nosocomial fungal infection has increased from 6% in 1980 to 10.4% in 1990 (Beck-Sagué *et al.*, 1993). The highest infection rates were found among burn/trauma, surgery, oncology and high-risk nursery patients (Beck-Sagué *et al.*, 1993). *Candida* species now rank fourth among the most commonly isolated organisms in BSI (Pittet *et al.*, 1997; Banerjee *et al.*, 1991; CDC NNIS System, 1996). While *Candida albicans* (~60%) and *Candida* species (~20%) are responsible for most fungal infections (Beck-Sagué *et al.*, 1993; Goodrich *et al.*, 1991), up to 150 fungal species have been known to be primary pathogens of humans involving all body sites (Fromtling, 1995).

The mortality rate of fungemia, which is higher than for bacteremia, ranges from 30-60% (Beck-Sagué *et al.*, 1993; Goodrich *et al.*, 1991; Nucci *et al.*, 1997; Karlowsky *et al.*, 1997). Tissue involvement can occur in up to 36% of fungemic episodes and has been associated with a very high mortality rate of 47-88% (Goodrich *et al.*, 1991; Goodman *et al.*, 1992; Winston *et al.*, 1993). Some organisms such as *Aspergillus* and *Fusarium* species have a mortality rate close to 100% (Goodrich *et al.*, 1991; Nucci *et al.*, 1997).

Table 1 shows the 10 most commonly isolated organisms from blood cultures at the Health Sciences Centre in Winnipeg in 1997. The total number of blood cultures submitted was 15,192 with 1,910 being positive in 1,295 patients. These organisms constitute approximately 82% of all species that were isolated.

Table 1. **Top 10 organisms* isolated from blood cultures in 1997 at the Health Sciences Centre, Winnipeg.**

Species	# blood cultures (%)
1. Coagulase-negative staphylococci	852 (44.6)
2. *E. coli*	189 (9.9)
3. *S. aureus*	108 (5.7)
4. Viridans streptococci	100 (5.2)
5. *Candida* species	95 (5.0)
6. *Streptococcus pneumoniae*	60 (3.1)
7. *Enterococcus faecalis*	45 (2.4)
8. *Klebsiella pneumoniae*	45 (2.4)
9. *Pseudomonas aeruginosa*	38 (2.0)
10. *Propionibacterium acnes*	35 (1.8)
Other species (~60 classifications*)	342 (17.9)

*Organisms are identified either to the species level (majority) or genus level.

Not all organisms represent true infection. When isolated from blood cultures, the following organisms generally (~100%) represent true infection: *E. coli, K. pneumoniae,*

S. aureus, P. aeruginosa, S. pneumoniae, E. cloacae, Serratia marcescens, Proteus mirabilis, Hemophilus influenzae, Candida spp., and *Cryptococcus neoformans*. Others such as *Enterococcus spp, Clostridium spp* and *Bacteroides fragilis* group usually signify an infection, however a small percentage may be considered contaminants (Weinstein *et al.*, 1997). Positive blood cultures considered contaminants can often result from improper phlebotomy technique or by using central lines for blood collection. *P. acnes* and *Corynebacterium spp.*, common skin residents, are almost always considered contaminants (Weinstein *et al.*, 1997). Others such as coagulase-negative *Staphylococcus* (CoNS) and viridans streptococci were previously always considered contaminants. However, we can now assume their pathogenicity in up to 21% and 39% of bacteremic cases in immunocompromised patients, respectively (Bochud *et al.*, 1994; Bow *et al.*, 1996; Elting *et al.*, 1997).

b. Bloodstream Infections in the Immunocompromised Host.

Advances in medical therapy such as chemotherapy, bone marrow and organ transplantation have resulted in an increase in immunocompromised patients. Consequently, there is greater opportunity for microbes, particularly opportunistic pathogens, to cause disease. There has therefore been an increase in the prevalence of nosocomial infections in the US as well as in our institution (Banerjee *et al.*, 1991; Beck-Sagué *et al.*, 1993; Karlowsky *et al.*, 1997) affecting primarily individuals with prolonged neutropenia. Despite the use of broad spectrum empiric antimicrobial therapy, infections remain a major cause of morbidity and mortality in neutropenic hosts, particularly when their absolute neutrophil count falls below 0.1×10^9/L (Bow and Ronald, 1993; Nucci *et al.*, 1997; Weinstein *et al.*, 1997). Febrile neutropenic episodes occur in 80% of patients

receiving chemotherapy (Bow *et al.*, 1996) and, depending on the antibacterial prophylaxis used or cytotoxic regimen administered, up to half the febrile neutropenic patients develop an established or occult infection (Hughes *et al.*, 1997). Of these, one quarter of patients with bacteremia and most patients with fungemia develop an additional focus of infection such as pneumonia, cellulitis, soft tissue infection, and hepatosplenic candidiasis (Bow *et al.*, 1995; Elting *et al.*, 1997). Most of the infections (~90%) are caused by Gram-positive organisms, as a result of chemotherapy, the presence of intravascular catheters and the administration of prophylactic antibiotics effective primarily against Gram-negative bacteria (Bow *et al.*, 1996).

c. Infection in the Neonate and Young Infant

Febrile infants often come into the hospital showing no source of bacterial infection, most having a self-limiting viral infection. However, the prevalence of a serious bacterial infection in febrile infants younger than 90 days ranges from 1.4%-17.3% (Baraff *et al.*, 1992). A treatable infection that is not recognized can have enormous consequences. Therefore, these patients are normally hospitalized for intravenous empiric antibiotics and observed for a period of 48 to 72 hours until blood culture results are available. The majority of these cultures are negative for bacteria or fungi since the febrile episode is usually of viral origin (Jones *et al.*, 1993).

In general, the magnitude of bacteremia in young children correlates with the severity of clinical disease: patients with meningitis or epiglottitis have >100 colony-forming units (CFU)/ml whereas those with occult bacteremia have low bacterial density, <10-15 CFU/ml (Bell *et al.*, 1985; Sullivan *et al.*, 1982). The majority of isolates responsible for occult bacteremia are *S. pneumoniae* (~80%) or *H. influenzae* (~20%)

(Lieu *et al.*, 1991). The ratio appears to be reversed if the CFU/ml is >100: *H. influenzae* (73%), *N. meningitidis* (20%) and *S. pneumoniae* (7%) (Sullivan *et al.*, 1982).

It remains uncertain how to best manage infants with occult bacteremia. Multiple management strategies aiming for clinical and cost-effectiveness have been developed. However, the decision of choice is to prescribe empiric antibiotic treatment to all infants at significant risk of occult bacteremia while awaiting blood culture results (Downs *et al.*, 1991; Lieu *et al.*, 1991; Isaacman *et al.*, 1996).

d. Risk Factors for and Outcome of Infection

The most important factor contributing to a favorable outcome of infection is initiation of early appropriate empiric antimicrobial therapy. Other positive factors include monomicrobial infections, susceptibility to antimicrobial agents, early absolute neutrophil count recovery in immunocompromised patients, catheter removal if present, and subsequent modification of initial therapy following blood culture results (Elting *et al.*, 1997; Spanick *et al.*, 1997; Weinstein *et al.*, 1997).

Risks factors associated with poor outcome include septic shock, bacteremia due to certain organisms including Gram-negative bacteria, *S. pneumoniae* and fungi, polymicrobial bacteremia and organisms resistant to initial empiric antimicrobial therapy (Nucci *et al.*, 1997; Pittet *et al.*, 1997; Weinstein *et al.*, 1997; Elting *et al.*, 1997). Deep tissue infections have a worse prognosis than bloodstream infection alone (Elting *et al.*, 1997). Other factors indirectly associated with poor outcome of bacteremia include advanced age, length of hospital stay prior to acquisition of infection and malignancies or diseases of the gastrointestinal tract (Pittet *et al.*, 1997, Weinstein *et al.*, 1997).

e. Antimicrobial Therapy

Initial empiric treatment is successful in treating most infections. However, due to the possible presence of organisms resistant to the selected antimicrobials, no antibiotic regimen is ideal. A dramatic increase in acquired resistance to antibiotics has been seen in most common pathogens including *Enterobacteriaceae* (eg: *E. cloacae*, *K. pneumoniae* and *E. coli*), *P. aeruginosa*, *S. aureus*, and *Enterococcus* species (Schaberg *et al.*, 1991). Furthermore, resistance to penicillin and other antibiotics in *S. pneumoniae* has become a serious problem, particularily in children (Jacobs *et al.*, 1998; Breiman *et al.*, 1994). An attempt to minimize the increasing acquisition of resistance is possible with minimal use and prescription of antibiotics.

Antibiotics are also used as prophylaxis in patients at risk of infection. However, quinolone use in this context has been associated with the isolation of quinolone-resistant bacteria (Richard *et al.*, 1994), while increased vancomycin use has been associated with vancomycin-resistant organisms (Shay *et al.*, 1995; Spanik *et al.*, 1997). Following prophylactic treatment, ~70% of immunocompromised patients are colonized with opportunistic yeasts (Bow *et al.*, 1996; Wingard *et al.*, 1991), increasing the risk of fungal infection. Consequently, the use of prophylactic antibiotics appears to be an important factor in the occurrence of superinfections (occurring during antibiotic therapy) caused not only by resistant organisms, but by fungi as well (Nucci *et al.*, 1997, Spanik *et al.*, 1997). Antifungal prophylaxis was previously not routinely administered; however due to the serious nature of invasive fungal disease and the availability of azole antifungals, its use is being increasingly advocated in this context (Winston *et al.*, 1993; Hughes *et al.*, 1997). An increase in the empiric use of these agents has brought forth a rise in the

prevalence of non-albicans *Candida* pathogens (Nguyen *et al.*, 1996; Price *et al.*, 1994; Wingard *et al.*, 1995), as well as an increase in resistance to antifungal agents, particularly the azoles (Price *et al.*, 1994; Rex *et al.*, 1995; Nguyen *et al.*, 1996) and amphotericin B (Nguyen *et al.*, 1996).

2. The Dilemma Surrounding Blood Culture Systems

Blood culture testing, despite being the best method to identify or confirm bloodstream infections, is far from ideal. Maximal microbial recovery from blood cultures is dependent upon blood volume, which may not always be adequately acquired especially where neonates and infants are concerned. In adults, the likelihood of detection increases proportionally with the volume of blood collected, ~3% per ml (Mermel and Maki, 1993). Only 70-80% of cultures will be positive per set of blood cultures; therefore 2 or 3 blood cultures per episode (20-30 ml of blood per venipuncture) need to be collected for maximal microbial recovery (Weinstein *et al.*, 1997; Reimer *et al.*, 1997; Washington and Ilstrup, 1986). Even with a sufficient amount of sample, a negative blood culture does not exclude bacteremia, especially among patients with prior antibiotic therapy.

Collection from different sites in the body eliminates a misdiagnosis due to contamination, which is often the case when *S. epidermidis*, *Corynebacterium* species and *P. acnes* organisms are isolated. If the infection is true, all blood cultures taken in series are positive unless the infection is intermittent or short-lived. If contamination occurs, different organisms are isolated from one set to another (Weinstein *et al.*, 1997).

Anaerobic blood cultures are necessary if an infection due to anaerobic organisms, such as *B. fragilis*, are suspected. In addition, some important fastidious pathogens require special growth conditions or appropriate media that are not provided by standard blood cultures systems. Some examples are *Helicobacter pylori, Mycoplasma spp.* and nutritionally variant streptococci (Reimer *et al.* 1997). These can only be detected or identified when a request is made for identification of a special organism in correlation with clinical findings.

The presence of antibiotics can have an important negative effect on detection. The diluting effect of the blood culture media may sometimes be sufficient to eliminate the effect of antimicrobials, while components such as penicillinase can be added to a blood culture bottle, increasing the risk of contamination. The addition of sodium polyanetholesulfate (SPS), a common additive used to increase bacterial growth by inactivating complement, has been shown to inhibit the growth of many organisms including *Neisseria meningitidis, N. gonorrhoeae, Francisella tularensis* and *Moraxella catarrhalis* (Reimer *et al.*, 1997).

The determination of metabolic and morphologic characteristics for the identification of fungi may take days to weeks. Morphology is often the only means of identification with many molds, which makes the task of identification difficult, particularly if there is a lack of expertise. This often renders specific diagnosis and antifungal therapy administration decisions difficult as well. In addition, blood culture systems may fail to detect as many as 45-75% of disseminated candidiasis cases (Goodrich *et al.*, 1991; Thaler *et al.*, 1988) and most cases of invasive aspergillosis (Wald *et al.*, 1997). When a blood culture result is positive for opportunistic fungi, far too often

it is obtained just prior to death, when it is too late. Therefore, the clinical picture remains the primary diagnostic measure while the microbiology laboratory plays a secondary role in the management. Consequently, this leads to the empiric use of broad-spectrum antibiotic or antifungal therapy and the choice of treatment is speculative based on the most probable pathogen involved. Studies have shown that early appropriate antimicrobial therapy given early is associated with a lower attributable mortality rate (10.4%) than if given once blood culture results are known (25.8%) (Weinstein *et al.*, 1997).

Despite improvements made in overcoming the problems associated with blood cultures, their overall sensitivity remains poor and the time to detection and identification is too lengthy, normally ranging from 2 to 5 days for most organisms or longer for fastidious organisms. Given the advantages of early diagnosis of infection and appropriate antibiotic administration, it is of great importance to use rapid, sensitive and specific methods of bacterial and fungal identification.

3. The Potential of Molecular Diagnostics

Early detection of infection has a great impact on the successful clinical outcome of most infectious diseases. However, current diagnostic tests are not rapid, sensitive or specific enough to identify or exclude the cause of a febrile episode. While no method has yet replaced blood culture testing, molecular diagnostics is increasingly playing an important role in rapid detection and identification of pathogenic organisms in clinical samples. With the recent advent of DNA probes and nucleic amplification techniques such as the polymerase chain reaction (PCR), many situations in which the detection and

identification of certain organisms had been difficult, slow or impossible have now become easier. DNA targets can be broad-range as in the differentiation of Gram-negative organisms from Gram-positive organisms or, as they are most often, species-specific. Some examples are the use of probes for the detection of *Mycobacterium tuberculosis*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* directly from clinical specimens to obtain a more rapid diagnosis. The sensitivity of probes ($\sim 10^4$ detectable copies), however, is not as sensitive as nucleic amplification techniques, which can detect as little as 1 single copy (Persing, 1993). Furthermore, the utilization of probes is costly and can be time-consuming.

PCR assays eliminate the use of radioactive detection and permit automated analysis. By carefully choosing the appropriate oligonucleotides, one can target almost any part of a genome for DNA amplification. PCR has rendered DNA sequencing considerably easier than the traditional method of cloning the targeted gene into a plasmid for replication. One of the few drawbacks of using PCR is that the target sequence of the primers must be known in order to synthesize them. However, once the primers have been selected and the amplification conditions have been optimized, PCR is a highly sensitive and specific method of DNA amplification. More and more, DNA amplification methods are taking place in diagnostic laboratories for the detection of species specific genes or antibiotic resistance markers.

Whereas conventional blood culture methodology requires a large volume of blood, proper dilution of antibiotics, and organism viability to permit bacterial growth for detection, PCR methods require only the presence of DNA and the absence of PCR inhibitors. Therefore, using PCR methods to directly identify organisms in the blood is

not only simple, but can have a great impact on patient care and reducing overall health-care costs.

To use probes or PCR for identification of microorganisms from clinical specimens, it is important to determine which genes are appropriate for specific identification. If one only wants to determine the presence of one species, a unique gene belonging to that species is the ideal target for detection. For the purpose of detection of all bacteria and fungi in clinical specimens, one needs to target a gene common to all and, if species identification is desired, unique characteristics within that gene are necessary to differentiate between species.

There is genetic variation that exists at the molecular level in bacterial and fungal species, which offers an alternative to culturing for detection and identification of these organisms. The ribosomal genes are a primary example, demonstrating conserved sequence regions ideal for primer targeting as well as regions of variability useful for species identification. The use of amplification techniques, with subsequent probing of the amplicons with species-specific probes, has been utilized to overcome the problems of sensitivity, specificity and delay encountered with conventional methodology (Davis and Fuller, 1991; Greisen *et al.*, 1994; Holmes *et al.*, 1994; Makimura *et al.*, 1994; Davidson *et al.*, 1995; Fujita *et al.*, 1995; Polanco *et al.*, 1995; Sandhu *et al.*, 1995; Sanche *et al.*, 1996; Yamakami *et al.*, 1996; Einsele *et al.*, 1997; Hee Shin *et al.*, 1997; Van Burik *et al.*, 1998). Although these methods denote progress in the field of diagnostics, further work needs to be done to render these less laborious, costly and time consuming. These methods are highly sensitive, specific, and more rapid than conventional blood culture testing. However, a probe will only hybridize to its

corresponding target species. Therefore it is not an efficient approach for general species identification, particularly with the large variety of bacteria and opportunistic fungi.

In general, the limitations of the present molecular detection methodologies include at least two of the following: time-consuming techniques, costly use of probes, no method for the comprehensive identification of all organisms and lack of clinical studies. However, all demonstrate the highly sensitive and specific nature of PCR technology. Among the most recent advancements that have included clinical studies, Hee Shin *et al.* have developed a PCR-enzyme immunosorbent assay against the five most common *Candida* species from positive blood culture specimens of suspect *Candidemia* patients, resulting in their identification within 7 hours. Einsele *et al.* have developed a highly sensitive probe system which can detect most *Candida* and *Aspergillus* species as part of a clinical study involving febrile neutropenic patients. Van Burik *et al.* have developed a panfungal PCR assay useful as a rapid and sensitive screening method for the detection of fungal infection in bone marrow transplant patients.

Molecular diagnostic research continues to be a priority in the health-care system, as we strive to develop methods that will positively impact on patient care. In addition to being rapid, sensitive and specific, the ideal diagnostic method would identify all bacterial and fungal species in any given clinical specimen.

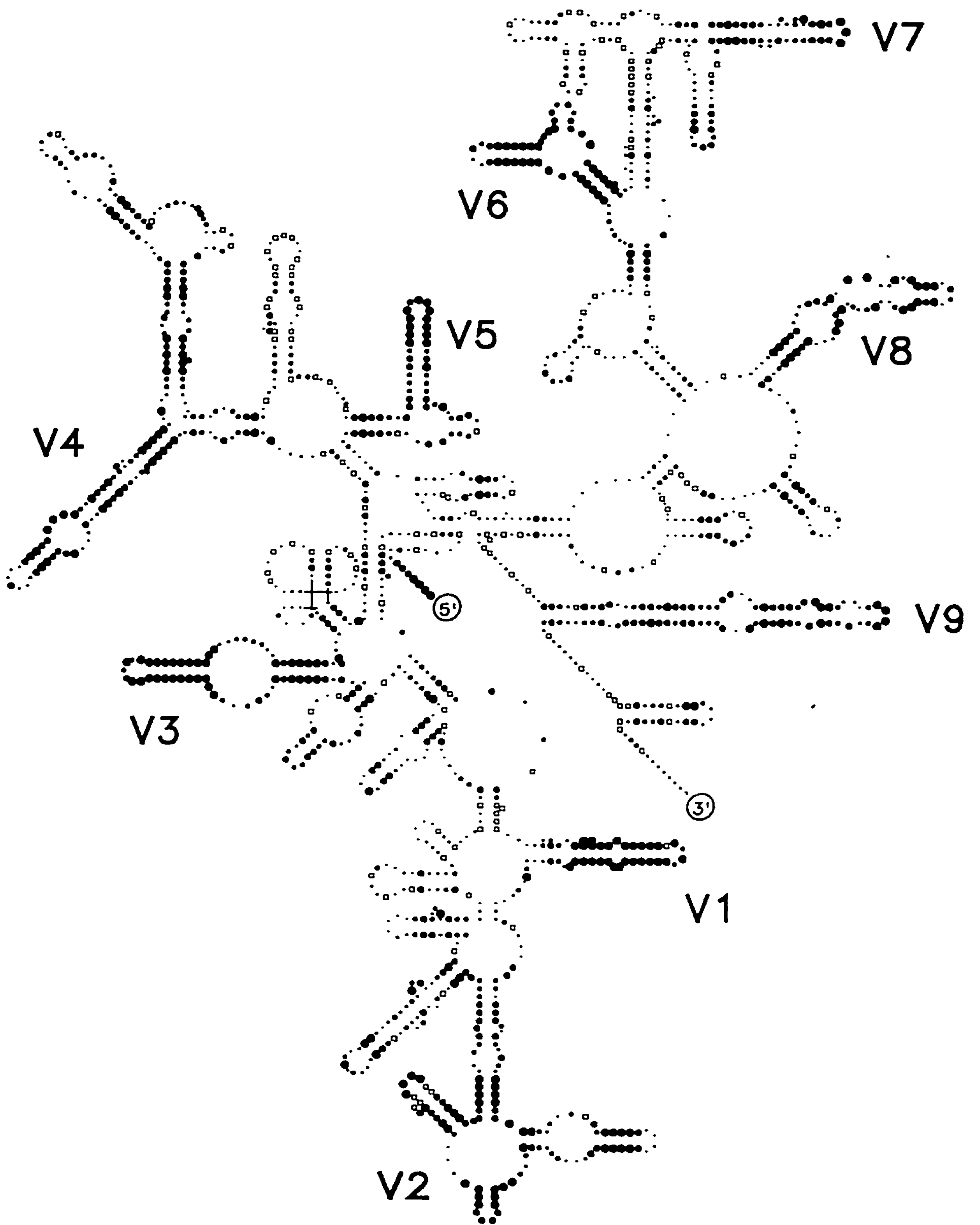
4. The Use of the 16S rRNA Gene in Bacteria for Identification

a. Evolution Tool

All prokaryotes possess the 70S ribosome which consists of 2 subunits: a large subunit of 50S and a small subunit of 30S, each of which can be broken down into ribosomal RNA (rRNA) and proteins. The large subunit of 50S consists of 31 proteins, a 23S rRNA and a 5S rRNA. The 16S rRNA, along with 21 proteins, makes up the small 30S subunit (Lewin, 1994). The role of the ribosome is to execute protein biosynthesis. *E. coli* carries approximately 19,000 ribosomes in its cellular content, which accounts for 10% of its protein content and 80% of total cellular RNA mass (Lewin, 1994).

Previously, microbiologists depended on morphology and physiology of bacteria for phylogenetic analysis and identification. However, the simplicity of these organisms can render classification difficult, especially among closely related species, biochemically inert bacteria or unculturable organisms such as the Whipple's bacillus. Bacterial geneticist Carl Woese has redone the prokaryotic phylogenetic tree on the basis of the 16S rRNA sequences comparison (Woese, 1989). Today, his concept is most widely accepted.

The utility of using the 16S rRNA gene for the purpose of bacterial identification derives from several advantages. First, the gene is generally present in 4-7 copies in the bacterial genome (Bentley and Leigh, 1995), depending on the species. Composed of approximately 1,500 base pairs (bp), the gene comprises regions of conserved sequences that are similar among bacteria and regions of variability termed signature sequences, which serve to differentiate at the species level. The sequence of the 16S rRNA gene acquires mutations slowly over time and is therefore considered a stable property (Woese,



[a]Reproduced from Neefs *et al.*, 1993.

Figure 1. **Secondary structure model for bacterial 16S ribosomal RNA[a].** Each nucleotide site is divided into five categories of variability, indicated by full circles of increasing diameter corresponding to increasing variability. Conserved sites are indicated as hollow squares. Clusters of hypervariable sites are designated areas V1 to V9.

1989). The 16S rRNA molecule has many regions of self-complementarity that are capable of forming double-helical regions. In general, all species adopt the same basic secondary structure of the 16S rRNA (Figure 1). It seems the secondary structure is more highly conserved than the primary structure because, most often, when mutations do occur in the double-stranded region, they are compensatory to maintain this structure.

b. Single-Stranded Conformation Polymorphism

Single-Stranded Conformation Polymorphism (SSCP) is a relatively recent method developed in 1989 (Orita *et al.*, 1989). This method is now commonly used as a tool for mutation detection for many genes amplified by PCR. The principle of SSCP is based on DNA sequence variation, which has an effect on the molecule's conformation under non-denaturing conditions. The method of PCR-SSCP begins with amplification of a known DNA segment. The PCR product is then heated for strand separation and analyzed by electrophoresis on a non-denaturing gel. Using a non-denaturing gel permits the single-stranded DNA fragments to adopt a certain conformation based on the DNA sequence and maintain this configuration throughout electrophoresis. This causes variation in electrophoretic mobility between PCR products of different sequences, regardless of the fact that the fragments may be of the same size. One cannot predict how mobility is affected in relation to the secondary structure, only that it differs among fragments of unique primary sequence.

The method of SSCP is very sensitive to many variables, and optimization of factors and conditions is important for each purpose (Hayashi and Yandell, 1993). Under the best conditions, a difference of one bp between two fragments can be detected. A compilation of SSCP experiments have demonstrated that more than 90% of sequence

variations of fragments 200 bp or smaller are detectable, whereas mutations will only be detected in 80% of 300-350 bp fragments. Better results are usually obtained by: 1) lower temperatures during electrophoresis, 2) the addition of glycerol in the buffer and polymer, and 3) an increase in gel concentration (Glavac and Dean, 1993; Iwahana *et al.*, 1994; Hebenbrock *et al.*, 1995).

Sequencing of the 16S rRNA gene of an organism for the purpose of identification holds great promise in its accuracy although, at present, it isn't recommended for batch identification due to cost and time constraints. In theory, since each bacterial species has a unique 16S rRNA sequence, all organisms can be differentiated from each other using PCR-SSCP. As SSCP is normally used for mutation detection, any variation in mobility from the "normal" PCR product (wild type vs. mutant) is satisfactory. For this method to be used as a diagnostic tool in microbiology, patterns of mobility need to be defined and therefore an internal standard is necessary for correct SSCP pattern determination. Widjojoatmodjo *et al.* have previously shown this method as being a promising option for use in molecular diagnostics. They have performed SSCP on 16S rDNA PCR amplified fragments using an automated slab gel sequencing system and, with few exceptions, have obtained a different peak pattern in 47 bacterial species spanning a broad range of Gram-negative and Gram-positive organisms of clinical interest. With the advent of capillary electrophoresis, this method may possibly be used in a time- and cost-effective manner for the identification of microorganisms in clinical samples by using the variability of the 16S rDNA sequence to obtain different peak patterns.

5. The Use of the ITS2 Region in Fungi for Identification

a. Evolution Tool

Eukaryotes, including all fungi, possess the 80S ribosome, which is larger and more complex than its prokaryotic counterpart while maintaining basically the same function and structure. The eukaryotic ribosome contains the 18S rRNA and 33 proteins in its small subunit of 40S and the 28S, 5.8S and 5S rRNAs with 49 proteins in its large subunit of 60S (Lewin, 1994). The ribosomal genes are found in copy tandem repeats of 50-100 in *C. albicans* on chromosome seven (Magee *et al.*, 1987; Thrash-Bingham and Gorman, 1992). Like bacterial ribosomal genes, PCR primers can target conserved regions of fungal rRNA genes, amplifying parts of the genes or the non-coding regions in between (White *et al.*, 1990). Sequence comparisons of these genes and intergenic regions have been useful in phylogenetic analyses of a variety of fungal groups. The intergenic transcribed spacer (ITS) regions evolve at a faster pace than the coding regions and therefore demonstrate a higher level of sequence variability among species in comparison with the ribosomal genes (James *et al.*, 1996). Not only is interspecies variability manifested in the sequence but, unlike the ribosomal genes themselves, ITS1 and 2 regions show also substantial variation in sequence length among different species (Lott *et al.*, 1993; Williams *et al.*, 1995).

The ITS1 region, situated between the 18S and 5.8S rRNA genes, has shown instances of intraspecies variation in *C. parapsilosis* and *Leptosphearia maculans* whereas the ITS2 region is more stable and shows little intraspecies variability (Morales *et al.*, 1993; Lin *et al.*, 1995). Lott *et al.* have utilized the sequence variability of the ITS2 region between the 5.8S and 28S rRNA genes of a variety of *Candida* species for

phylogenetic analyses in the hopes of characterizing species-specific sequences in *Candida* species for use as diagnostic probes. They have not encountered instances of intraspecies variability among *C. albicans* (10 strains) and *C. parapsilosis* (5 strains).

b. Fragment Length Analysis

Utilizing the apparent variability in length of the ITS2 region to make specific diagnosis of pathogenic fungal isolates appears very promising. PCR products can be detected and sized in a number of ways, the most employed method being by ethidium bromide agarose gel electrophoresis along with a known size marker in an adjacent lane. Even though a higher agarose concentration allows for better fragment separation, the size differences between some of the amplicons from different species can be quite small, making separation by agarose gel electrophoresis difficult. Precise fragment length determination can be obtained by polyacrylamide gel electrophoresis, yet this method is time consuming and lengthy. A more rapid method includes making use of an automated capillary electrophoresis sequencer in which the ITS2 regions amplified with fluorescent primers are run under denaturing conditions for accurate fragment size determination. The same capillary electrophoresis system can be used for SSCP pattern determination of bacterial 16S rDNA gene fragments amplified with fluorescent primers and run using a non-denaturing polymer.

4. Thesis Objective

With the overall goal of better patient care, the ultimate objective of this thesis was to be able to rapidly identify pathogenic organisms directly from sterile fluids such as blood, using the 16S rRNA gene for bacteria or the ITS2 genetic region for fungi. To accomplish this, specific objectives were devised as follows:

- To explore the utility of the interspecies sequence differences of the 16S rRNA gene for the rapid identification of all bacteria using SSCP.
- To explore the utility of the interspecies length variation of the ITS2 region for the identification of fungi.
- To determine the sensitivity and specificity of the PCR protocols developed for the universal detection of these genes from bacteria and fungi
- To determine the specificity of the developed methods of SSCP, sequencing (with the 16S rRNA gene) and fragment analysis (with the ITS2 region) to directly identify bacterial or fungal species from blood cultures.
- To compare time and cost-effectiveness of the molecular methods developed with conventional methodology.

B. MATERIALS AND METHODS

1. Bacterial Preparation

a. Strains

For preliminary testing of specificity and sensitivity of the PCR methods developed, reference strains of *E. coli* (ATCC 25922), *S. epidermidis* (ATCC 12228) or *S. aureus* (ATCC 25923) were used. For the determination of SSCP patterns of the most common blood culture isolates, ATCC strains or CAP (College of American Pathologists) strains were used for controls, as well as previously identified blood culture isolates which had been stored in skim milk at –80°C. All stains were subcultured onto Trypticase soy-5% sheep blood agar and incubated at 37°C for 24 hours prior to DNA extraction. Biochemical testing of all blood culture isolates was determined by conventional manual and automated methods. Manual methods included biochemical testing as suggested in the Manual of Clinical Microbiology from the American Society for Microbiology (Ferraro *et al.*, 1995). Automated analysis consisted of using Dade MicroScan® (West Sacremento, CA) panels for Gram-negative bacilli and Gram-positive cocci.

The patient specimens used for the molecular identification of bacteria using SSCP were all blood cultures that became positive from December 8, 1997, to March 30, 1998. Our laboratory uses the BacT/Alert blood culture system (Organon Teknika Corp., Durham, NC) which is a continuous monitoring, colorimetric CO_2 microbial growth detection system. As part of the routine identification protocol, a Gram-stain was performed on all blood cultures that became positive. The only information shared between conventional and molecular identification was whether the organism present

were bacterial and/or fungal in nature (Figure 2). The hospital clinical laboratory technologist proceeded with conventional testing and the final report was made known to the researcher only after all specimens had been identified by molecular methods.

b. DNA Isolation

***α*. Extraction from Culture Colonies**

The isolation of DNA for PCR amplification of known Gram-positive organisms was performed as follows: one or two colonies were taken from a 24 hour subculture, suspended in 1 ml of saline and centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in 300 μl of lysis solution (0.1M NaOH, 2M NaCl, 0.5% sodium dodecyl sulfate (SDS)) and boiled for 15 minutes. After cooling, 200 μl of 0.1M Tris-HCl (pH 8.0) was added. Extraction of genomic DNA was done by the addition of 500 μl of cold phenol-chloroform-isoamyl alcohol in a 25:24:1 ratio followed by centrifugation at 13,000 rpm for 10 minutes. The aqueous (top) layer was transferred into a new tube and 1 ml of cold 100% ethanol was added for DNA precipitation at –80°C for a minimum of 30 minutes followed by centrifugation at 4°C for 15 minutes at 13,000 rpm. The supernatant was removed and the pellet was air-dried. The DNA pellet was resuspended in 30 μl of sterile distilled H_2O. The lysate can be used as DNA template for amplification after 30 minutes or stored at –20°C for future use. Routinely, 10 μl of the lysate was used for PCR.

For the DNA extraction of known Gram-negative organisms, the above method could be used as well. However, a more rapid method was available, and was used in this study. One or two colonies from a 24 hours subculture were suspended in 100 μl of distilled H_2O. The suspension was then heated at 95°C for 5 minutes, then cooled.

Routinely, 10 µl of this lysate was used for PCR. The lysates were then stored at –20°C for future use. All DNA extraction procedures, as well as PCR preparations, were performed using positive-displacement pipettes to minimize sample to sample contamination.

β. Extraction from Whole Blood

Whole blood specimens were collected in tubes with sodium citrate or ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The blood was mixed, and 1-2 ml was removed and added to a sterile glass tubes (for better visualization after centrifugation). The specimen was centrifuged at 700 rpm for 5 minutes and the supernatant (plasma) was carefully removed and added to a 1.5 ml centrifuge tube. The supernatant was centrifuged at 13,000 rpm for 10 minutes while the red blood cells were discarded. The supernatant was discarded and the extraction proceeded as above by adding 300 µl of lysis solution. This method was used for both Gram-positive organisms as well as Gram-negative organisms.

γ. Extraction from Blood Cultures

The protocol used for the isolation of bacterial DNA from blood cultures was provided by Dr. Fredricks, Stanford University, Stanford, CA (personal communication, 3) with slight modification. One hundred microlitres of blood culture specimen was added to 100 µl of lysis buffer (5M guanidine hydrochloride, 100 mM Tris-pH 8.0) and 400 µl of H_2O. The mix was vortexed for 15 seconds and 800 µl of benzyl alcohol was then added. The following step, being the most crucial as it successfully removes the SPS found in blood culture media, consists of mixing the sample end over end ~500 times (~5 minutes). The mix was then centrifuged 13,000 rpm for 10 minutes and the

aqueous (top) layer was transferred to another 1.5 ml tube. Forty microlitres of 3M sodium acetate and 440 μl of isopropanol was added and mixed briefly. The sample was then centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the precipitated DNA was washed with 1 ml of 70% alcohol, followed by centrifugation at 13,000 rpm, for 15 minutes, at 4°C. The supernatant was removed and the pellet air-dried for 30 minutes, followed by resuspension in 100 μl of H_2O. Routinely, 10 μl was used for PCR.

c. Polymerase Chain Reaction

PCR amplification was used for a variety of purposes, such as for detection, SSCP or sequencing, employing different primer sets for the amplification of different parts of the 16S rRNA gene. The use of non-labeled primers served for the purpose of PCR product detection on ethidium bromide stained agarose gel or for sequencing template. The PCR amplification of the 16S rRNA gene using fluorescently-tagged primers was used for SSCP analysis with the ABI PRISM 310™ Genetic Analyzer. Table 2 indicates all universal primers used in our PCR or sequencing reactions, all of which can be fluorescent, non-fluorescent, or used for sequencing.

α. PCR Protocol Using Non-labeled Primers

A 50 μl PCR reaction mixture contained 10 μl of DNA template, 5 μl of 25 mM $MgCl_2$-10X PCR buffer, 1.25 mM each dCTP, dGTP, dATP and dUTP:dTTP in a 8:1 ratio, 0.5 μl of 100 mM each primer, 0.5U of uracil DNA glycosylase (UDG) (Life Technologies, Burlington, ON), 2.5U of Taq DNA polymerase (Pharmacia Biotech, Baie d'Urfé, Québec) and 25 μl of sterile distilled H_2O. The PCR reaction was performed using a Perkin-Elmer GeneAmp™ PCR System 9600 with cycles of 37°C for 10 minutes

for UDG activation, 95°C for 10 minutes for UDG inactivation and template denaturation, 30 cycles of 95°C, 55°C and 72°C for 1 minute each and incubated at 72°C for 10 minutes for final extention.

Table 2. Universal 16S rDNA primers for PCR

Primer	Primer Sequences	Location[a]	Reference
8FPL(f)[b]	5'-agt ttg atc ctg gct cag-3'	8-27	Eden *et al.*, 1991
515(f)	5'-tgc cag cag ccg cgg taa-3'	515-533	Relman *et al.*, 1992
91E(f)	5'-tca aa(t/g) gaa ttg acg ggg gc-3'	911-930	Relman *et al.*, 1992
RW01(f)	5'-aac tgg agg aag gtg ggg at-3'	1170-1189	Greisen *et al.*, 1994
806R(r)[c]	5'-gga cta cca ggg tat cta at-3'	806-787	Relman *et al.*, 1992
13B(r)	5'-agg ccc ggg aac gta ttc ac-3'	1390-1371	Relman *et al.*, 1990
DG74(r)	5'-agg agg tga tcc aac cgc a-3'	1522-1540	Greisen *et al.*, 1994

[a]*E. coli* 16S rRNA position (Brosius *et al.*, 1978)
[b](f): primer annealing resulting in DNA extension in the forward (5'-3') direction
[c](r): primer annealing resulting in DNA extension of the complementary strand: reverse (3'-5') direction.

β. Multiplex PCR Protocol Using Fluorescent Primers

A 50 µl PCR reaction mixture contained 10 µl of DNA template, 5 µl of 25 mM $MgCl_2$-10X PCR buffer, 1.25 mM each dCTP, dGTP, dATP and dTTP, 0.5 µl of 100 mM each fluorescent primer (5'HEX-13B, 6-FAM-RW01, 5'HEX-806R and 6-FAM-515) (Life technologies), 2.5U of Taq DNA polymerase (Pharmacia Biotech) and 25.5 µl of sterile distilled H_2O. The PCR reaction was performed using a Perkin-Elmer GeneAmp™ PCR System 9600 with a cycle of 95°C for 5 minutes, 30 cycles of 95°C, 55°C and 72°C for 1 minute each and incubated at 72°C for 10 minutes for final extension.

γ. Primer Specificity

The specificity of the universal 16S rRNA gene primers was determined by PCR amplification using the 806R/8FPL primer pair (non-fluorescent) with human genomic DNA (1 ng and 10 pg) and *C. albicans* (1 ng and 10 pg). This reaction was performed in parallel with variable concentrations of *E. coli* genomic DNA (1 ng, 10 pg, 1 pg, 10 fg), and with a routine concentration of *E. coli* DNA (having used 1-2 colonies for the DNA lysate preparation) for positive control. Conditions of the PCR reaction were as described in section 1. c. α.

δ. Primer Sensitivity

Suspensions of 10^4, 10^3, 10^2, and 10 cfu/ml *E. coli* ATCC 25922 organisms were made in whole blood, of which 1 ml was used for DNA extraction as described in section 1. b. β. Conditions of the PCR reaction using the 806R/8FPL primer pair were as described in section 1. c. α.

ε. Prolonged Sample Storage

The effect of prolonged storage was determined by preparing individual suspensions of 10^2 cfu/ml of *E. coli* and *S. epidermidis* in 1 ml whole blood. The samples were stored at 4°C for 1, 10, 14 and 21 days. The viable colony count was determined on day 0 and on the days the PCR lysates were prepared for PCR amplification using the extraction method from whole blood described in section 1. b. β. Conditions of the PCR reaction using the 806R/8FPL primer pair were as described in section 1. c. α.

ζ. Addition of Antibiotics

For each antibiotic tested, serial dilutions of *S. epidermidis* and *E. coli* ranging from 10^8 to 10^1 cfu/ml was prepared in 1 ml whole blood and processed for DNA

extraction from whole blood described in section 1. b. β. following the addition of antibiotics. The levels of protein-bound antibiotics, which were added to each dilution, corresponded to normal levels found in patients following antibiotic treatment: 1.5 μg/ml of gentamicin, 4 μg/ml of cephalexin, 0.2 μg/ml of ciprofloxacin, 7.5 μg/ml of ceftriaxone, 2 μg/ml of tetracycline, 0.25 μg/ml of clindamycin and 0.25 μg of erythromycin. Conditions of the PCR reaction using the 806R/8FPL primer pair were as described in section 1. c. α.

d. PCR Product Detection and Identification

α. Agarose Gel Electrophoresis

Detection of PCR amplified product was performed by electrophoresis on a 1-2% agarose gel made with 0.5X TBE buffer and stained with ethidium bromide. A volume of 20 μl of PCR product and 2.2 μl of Ficoll dye was loaded in each lane. A 1 kilobase (kb), 100 bp or a 123 bp ladder was also run in parallel for approximate PCR product band sizing. Electrophoresis conditions were 100 volts for 45 minutes in 0.5X TBE buffer.

β. Southern Hybridization Protocol

i. PCR amplification

PCR amplification of *E. coli* was performed as described above using the universal non- fluorescent DG74/RW01 primer pair. A 1% agarose gel was prepared and 20 μl of sample containing PCR product of varying amounts (20 μl, 10 μl, 5 μl, 4 μl, 3 μl, 2 μl and 1 μl) diluted in H_2O was added to each lane.

ii. Transfer of DNA to membrane

To prepare the gel for hybridization, the PCR product in the gel is cross-linked in UV light for 5 minutes. The gel is washed three times for 20 minutes in a solution of

0.5N NaOH/1.5M NaCl, followed by a rinse in distilled H_2O for 3 minutes. The gel is then washed twice for 20 minutes in a solution of 0.5M Tris/1.5M NaCl, then rinsed again in distilled H_2O. The transfer of DNA from the gel onto a nylon membrane (Boehringer Mannheim, Indianapolis, IN) was set up in 1 l of 10X SSC (1:2 dilution in distilled H_2O of 20X SSC stock: 3 M NaCl, 0.3 M sodium citrate, pH adjusted to 7.0 with HCl). Two strips of 3MM paper were placed across a glass plate set over a dish containing the 10X SSC solution in which the ends of the paper immerged. The paper was then soaked with the 10X SSC solution. The following stack, cut into the size of the gel, was assembled, with each piece soaked and bubbles removed between layers: three pieces of 3 MM paper, the gel, the nylon membrane, a "frame" of parafilm pieces draping over the 3 MM paper and three more pieces of 3 MM paper. Finally, a stack of paper towels was placed on top and compressed using a heavy weight (eg.: book). A plastic cover was used to prevent evaporation and the transfer was left overnight.

The membrane is removed from the transfer and immediately cross-linked with UV lighting for 3 minutes. The membrane was then soaked briefly into 2X SSC to reduce salt content and baked in an 80°C incubator for 2 hours.

iii. Preparation of Digoxigenin Labeled Probes

Probe synthesis of RDR245 and RDR278 (100 μM) (Table 3) was performed on a on a Beckman Oligo 1000 DNA Synthesizer (Beckman). Probes were labeled with Digoxigenin-11-dUTP using the Genius 6 Oligonucleotide Tailing Kit (Boehringer Mannheim). Basically, the following order of reagents was added to a sterile microfuge tube: 5X reaction buffer (4 μl), $CoCl_2$ (4 μl), DIG-dUTP (1 μl), probe (1:5) (5 μl), dATP (1 μl), terminal transferase (1 μl), and H_2O (4 μl). The mix was incubated at 37°C for 15

minutes, then put in ice. One microliter of 200 µM EDTA was added to terminate the labeling reaction. The probes, which have been used for Southern hybridization and for dotblot analysis, are described in Table 3.

Table 3. PCR probes for the detection of bacteria.

Probe	Sequence	Position	Reference
RDR245[a]	5'-gta caa ggc ccg gga acg tat tca ccg-3'	1369-1395	Greisen *et al.*, 1994
RDR278[b]	5'-gac gta agg gcc atg agg act tga cgt c-3'	1190-1217	Greisen *et al.*, 1994

[a]Universal 16S rDNA bacterial probe
[b]Gram-negative 16S rDNA probe

iv. Prehybridization

The membrane was placed in a plastic bag in which 20 ml of standard buffer was added (distilled H_2O, 12.88 ml; 20X SSC, 5 ml; 20% sarcosyl, 0.1 ml; 20% SDS, 0.02 ml; 10% blocking reagent, 2 ml). The bag was sealed and incubated for 1 hour at 60°C, equal to the subsequent hybridization temperature.

v. Hybridization

Following prehybridization, the buffer was poured out of the bag. The hybridization buffer containing the DIG-labeled probe was added and the bag was sealed. The blot was incubated at 60°C for 1 hour to allow probe hybridization. All subsequent washes were performed on a rotator. The membrane was washed twice for 5 minutes each in 2X wash solution (distilled H_2O, 89.5 ml; 20X SSC, 10 ml; 20% SDS, 0.5 ml), then washed twice in 0.5X wash solution (distilled H_2O, 97 ml; 20X SSC, 2.5 ml; 20% SDS, 0.5 ml), 5 minutes each. The membrane was washed for 2 minutes in washing buffer, then incubated in blocking solution for 45 minutes prior to detection.

vi. Detection

Five microlitres of anti-DIG-AP was added (1:10 000 dilution) and the blot was incubated for 30 minutes. The membrane was washed twice, 20 minutes each in washing buffer, then equilibrated for 2 minutes in detection buffer. A small amount of dilute CSPD (1:100), enough to cover the membrane, was added and incubated for 5 minutes. The excess was drained off, the membrane was sealed in a hybridization bag and incubated at 37°C for 10 minutes. Exposure to an X-ray film may varied from 15-30 minutes or longer.

γ. Dotblot Analysis

i. Universal 16S rDNA Probe Detection

PCR amplification of *E. coli* was performed using the DG74/RW01 primer pair as described in section 1. c. α. The PCR product was then processed as follows:

Mix:				
	a)	10 µl PCR product	6.6 µl (1 M NaOH)	0.5 µl (200 mM EDTA)
	b)	5	3.3	0.26
	c)	4	2.6	0.21
	d)	3	2	0.15
	e)	2	1.3	0.1
	f)	1	0.66	0.05

Each mix was then boiled 15 minutes for DNA denaturation and immediately placed on ice. A nylon membrane was prepared and soaked in H_2O for 10 minutes. A manifold was assembled with the membrane on top of a filter paper. Five hundred microlitres of H_2O was washed through the wells and samples were applied in various amounts of each mix: 5, 4, 3, 2, and 1 µl. Excess was rinsed with 500 µl of 0.4 M NaOH and the manifold was then taken apart. The membrane was rinsed in 2X SSC for 2 minutes then air-dried and baked at 80°C for 30 minutes. Hybridization and detection

procedures were similar to the Southern hybridization protocol described above in sections 1. d. β. iv-vi. The DIG-labeled probe RDR245 was used for detection.

ii. Gram-Negative Bacteria Probe Detection

PCR amplification of *E. coli* and *S. aureus* using the DG74/RW01 primer pair was performed as described in section 1. c. α. The PCR product was then processed as follows for each organism:

Mix:	5 µl (PCR product)	3.3µl (1 M NaOH)	0.26 µl (200 mM EDTA)
	3 µl	2 µl	0.15

Subsequent steps were performed as described above. Hybridization temperatures were tested at 62°C and 65°C. The DIG-labeled probe RDR278 was used for detection.

δ. SSCP of 16S rRNA Genes

The multiplex PCR reaction with fluorescent primer pairs 515/806R and RW01/13B was preformed as described in section 1. c. β. The PCR product was diluted 1:10 in $T_{10}E_1$ buffer of which 1 µl was added to 11.5 µl of SSCP mix (10.5 µl formamide, 0.5 µl of 3N NaOH, and 0.5 µl of GeneScan®-500 (ROX) Size Standard (Applied Biosystems Division/Perkin-Elmer, Foster City, CA). The samples were heated at 95°C for 2 minutes for denaturation and immediately cooled on ice prior to loading on the ABI PRISM 310 Genetic Analyzer. Subsequent preparation was performed in accordance to the manufacturer's instructions (PE Applied Biosystems). The non-denaturing 4% SSCP polymer (2.85 ml of 7% GeneScan Polymer (PE Applied Biosystems), 500 µl 10X TBE, 500 g glycerol, and taken to 5 ml with distilled H_2O) was used as the replaceable polymer matrix. The anode/cathode buffer was made of 10% glycerol and 1X TBE. Electrophoresis conditions were 13 kV at 30°C.

ε. Sequencing of the 16S rRNA Gene

i. Sequencing Reaction and Analysis

PCR amplification was performed using non-fluorescent universal 16S rDNA gene primers 91E and 13B. Sequencing reactions were performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The primer used for the sequencing reaction was 91E or 13B diluted 1:100 in Tris-EDTA buffer. A 20 µl sequencing reaction mix contained 8 µl of Reaction Mix (PE Applied Biosystems), 3.2 µl of one primer, 5 µl of purified PCR product and 3.8 µl H_2O. This method is based on the use of ddNTP terminators, of which each C, A, T or G is labeled with a different dye: blue, green, red and black, respectively. Cycle sequencing was performed on the GeneAmp PCR System 9600 and analysis was performed with the ABI PRISM™ 310 Genetic Analyzer and the Sequence Analysis software in accordance with the manufacturers' instruction.

ii. Basic Local Alignment Search Tool (BLAST)

Each resulting sequence obtained from the 310 Sequencing Software was entered into an internet program, BLAST Search, for identical matching with submitted sequences from GenBank, EMBL, DDBJ, and PDB databases. This program can be accessed from the NCBI homepage: http://www.ncbi.nlm.nih.gov. Sequence alignment comparisons are then given with the highest probability most likely to be the identification of the organism sequenced, as long as its sequence has previously been entered into one of the mentioned databanks.

2. Fungi Preparation

a. Strains

For the testing of primer sensitivity and specificity, a reference strain of *C. albicans* (ATCC 10231) was used. For the determination of the ITS2 PCR fragment length, a wide collection of reference strains, ATCC or CAP specimens were used, as well as strains obtained from the Health Sciences Centre Microbiology Laboratory stock culture collection. Yeast organisms were grown on Sabouraud dextrose agar plates (BBL, Becton-Dickinson, Cockeysville, Md.) for 24 hours at 37°C and molds were grown on potato dextrose agar (McGinnis, 1980) for up to 7 days. Species identification was established using the API 20C kit (BioMerieux, Hazelwood, Mo.) or by conventional morphological analysis as previously described (Larone, 1995).

b. DNA Isolation

α. Extraction from Culture Colonies

Mold scrapings or 1-2 yeast colonies were suspended in 1 ml of $T_{10}E_1$ buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) followed by centrifugation at 13,000 RPM for 5 min. The pellet was resuspended in 200 µl of 50 mM NaOH, vortexed and incubated at 95°C for 10 min. The mixture was then neutralized with 200 µl of 0.1 mM Tris-HCL (pH 7.0) and centrifuged 5 min at 13,000 rpm. The pellet was resuspended in 500 µl sterile H_20 and centrifuged at 13,000 rpm for 5 min. The supernatant was removed, followed by the addition of 200 ml of Yeast Cracking Buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 20 mM TRIS pH 8.0, 10 mM EDTA pH 8.0). Extraction of genomic DNA was achieved by addition of glass beads to 3/4 of the liquid volume followed by the addition of 200 ml of cold phenol-chloroform-isoamyl alcohol in a 25:24:1 ratio. The mix was

subjected to constant vortexing for 30 min. The sample was then centrifuged at 13,000 rpm for 5 min. The aqueous (top) layer was transferred into a new tube and 1 ml of cold (-20°C) 100% alcohol was added for DNA precipitation. The sample was centrifuged at 13,000 rpm for 2 minutes. The supernatant was removed and the pellet was resuspended in 400 µl of $T_{10}E_1$ buffer and 30 µg of RNase A (Sigma, St. Louis, Mo.) for a one hour waterbath incubation at 35°C. We then added 10 µl of 3 M sodium acetate and 1000 µl of cold 100% alcohol. The sample was centrifuged at 13,000 rpm for 2 min., the supernatant removed, and the pellet air-dried. The DNA pellet was resuspended in 50 µl of TE buffer. The lysate can be used as DNA template for amplification after 30 min. or stored at -20°C for future use.

β. Simplified Extraction Method for Yeast

The previous method, which can be used with all fungi we have tested, is necessary for the extraction of DNA from certain molds such as *Aspergillus terreus*, *Penicillium sp.*, *Paecilomyces sp.* and *Sporothrix schenckii*. Steps can be omitted or reduced in time if the organism is known to be a yeast: resuspension and incubation of the fungal pellet in 50 mM NaOH is not necessary; the 30 minutes vortexing step with glass beads can be reduced to 3 times for 30 seconds and held on ice for short periods in between; and the RNase A incubation period is only 5 minutes.

γ. Extraction from Whole Blood

Whole blood specimens were collected in tubes with sodium citrate or EDTA as an anticoagulant. The blood was mixed, and 1 ml was removed and added to a sterile glass tube for better visualization. If required, the blood specimen was seeded with yeast at this point. The specimen was centrifuged at 700 rpm for 5 minutes and the supernatant

(plasma) was carefully removed and added to a 1.5 ml centrifuge tube. The red blood cells were discarded and the supernatant was centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the extraction proceeded as in section 2. b. α/β. starting with the addition of 200 μl of Yeast Cracking Buffer.

δ. Extraction from Blood Cultures

The extraction of fungal DNA from blood culture specimens was performed as described for bacteria in section 1. b. γ.

c. PCR Amplification

α. PCR Protocol

The following protocol was used for all PCR reactions performed with fungi in this thesis. The primers used for universal fungal amplification were ITS4 (reverse primer [5'-tcc tcc gct tat tga tag c-3']) obtained from White *et al.* and ITS86 (forward primer [5'-gtg aat cat cga atc ttt gaa c-3']) (Life Technologies) derived from sequence comparison of various fungi from GenBank databases using the PCGene software (University of Geneva, Geneva, Switzerland). Primer ITS86 was fluorescently labeled with a phosphoramidite dye: 5'HEX (green). The 50 μl PCR reaction mixture contained 5 μl of DNA template, 5 μl of 25 mM MgCl2-10X PCR buffer, 1.25 mM deoxynucleoside triphosphate; dATP, dGTP, dCTP and a 8:1 ratio of dUTP:dTTP, 0.5 μl of 100 mM each primer, 0.5U of UDG (Gibco BRL), 2.5U of Taq DNA polymerase (Pharmacia Biotech) and 30 ml of sterile distilled H_2O. The PCR reaction was performed in a Perkin-Elmer GeneAmp PCR System 9600 (PE Applied Biosystems) with cycles of 37°C for 10 min (UDG activation), 94°C for 10 min (UDG inactivation), 30 cycles of 94°C, 55°C and 72°C for 1 minute each and incubated at 72°C for 10 min for final

extension. Amplification of all fungi tested using these primers yielded fragments of 200-500 bp in length. When determining the presence and identification of fungi from blood culture specimens, the UDG was omitted from the PCR reaction. On that account, we could use the same PCR conditions as for the multiplex PCR amplification of bacteria described in section 1. c. β. and therefore, the same thermocycler.

β. Primer Specificity

A variety or bacterial organisms were chosen to determine the specificity of the fungal primers: *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853) and *C. perfringens* (ATCC 13124). The primers were also tested against genomic DNA deriving from human whole blood, concentrated leukocytes and liver tissue. Conditions of the PCR reaction were as described above in section 2. c. β.

γ. Primer Sensitivity

The determination of the sensitivity of detection of *C. albicans* organisms in whole blood collected in EDTA tubes was as follows: for each *C. albicans* concentrations (10^7 to 1 cfu/ml), 1 ml of blood was transferred into a glass centrifuge tubes and centrifuged at 700 RPM for 5 min. The plasma was transferred to a 1.5 ml plastic tube and centrifuged at 13,000 RPM for 5 min and DNA extraction from whole blood was performed as described in section 2. b. γ.

d. PCR Product Detection and Identification

α. Agarose Gel Electrophoresis

Detection of PCR amplified product was performed by electrophoresis on a 2% agarose gel stained with ethidium bromide. A volume of 20 μl of PCR product and 2.2 μl

of Ficoll dye was loaded in each lane. Electrophoretic conditions were 100 volts for 45 min in 0.5 X TBE buffer. A 123 bp ladder was also run in parallel for approximate PCR product band sizing.

β. Southern Hybridization Protocol

PCR amplification of various *Candida* species was performed as described above. A *C. albicans* species-specific probe (5'acc gct ggg ttt ggt gtt ga 3'), targeted towards the 5' end adjacent to the 5.8S rRNA gene, was synthesized and DIG-labeled as performed for bacterial probes in section 1. d. β. This probe was developed based on sequence comparisons of the ITS2 region in common *Candida* species (Lott *et al.*, 1993). The PCR product was run on an agarose gel and subsequently transferred onto a nylon membrane as previously described. Prehybridization and hybridization was performed as described in section 1. d. β., both incubation times being 1 hour at 65°C. The detection of *C. albicans* was performed as described in section 1. d. β.

γ. Fragment Analysis of ITS2 Regions

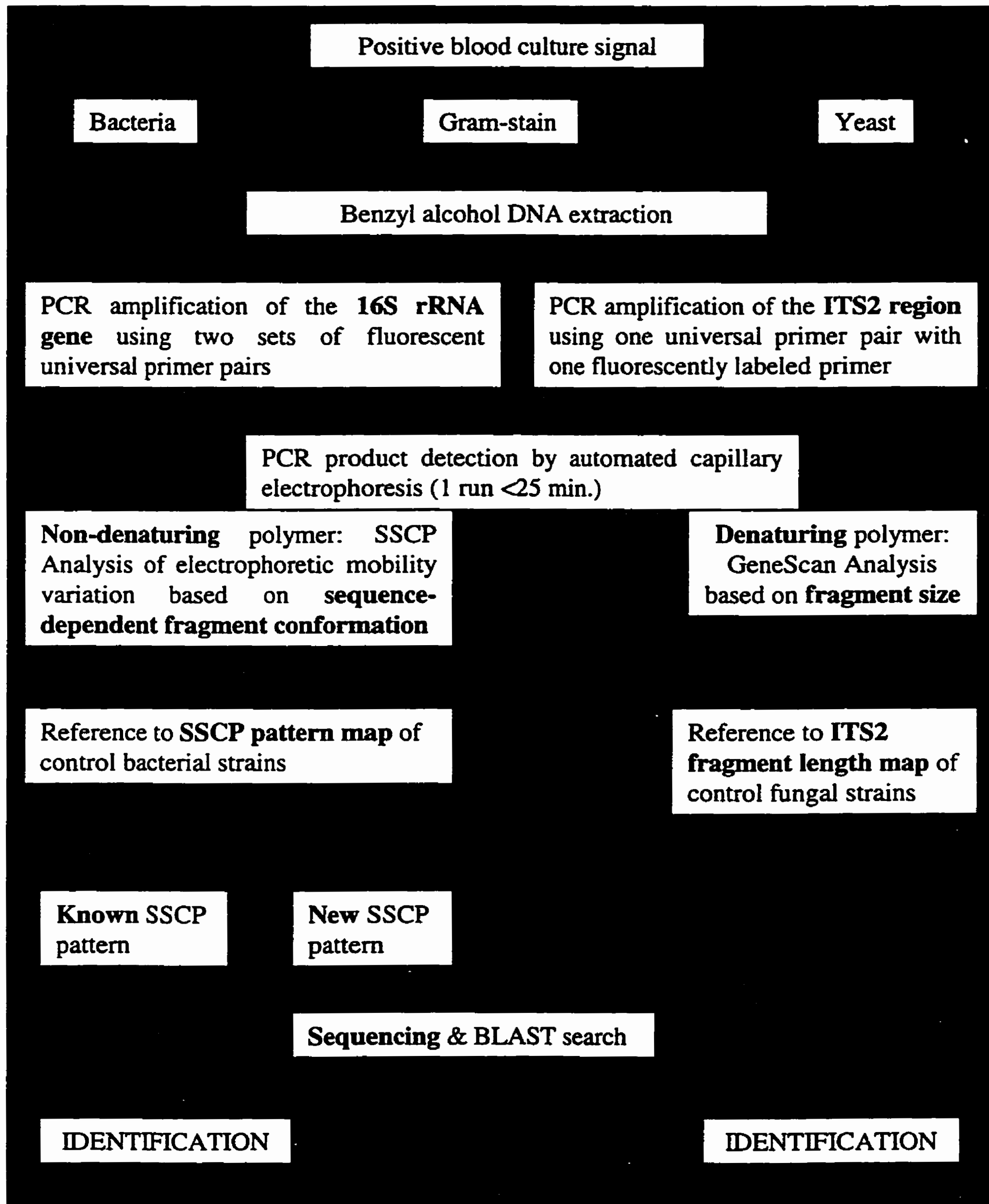
PCR product was used for analysis on the ABI PRISM™ 310 Genetic Analyzer for precise bp length determination of the PCR fragments containing the ITS2 regions. Fragment analysis was done using the ABI PRISM 310 GeneScan™ Analysis Software (PE Applied Biosystems). Sample preparation for capillary electrophoresis involved diluting a small amount of PCR product 1:25 in TE buffer, of which 1 µl was added to the capillary electrophoresis mix (12 µl deionized formamide and 0.5 µl GeneScan®-500 (ROX) Size Standard (PE Applied Biosystems). A lesser dilution could be performed for a more intense signal if necessary. Subsequent preparation was done following the manufacturer's instructions with reference to using Performance Optimized Polymer 4

(POP4) for microsatellite analysis (PE Applied Biosystems). POP4 was used as the replaceable polymer matrix. Electrophoresis conditions were based on the manufacturer's instructions.

3. Time and Cost Analysis

The time to conventional and molecular identification of bacteria or fungi present in blood culture samples was determined from the period the blood culture detection system (BacT/Alert) gave a positive signal. This was given in days for conventional testing, and in hours for molecular testing. The time to molecular identification was not determined in real time but in a hypothetical scenario, as testing was performed by one person only for research purposes.

The cost of all reagents used as well as labor costs ($19.63/hour: peak of wage scale for a General Duty Technologist, Health Sciences Centre, Winnipeg) was determined for conventional testing and molecular testing. Four basic algorithms for work-up were represented for conventional testing: yeast, anaerobes, Gram-positive cocci and Gram-negative bacilli. Molecular testing cost was determined for SSCP identification and for sequencing as well.



Positive blood culture signal
Bacteria
Gram-stain
Yeast
Benzyl alcohol DNA extraction
PCR amplification of the 16S rRNA gene using two sets of fluorescent universal primer pairs
PCR amplification of the ITS2 region using one universal primer pair with one fluorescently labeled primer
PCR product detection by automated capillary electrophoresis (1 run <25 min.)
Non-denaturing polymer: SSCP Analysis of electrophoretic mobility variation based on sequence-dependent fragment conformation
Denaturing polymer: GeneScan Analysis based on fragment size
Reference to SSCP pattern map of control bacterial strains
Reference to ITS2 fragment length map of control fungal strains
Known SSCP pattern
New SSCP pattern
Sequencing & BLAST search
IDENTIFICATION
IDENTIFICATION

Figure 2. Proposed algorithm for species identification of bacteria and fungi from blood culture positive specimens. A schematic representation of the process by which we proposed to rapidly and specifically identify bacterial and fungal organisms from blood culture positive specimens is represented.

C. RESULTS

To assess the utility of the bacterial 16S rRNA gene of bacteria and the fungal ITS2 region of fungi in clinical specimens, the specificity and sensitivity of each PCR protocol necessary for the detection of these fragments was determined. The potential effect of prolonged storage and the presence of antibiotics in a PCR reaction was also determined. Various methods of PCR product detection and identification have been assessed including agarose gel electrophoresis, Southern hybridization, dotblot analysis and PCR using fluorescently-tagged primers for analyzed on the ABI PRISM™ 310 Genetic Analyzer, an automated capillary electrophoresis system. For specific identification of the bacteria found in blood, SSCP analysis was performed using reference strains representative of 25 species that encompass approximately 87% of bacterial species isolated from blood cultures at the Health Sciences Centre, Winnipeg, from 1992-1997. For specific identification of fungal blood pathogens, fragment analysis was performed on the amplified product containing the ITS2 region of 47 fungal species including *Candida* species. To evaluate in part the clinical utility of this method, the molecular identification of 304 consecutive blood culture positive patient specimens and 103 seeded blood cultures with yeast was determined in a double-blinded fashion.

PART I. Bacterial Identification Using the 16S rRNA gene

1. PCR Amplification of the 16S rRNA Gene Using Universal Primers

a. Amplification of Segments of the 16S rDNA Using Different Primer Sets

Five combinations of universal 16S rDNA primer pairs have been used throughout the thesis, of which the PCR product resulting from four of the pairs are

shown in Figure 3. Each is equally useful for detection. Primer pair 8FPL/806R was most often used for the determination of sensitivity and specificity. Primer pairs RW01/13B and 515/806R were chosen for SSCP analysis and primers 91E/13B amplified the fragment we utilized for sequencing. We have also used the DG74/RW01 primer pair to amplify a fragment that was used for detection using probes.

b. Primer Specificity in Whole Blood

The specificity of 16S rDNA universal primers against human genomic DNA was determined using primer pair 8FPL/806R. No cross-reaction was demonstrated when various concentrations of human or fungal (*C. albicans*) DNA were used as template (Figure 4).

c. Primer Sensitivity in Whole Blood

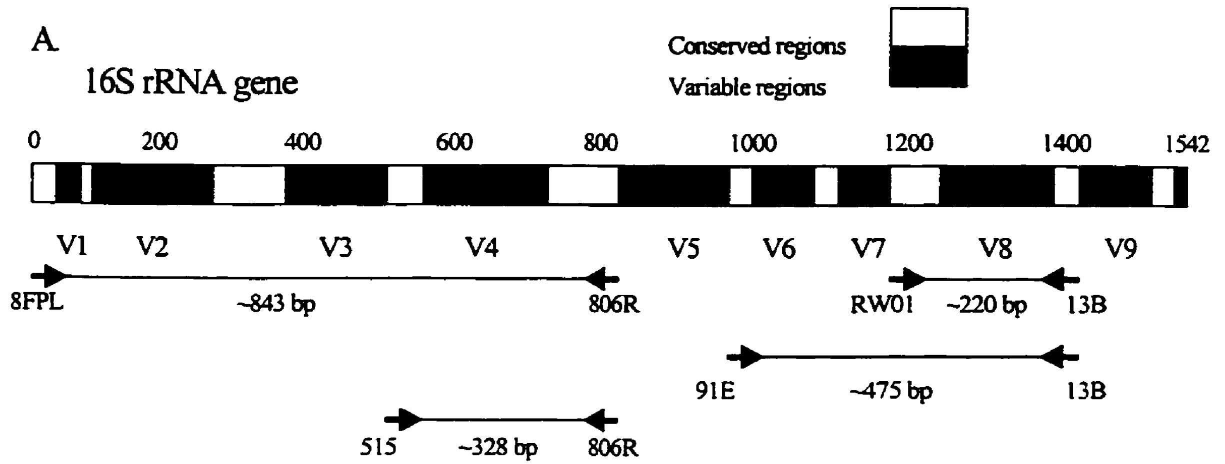
The sensitivity of the universal primers 8FPL/806R was tested with concentrations of 10^4 to 10^1 CFU/ml of *E. coli* organisms. All resulted in successful amplification, indicating that sensitivity of detection was at least as low as 10 CFU/ml (Figure 5).

d. Sensitivity of Detection After Prolonged Storage at 4°C

The integrity of bacterial DNA upon prolonged storage of whole blood at 4°C was not significantly diminished for up to 21 days (Figure 6). The initial viable colony counts of *S. epidermidis* and *E. coli* were 2×10^2 and 10^2, respectively. No colonies were grown using an aliquot of *S. epidermidis* suspensions at 10, 14 and 21 days. However, PCR amplification was successful and did not show any significant differences in the detection of the organisms. The viable colony counts for *E. coli* were determined to be 2×10^2 at 10 days, 8×10^2 at 14 days and 2×10^2 at 21 days.

e. Sensitivity of Detection in the Presence of Antibiotics

The sensitivity of the universal primers in the presence of various antibiotics in the blood sample did not decrease in the presence of erythromycin, as successful amplification was detected up to 10 CFU/ml using both *E. coli* and *S. epidermidis* as DNA templates (Figure 7). The same results have been obtained with the presence of protein-bound antibodies including gentamicin, cephalexin, ciprofloxacin, ceftriaxone, tetracycline, and clindamycin (data not shown).



B.

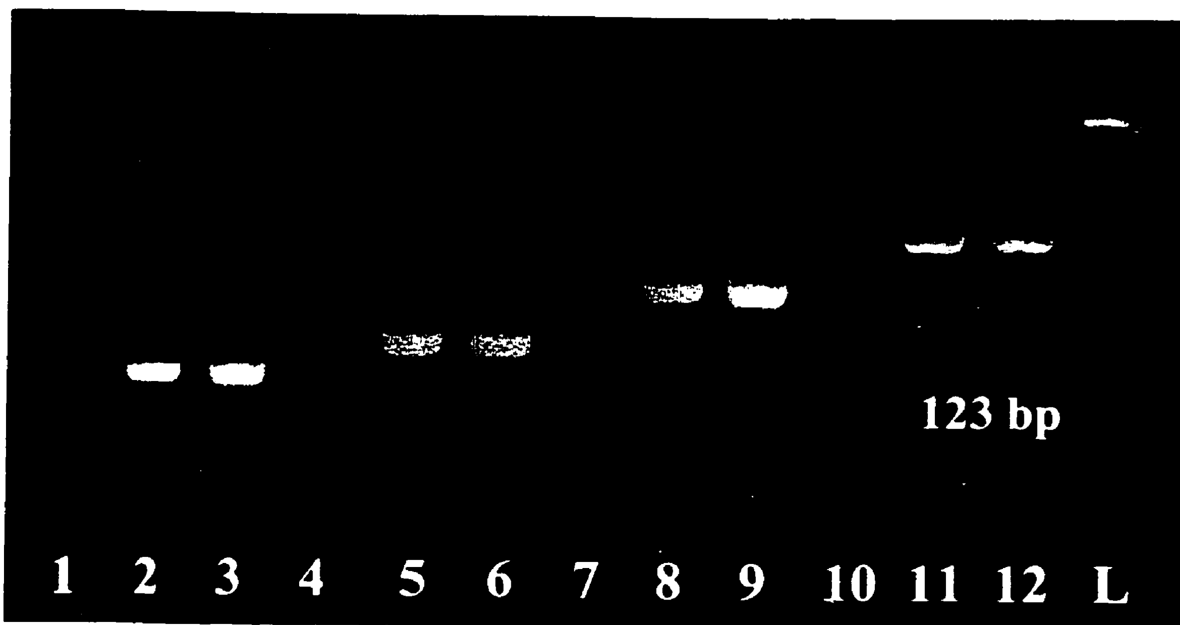


Figure 3. **Amplification of the 16S rRNA gene using different universal primer combinations.** Four sets of non-fluorescent universal primer pairs have been used in four separate PCR reactions, under the same conditions, as described in Materials and Methods. (A) A schematic representation of the primer target areas and resulting amplified fragment. (B) Lanes 1-3: RW01/13B; lanes 4-6: 515/806; lanes 7-9: 91E/13B; lanes 10-12: 8FPL/806R. *E. coli* ATCC 25922 (lanes 3, 6, 9, 12), *S. epidermidis* ATCC 12228 (lanes 2, 5, 8, 11) and sterile H_2O for contamination control (lanes 1, 4, 7, 10) served as PCR template.

Figure 4. **Specificity of universal 16S rDNA primers against fungal and human DNA.** PCR amplification using non-fluorescent 8FPL and 806R was performed as described in Materials and Methods. Human genomic DNA was used as template in the following concentrations: 1 ng (lane 1); 10 pg (lane 2). Genomic DNA from *C. albicans* was used in the following concentrations: 1 ng (lane 3); 10 pg (lane 4). Various concentrations of *E. coli* ATCC 25922 were used for comparison: 1 ng (lane 6); 10 pg (lane 7); 1 pg (lane 8); 10 fg (lane 9). Lane 5: concentrated *E. coli* (positive control); lane 10: H_2O contamination control.

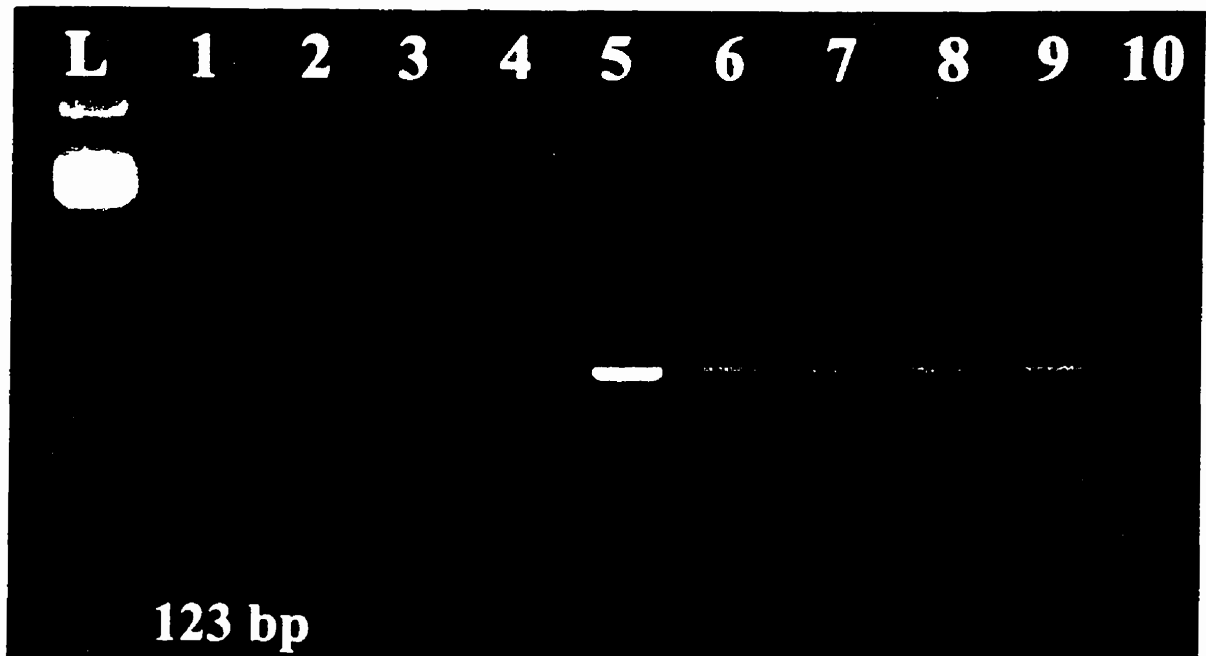


Figure 5. **Sensitivity of universal 16S rDNA primers in whole blood.** PCR amplification of variable *E. coli* ATCC 25922 suspensions using non-fluorescent 8FPL and 806R was performed as described in Materials and Methods. Lane 1: 10^4 CFU/ml; lane 2: 10^3 CFU/ml; lane 3: 10^2 CFU/ml; lane 4: 10^1 CFU/ml; and lane 5: H_2O contamination control.

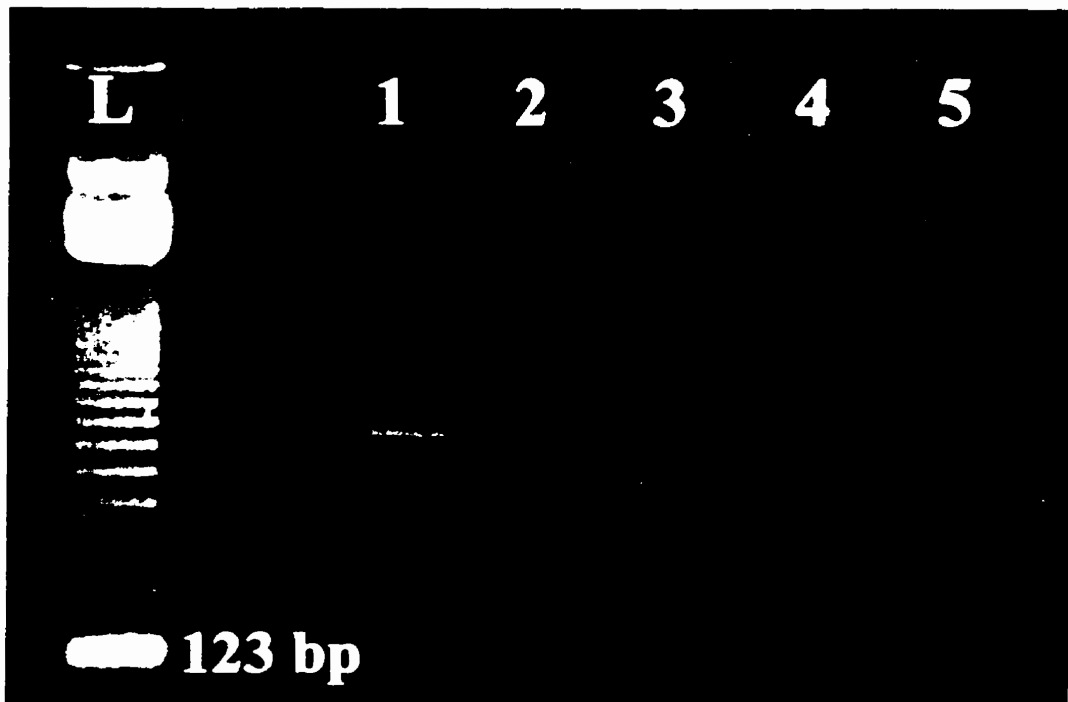


Figure 6. Sensitivity of 16S rDNA amplification after prolonged storage at 4°C.

E. coli ATCC 25922 (lanes 1, 4, 7 and 10) and *S. epidermidis* ATCC 12228 (lanes 2, 5, 8, and 11) were amplified following storage at 4°C for 1 day (lanes 1-2), 10 days (lanes 4-5), 14 days (lanes 7-8) and 21 days (lanes 10-11) as described in Materials and Methods. Lanes 3, 6, 9 and 12: H_2O contamination controls.

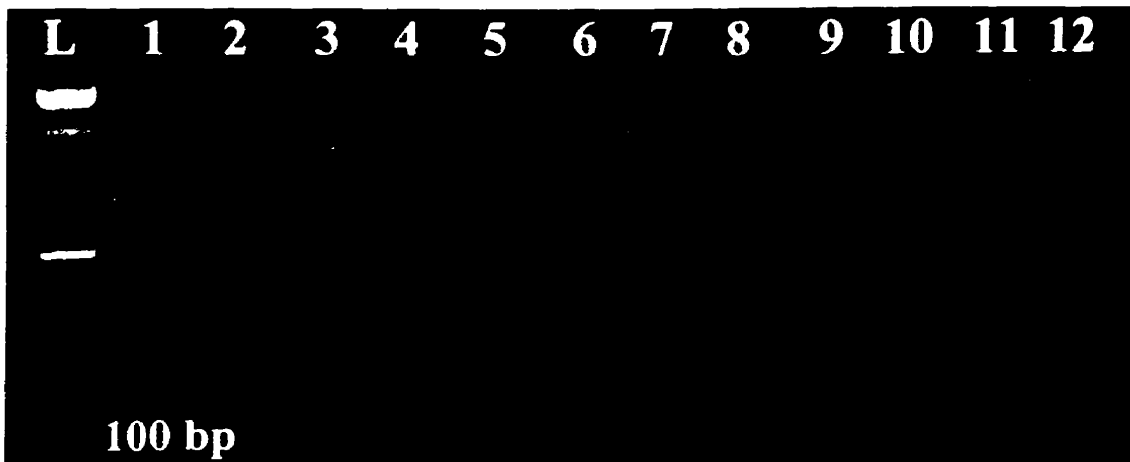
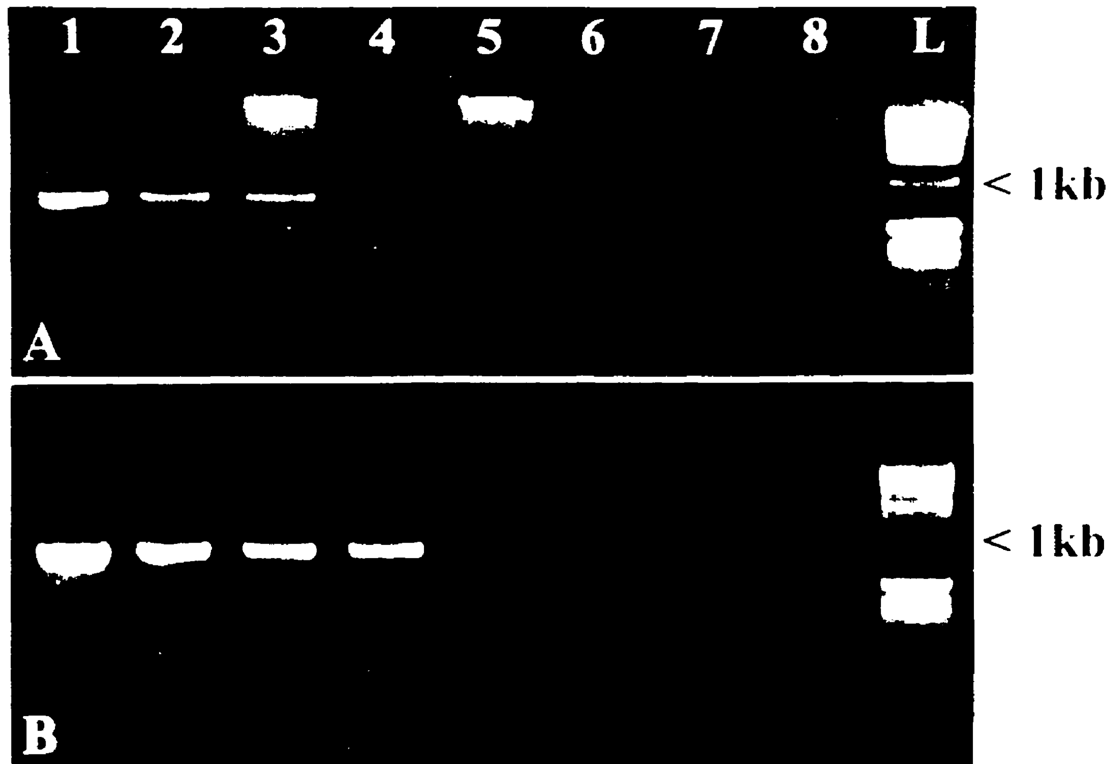


Figure 7. **Sensitivity of 16S rDNA amplification in the presence of erythromycin.** PCR amplification of *E. coli* ATCC 25922 **(A)** and *S. epidermidis* ATCC 12228 **(B)** using non-fluorescent primers 8FPL and 806R was performed as described in Materials and Methods. Lane 1: 10^8 CFU/ml; lane 2: 10^7 CFU/ml; lane 3: 10^6 CFU/ml; lane 4: 10^5 CFU/ml; lane 5: 10^4 CFU/ml; lane 6: 10^3 CFU/ml; lane 7: 10^2 CFU/ml; and lane 8: 10^1 CFU/ml. H_2O contamination control was negative (not shown). A 1 kb ladder was used for approximate band sizing.



2. Detection of Amplified Product With the Use of Probes.

a. Universal Probes

We have used the same probe, a universal probe (RDR245) which targets the bacterial 1369-1395 bp region of the 16S rRNA gene for detection for both Southern blot protocol (Figure 8) and a dotblot hybridization protocol (Figure 9). The Southern blot protocol was very time consuming (minimum of 2 days to perform) and laborious. We were not able to determine the utility of using probes for increased sensitivity.

b. Gram-Negative Probes

We have compared the detection of bacteria using a Gram-negative probe against *E. coli* and *S. aureus* (Figure 10). With a hybridization temperature of 62°C for 1 hour, this Gram-negative probe hybridized to both *E. coli* and *S. aureus*. Further optimization was necessary, as there was only 1 bp difference in the target region between Gram-positive and Gram-negative organisms. An increase in the hybridization temperature to 65°C resulted in the hybridization of *E. coli* alone. However, the detection was not as sensitive, as only the spotted samples containing 5 μl of PCR product and not 3 μl were detected.

Figure 8. **Southern hybridization using a universal 16S rDNA probe.** PCR amplification of *E. coli* ATCC 25922 using the DG74/RW01 primer pair, electrophoresis **(A)**, transfer onto a nylon membrane and subsequent hybridization with RDR245 **(B)** was performed as described in Materials and Methods. Product was diluted to 20 µl. The amount of PCR product in each lane is as follows: lane 1: 1 µl; lane 2: 2 µl; lane 3: 3 µl; lane 4: 4 µl; lane 5: 5 µl; lane 6: 10 µl; and lane 7: 20 µl.

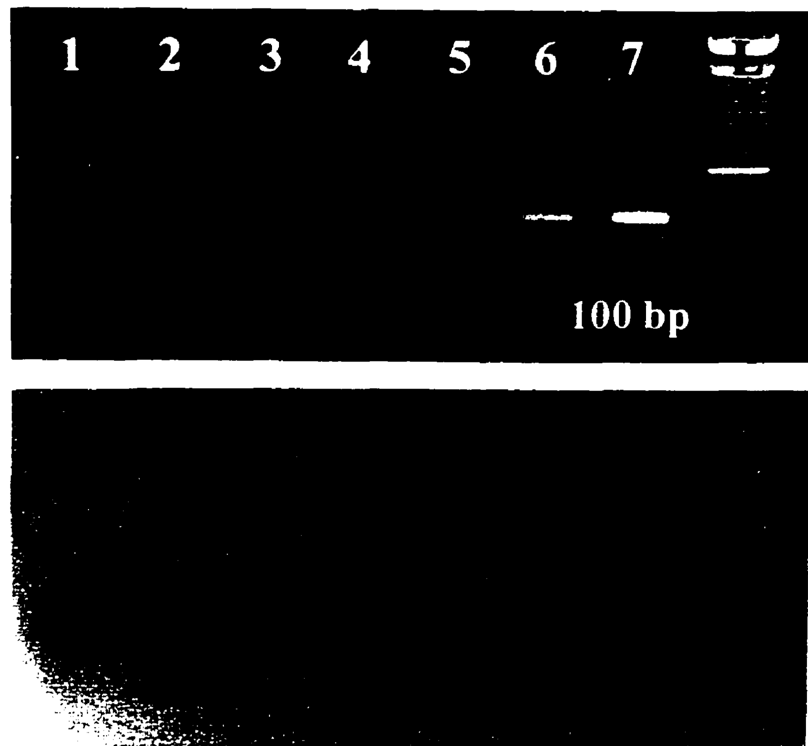


Figure 9. **Dotblot analysis using a universal 16S rDNA probe.** PCR amplification of *E. coli* ATCC 25922 using DG74/RW01 primer pair, blotting procedure and subsequent hybridization was performed as described in Materials and Methods. The amount of PCR product used in the 1 M NaOH/200 mM EDTA mix were as follows: column 1: 1 µl; column 2: 2 µl; column 3: 3 µl; column 4: 4 µl; column 5: 5 µl; and column 6: 10 µl. The amount of mix spotted onto the nylon membranes was as follows; row A: 5 µl; row B: 4 µl; row C: 3 µl; row D: 2 µl; and row E: 1 µl.

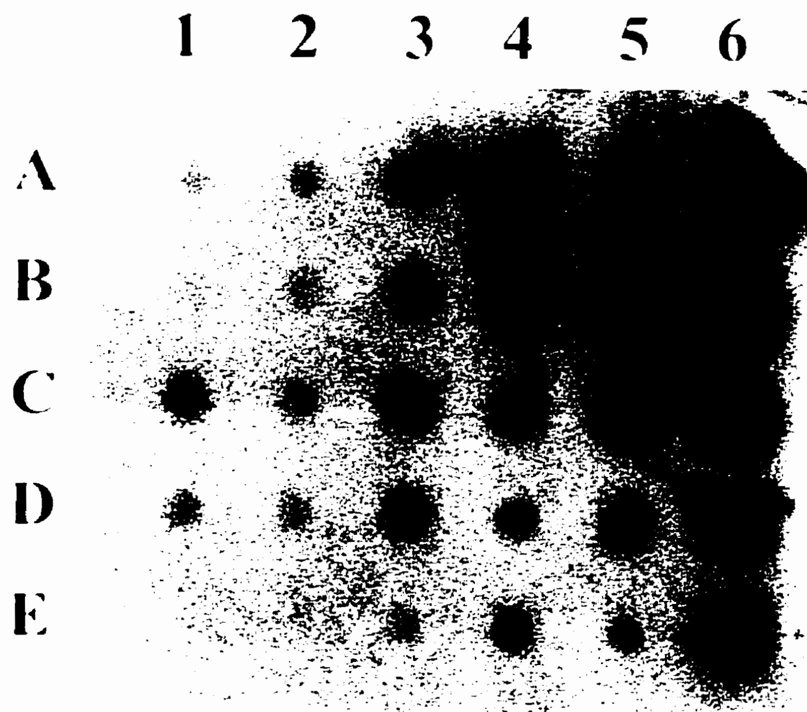
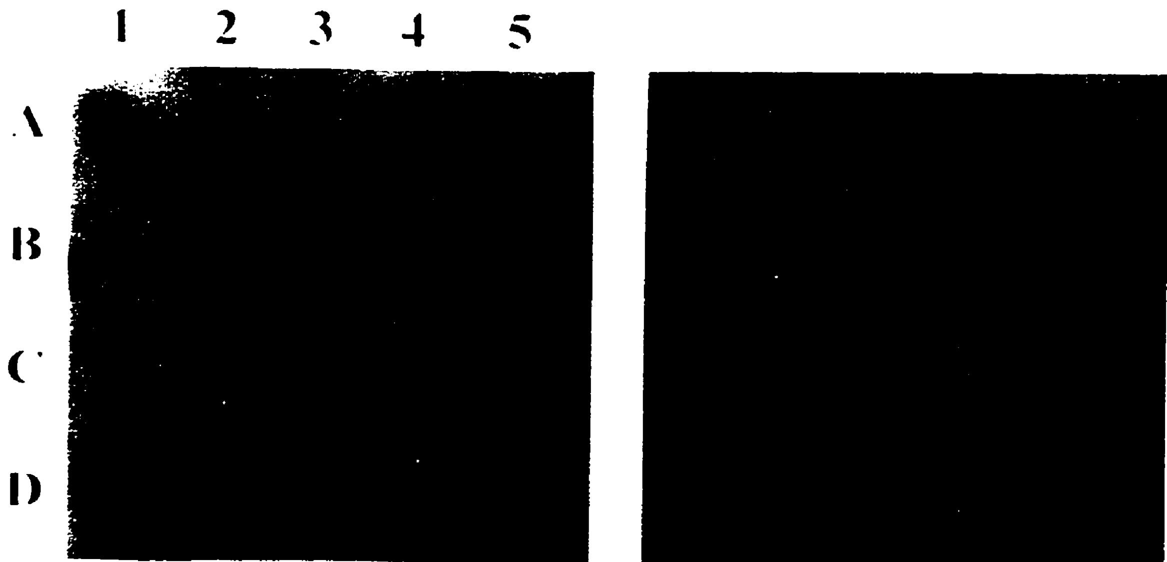


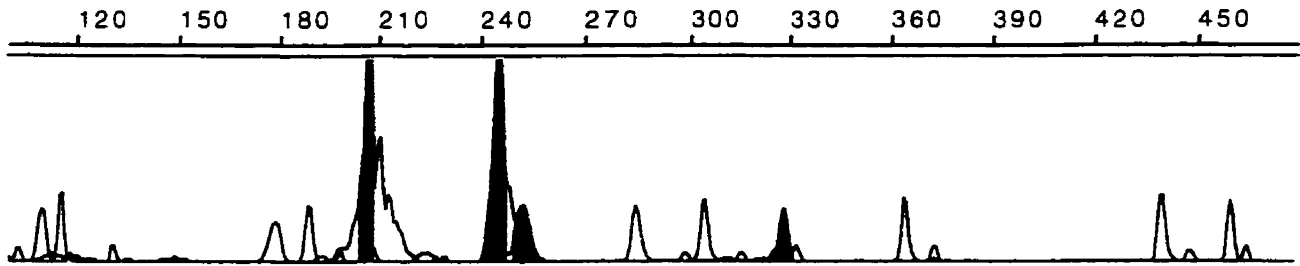
Figure 10. **Dotblot analysis using a Gram-negative 16S rDNA probe.** PCR amplification of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 using DG74/RW01 primer pair, blotting procedure and subsequent hybridization with RDR278 was performed as described in Materials and Methods. The hybridization temperatures were 62°C **(A)** and 65°C **(B)**. The amount of PCR product used in the 1 M NaOH/200 mM EDTA mix were as follows: row A: 5 µl of *E. coli*; row B: 3 µl of *E. coli*; row C: 5 µl of *S. aureus*; and row D: 3 µl of *S. aureus*. The amount of mix spotted onto the nylon membranes was as follows; column 1: 1 µl; column 2: 2 µl; column 3: 3 µl; column 4: 4 µl; and column 5: 5 µl.



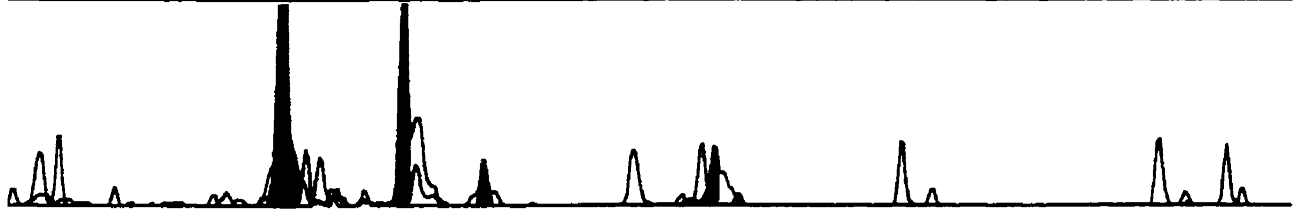
3. Species Identification Using SSCP Analysis and Sequencing

a. The Determination of SSCP Peak Patterns of Common Blood Culture Isolates

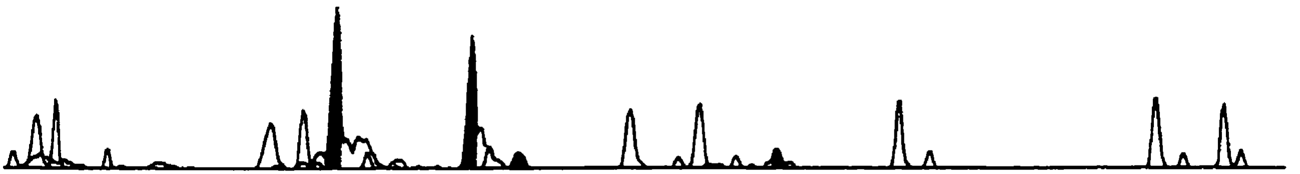
The SSCP pattern of the most common organisms isolated from blood cultures was determined. All organisms included constituted >85% of all organisms isolated from blood cultures at HSC. Other groups of common organisms, which include many genera such as diphtheroids or bacillus, were not included. The identification of these organisms, if the case need be, would be determined by sequencing, as we would expect a new SSCP pattern to present itself upon application of this method for identification. All control strains were run three times and subsequent strains at least once for confirmation of the species-specific nature of the pattern. If conventional identification of common isolates was only up to the genus level or group, such as for *Micrococcus*, CoNS, and Viridans streptococci , the most common species known to belong to these groups were chosen for the SSCP pattern determination. Figure 11 is a representation of the electropherograms of the five most common 16S SSCP patterns as analyzed by the ABI PRISM GeneScan® software. A total of twenty-five SSCP patterns have been determined. The SSCP pattern was defined as the average retention time ("size") of each of the 4 PCR amplified fragments upon non-denaturing electrophoresis in comparison with an internal "size" standard. As we are not using a denaturing polymer, "size" designation is not based on actual fragment length size but on the retention time of each fragment upon electrophoresis.



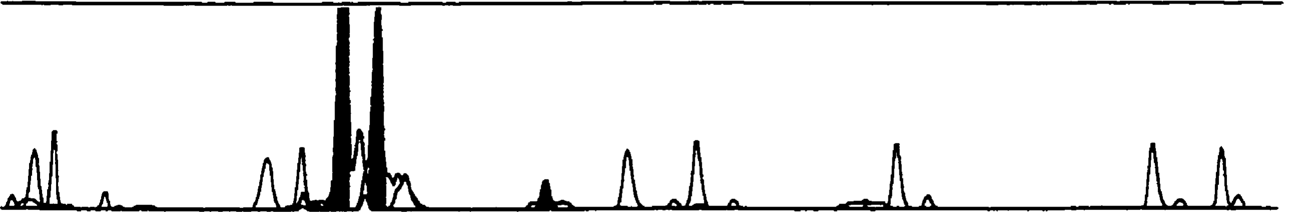
11 B: S. epidermidis (54618)
11 R: S. epidermidis (54618)
11 G: S. epidermidis (54618)



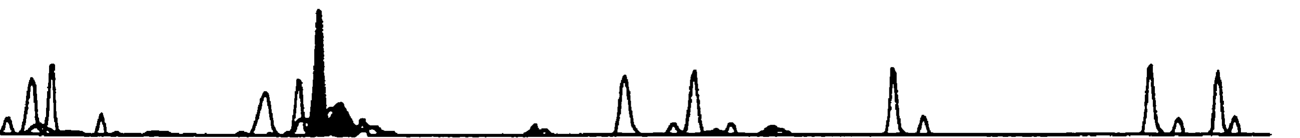
10 B: E. coli (54791)
10 R: E. coli (54791)
10 G: E. coli (54791)



13 B: S. aureus (57879)
13 R: S. aureus (57879)
13 G: S. aureus (57879)



9 B: S. pneumoniae (64218)
9 R: S. pneumoniae (64218)
9 G: S. pneumoniae (64218)



12 B: E. faecalis (56956)
12 R: E. faecalis (56956
12 G: E. faecalis (56956)

Figure 11. **SSCP peak patterns of the five most commonly isolated blood pathogens as analyzed by the ABI 310 PRISM Genetic Analyzer.** The multiplex PCR amplification was performed using two sets of fluorescent primers (6-FAM-RW01 and 5'HEX-13B; 6-FAM-515 and 5'HEX-806R) as described in section 1. c. β. The FAM label is demonstrated in blue and the HEX label in green. This resulted in alternating blue and green peaks upon SSCP analysis. An internal standard (ROX labeled-red) was included with each sample for precise retention time determination (x axis). The highlighted peaks indicate the retention time designated for each PCR fragment. The table below represents the data corresponding to each electropherogram. Retention times are indicated for each PCR fragment. Since the electrophoresis was performed using a non-denaturing gel, this is not indicative of the bp size of the fragment, but is relative to the numbers assigned to the standard peaks..

Species	RW01	13B	515	806R
S. epidermidis	207.46	245.80	252.71	328.95
E. coli	182.59	218.52	242.18	308.90
S. aureus	199.55	239.39	253.08	328.75
S. pneumoniae	201.87	212.20	261.98	~350-365
E. faecalis	195.81	201.97	259.73	328.88

Table 4. Determination of SSCP peak patterns of control strains. Control strains and/or previously identified blood culture isolates were used for the determination of species-specific SSCP peak patterns of common blood culture bacterial isolates.

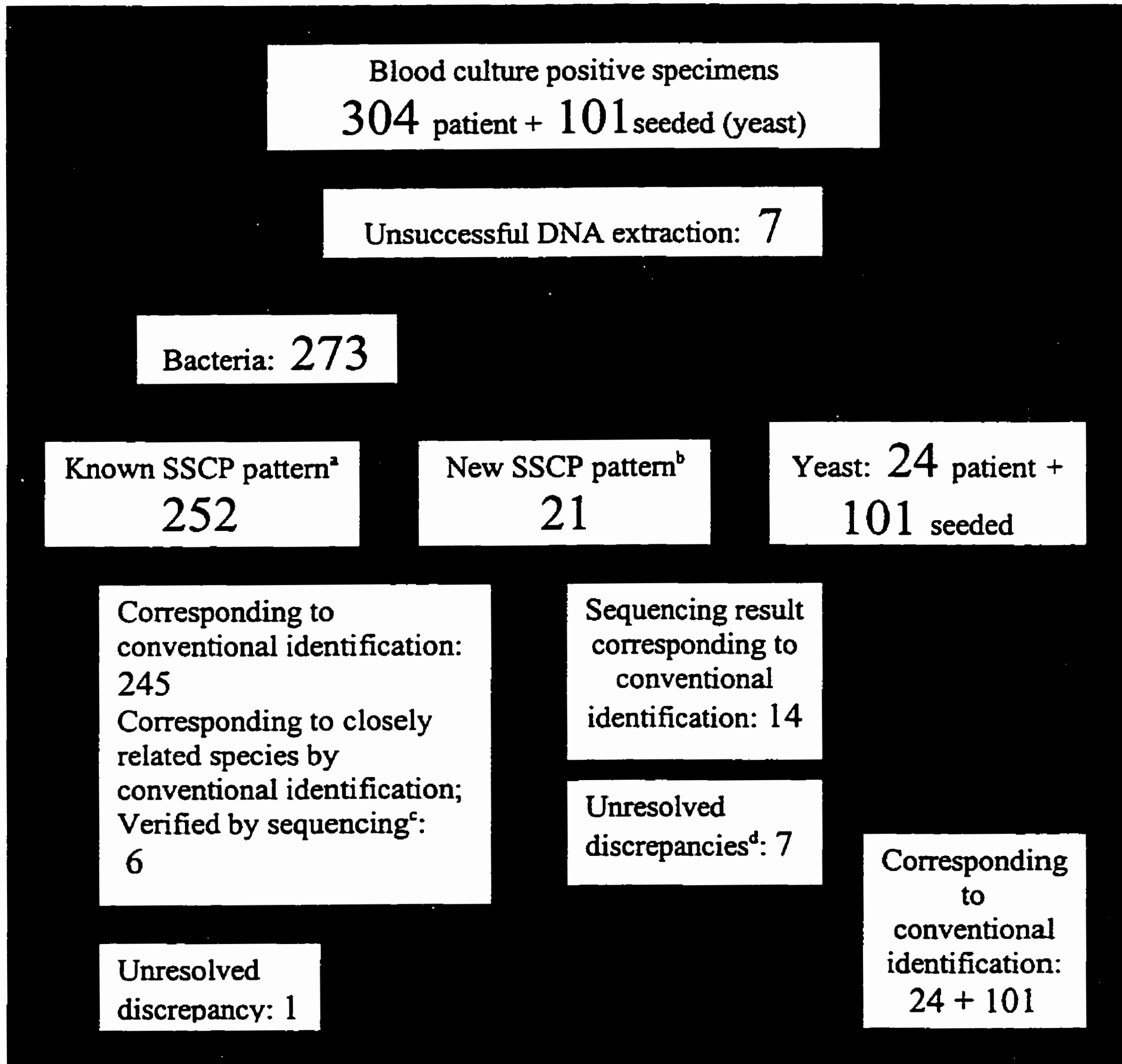
Species	strains	RW01	13B	515	806R
CoNS: ***S. epidermidis***	ATCC 12228; N421	207.6	243.9	253.5	328.4
S. hominis	ATCC 27844; N026	199.8	238.1	253.5	328.5
S. haemolyticus	ATCC 29970; N473	199.5	237.7	254.2	328.2
S. warneri	ATCC 27836; N042	202.0	232.1	254.6	327.3
S. capitis	ATCC 27840; N1812	197.3	238.5	253.9	328.3
S. auricularis	N914	199.9	237.4	253.8	327.4
S. simulans	ATCC 27848; P315	200.8	231.7	254.2	326.9
S. cohnii	ATCC 29974	204.4	235.3	253.6	328.8
S. aureus	ATCC 25923; N005	199.7	238.2	253.6	328.3
E. coli	ATCC 25922; N004	182.2	219.7	243.4	308.2
Viridans: ***S. mitis***	ATCC 9811; CAP95B22; N108	201.7	211.7	261.1	350-370
S. mutans	ATCC 35668; P1743	196.2	214.3	258.9	328.3
S. salivarius	ATCC 25975	197.5	221.0	258.1	326.5
S. pneumoniae	ATCC 49619; N465	201.4	211.3	261.8	350-370
E. faecalis	ATCC 51299; N047	195.5	202.3	260.5	327.8
K. pneumoniae	ATCC 13883; N078	187.7	227.2	234.7	318.6
P. aeruginosa	ATCC 27853; N207	183.4	230.5	252.6	313.1
E. cloacae	ATCC 23355; N138	187.4	227.4	235.1	318.9
M. luteus	ATCC 49732; N142	205.2	235.9	242.1	320.6
S. maltophila	ATCC 13637	194.1	227.6	249.8	329.9
P. acnes	ATCC 11827; P147; N125; TS178	181.8	226.3	-	-
Streptococcus B	ATCC 27956	199.9	213.9	259.7	326.7
Streptococcus A	ATCC 19615; N679	199.9	214.2	262.8	322.1
K. oxytoca	ATCC 49131	195.2	216.7	234.2	318.7
E. faecium	ATCC 35667; D-5; CAPB32; VRE 1523	192.9	211.3	260.2	328.4

Certain particularities pertaining to these patterns included the high fluorescent intensity of the RW01/13B fragments compared to the 515/806R fragments. In addition, some species were found to have an identical pattern: *S. aureus, S. hominis, S. haemolyticus*, and *S. auricularis*; *S. pneumoniae* and *S. mitis*; *E. cloacae* and *K. pneumoniae*. Unique to the *S. pneumoniae* and *S. mitis* pattern, one of their peaks (806R) was flat and wide, spanning over 350-370 of retention time. The multiplex PCR reaction of 4 strains of *P. acnes* did not result in amplification of the 515/806R fragment. One single SSCP pattern was observed between the control and stock isolates tested for each species.

b. Breakdown of the Results Obtained From All Blood Culture Positive Specimens for Molecular Identification.

All blood culture positive specimens from December 1997 to March 1998 (n=304) were processed for PCR amplification. The only information made known to the molecular analyst was whether the specimen contained bacteria, fungi, or both, provided by the Gram-stain. Molecular identification was performed for 277 blood cultures containing bacteria and 27 containing yeast. DNA extraction was unsuccessful for 4 and 3 of them, respectively. All others were detected by capillary electrophoresis having used the appropriate set of fluorescent primers for bacterial or fungal analysis. Three categories of sample results included fungal ITS2 detection, SSCP detection of a previously determined pattern, and SSCP detection of a new pattern (Figure 12).

Figure 12. Molecular identification results of all blood culture positive specimens. From December 1997 to March 1998, 304 blood culture positive specimens were obtained for molecular species identification. The breakdown of the consequent results are represented here.



[a]Table 5
[b]Table 7
[c]Table 5, see *
[d]Table 7, see c-g

c. Identification of Blood Culture Isolates Presenting a Known SSCP Pattern.

Of 273 blood culture containing bacteria for which the SSCP pattern detection was successful, a previously established SSCP pattern from Table 4 was discovered in 252 of them (92%). This data is shown in Table 5. The conventional identification result does not include the other species found in the samples that were mixed. Only the species corresponding to the SSCP pattern detected is indicated. However, if two SSCP patterns did in fact occur in one sample it is indicated in the table for both conventional and molecular testing.

Occasionally, a "new" species was indicated by conventional testing which was represented by a known SSCP pattern. These were subsequently sequenced to confirm the identity of the organism (Table 6). In general, closely related species would appear to have identical patterns, and for the most part the clinical significance was minor, for example: *Micrococcus*, *Gemella* and *Stomatococcus sp.*; *P. acnes*, *Propionibacterium sp.* and diphtheroids.

In two instances, the SSCP pattern determination did not correspond to the biochemical identification. In addition to sequencing of these two isolates, biochemical identification was repeated. One organism had an SSCP pattern and a sequencing result corresponding to *S. salivarius*, whereas the biochemical identification indicated this organism was *S. mutans*. The SSCP pattern of the control *S. mutans* strain is distinct from *S. salivarius*, and the *S. mutans* sequence from Genbank had many mismatches with the query sequence. We believe the organism is a *Streptococcus* bacteria belonging to either the *S. mutans* or *S. salivarius* groups, which comprise several species. We did not consider this case a discrepancy as this pattern corresponds to a viridans species.

Table 5. Blood Culture organisms corresponding to pre-determined SSCP patterns (total=252).

SSCP pattern identification	*n*	Identification by conventional testing
Staphylococcus epidermidis	58	*S. epidermidis* (57) CoNS (1)
E. coli	23	*E. coli* (23)
E. coli and *E. cloacae/K. pneumoniae*	1	*E. coli*
S. aureus/CoNS *group*	68	*S. aureus* (36) CoNS (2) *S. hominis* (9) *S. simulans* (1) *S. haemolyticus* (7) *S. auricularis* (11) *S. auricularis and hominis* (1) *S. carnosus* (1)
S. aureus/CoNS and *S. epidermidis*	1	*S. hominis and S. epidermidis*
S. warneri	14	*S. warneri* (7) CoNS (7)
S. capitis	6	*S. capitis* (5) *S. kloosii* (1)
S. capitis and *S. aureus*/CoNS *group*	1	*S. auricularis* and *S. hemolyticus*
S. capitis and *S. epidermidis*	1	*S. epidermidis*
S. pneumoniae/mitis	21	*S. pneumoniae* (12) Viridans (9)
S. pneumoniae/mitis and *S. aureus*/CoNS	3	*S. aureus and* Viridans (3)
E. cloacae/K. pneumoniae	10	*E. cloacae* (4) *K. pneumoniae* (4) *E. cloacae* and *K. pneumoniae* (1) *E. amnigenus* (1)*
E. faecalis	18	*E. faecalis* (17) *S. epidermidis* (low probability) and non-dextrose fermenter(1)*
P. aeruginosa	9	*P. aeruginosa* (9)
M. luteus	6	*Micrococcus sp.* (4) *Gemella sp.* (1)* *Stomatococcus sp.*(1)*
P. acnes	5	*P. acnes* (2) *Propionibacterium sp.*(1)* Diphtheroids (2)*
Streptococcus B	1	*Streptococcus* B
Streptococcus A	1	*Streptococcus* A
E. faecium	2	*E. faecium* (2)
S. salivarius	1	*S. mutans**
X. maltophila	2	*X. maltophila* (2)

* Isolates were subsequently sequenced to confirm species identity. See Table 7.

In the other example, an SSCP pattern that corresponded to *E. faeclis* was not identified as such by conventional testing. The mixed specimen in question indicated the presence of *S. epidermidis* and of a non-dextrose fermenter. This specimen probably did contain *E. faecalis*, but was not isolated and therefore never stocked as a blood culture isolate. The sequencing of this organism corresponded exactly to that of *E. faecalis*.

Table 6. Sequence analysis of known SSCP patterns associated with new/other organisms.

Known SSCP pattern	*n*	Bench ID	Sequencing result
E. cloacae/K. pneumoniae	1	*E. amnigenus*	*E. asburiae/Pantoea sp.*
P. acnes	2	Diphtheroids	*P. acnes, Actinomycete sp.*
M. luteus	1	*Stomatococcus sp.*	*Stomatococcus mucilaginosus*
M. luteus	1	*Gemella sp.*	*Gemella sp.*
E. faecalis	1	*S. epidermidis*; non-dextrose fermenter	*E. faecalis*

c. Identification of Isolates Presenting a New SSCP pattern with Rapid Sequencing and BLAST Search.

In the case of less common organisms or those such as diphtheroids for which we did not previously determine an SSCP pattern, sequencing of the V6-V8 region of the 16S rDNA has been performed subsequently to SSCP analysis as these presented new peak patterns. Once the sequence was determined, it was entered into the BLAST Search program on the internet for sequence matching. The closest sequence match in the query was determined to be the probable identification of the organism (Table 7). In certain cases, no perfect match was found. Interpretation was required to determine if the closest match was the right identification or if there were other problems associated with

sequence matching, such as the actual sequence not being in a databank or perhaps the sequences which had been submitted by other scientists were incorrect.

Table 7. Sequencing analysis of organisms presenting new SSCP patterns.

Bench identification	*n**	RW01	13B	515	806R	BLAST Search result
B. fragilis	2	186.1	215.3	248.7	318.1	*B. fragilis*
H. influenzae	1	190.0	232.7	267.9	325.6	*H. influenzae*
L. monocytogenes	1	195.1	220.4	258.1	314.0	*L. monocytogenes*
N. gonorrhoeae	1	196.3	223.4	256.7	315.6	*N. gonorrhoeae*
Diphtheroids,	1[a]	209.9	241.6			*Actinomyces neuii*
P. assacharolyticus	1	217.0	243.3	278.6	330.8	*P. hareii*
C. perfringens	1	209.3	235.1	249.1 255.7	336.4	*C. perfringens*
Corynebacterium sp.	3	185.6	222.5	241.9	315.3	*C. amycolatum/asperum*
Corynebacterium sp.	1	185.5	221.8	242.2	325.1	*C. amycolatum/asperum*
Corynebacterium sp.	1	192.6	225.8	233.6	326.9	*Corynebacterium sp.*
Non-dextrose fermenter	1	189.1	212.1	240.8	319.4	*Acinetobacter sp.*
S. paratyphi A	1	182.4	219.4	234.6	316.0	*S. paratyphi* B[b]
E. cloacae	3	187.8	217.7	235.7	317.5	*E. asburiae/Pantoae sp.*[c]
E. cloacae	1	182.2	226.1	233.5	318.2	*Enterobacter/Klebsiella sp*[d]
Citrobacter koseri	1	189.0	217.5	239.8	307.0	*K. planticola*[e]
S. kloosii	1	205.3	239.3	262.2	315.7	*Brachybacterium sp.*[f]
Unable to identify	1	210.1	226.5	232.4	306.2	*Arthrobacter sp.*[g]

*Only the first organism detected with the new pattern was sequenced. All subsequent organisms were identified by the SSCP pattern, which had been added to the map of previously determined SSCP patterns.

[a]As this specimen presented two new SSCP patterns, sequencing of each organism was performed from the blood culture stocks containing each isolated organism.

[b]100% match in BLAST search. Organisms listed with 1 mismatch: *S. paratyphi* A, *E. coli* (~20 strains), *Shigella* and *Salmonella spp.*

[c]This new pattern, subsequently named *Enterobacter*-1, was obtained for 3 other isolates also identified as *E. cloacae.*

[d]Pattern named *Enterobacter*-2

[e]No close match in BLAST search. No sequence for *C. koseri/diversus* 16S rDNA in Genbank was found .

[f]No close match in BLAST search.

[g]No close match in BLAST search.

e. Difficulties with *Enterobacter* Species Identification.

We have encountered identification problems resulting from new SSCP patterns emerging amongst organisms identified conventionally as *E. cloacae*. A total of 9 blood culture specimens resulted in an SSCP pattern corresponding to the *E. cloacae/K. pneumoniae* group. By conventional identification, 4 of those were *K. pneumoniae*, 4 were *E. cloacae*, and 1 was *E. amnigenus*. However, 3 other specimens conventionally identified as *E. cloacae* resulted in a new pattern which we called *Enterobacter*-1; one specimen resulted in another pattern which we called *Enterobacter*-2 (Table 7), and one specimen had the SSCP pattern corresponding to the *E. cloacae/K. pneumoniae* group and the *Enterobacter*-1 group in the same sample. Unfortunately, sequencing was not useful in the determination of the true identity of these organisms, as all specimens sequenced except one, including the *E. cloacae* ATCC strain, resulted in the BLAST Search identification as 100% match with *E. asburiae* and *Pantoea sp*. There is one submitted sequence of *E. cloacae* in Genbank representative of the V6-V8 region of the 16S rRNA gene, however, it presents 2 mismatches with the control strain as well as the other strains. As the entry of sequences into databanks is subject to human error, this does not indicate that the organisms we have sequenced are not *E. cloacae*. Biochemical identification was repeated on all questionable strains. Many of these specimens contained multiple isolates and therefore biochemical identification may have been repeated on more than one stocked strain. Table 8 is a representation of all the results acquired in the investigation of the *Enterobacter* group identity.

Table 8. *Enterobacter* group identification

	Conventional identification	SSCP pattern	BLAST Search result	Microscan re-identification
1	*E. cloacae*	Enterobacter-1	E. asburiae/Pantoea	*E. cloacae* (99.9%)
2	*E. cloacae*	Enterobacter-1	E. asburiae/Pantoea	N/D
3-a* 3-b	*E. cloacae* *K. pneumoniae*	Enterobacter-1	E. asburiae/Pantoea	*E. cloacae* (99.9%) *K. pneumoniae* (99.9)
4	*E. cloacae*	Enterobacter-1	E. asburiae/Pantoea	N/D
5	*E. amnigenus*	*E. cloacae*	E. asburiae/Pantoea	*E. amnigenus* (99.8%)
6-a 6-b	*E. cloacae* *E. cloacae*	Enterobacter-2	Enterob./Klebs sp.	*E. cloacae* (99.9%) *E. cloacae* (99.9%)
E. cloacae ATCC 23355		*E. cloacae*	E. asburiae/Pantoea	N/D

*a and b are two strains stocked from the same blood culture specimen.
N/D: not done

f. Identification in Mixed Cultures.

Out of 304 blood culture specimens, bacterial and/or fungal, 48 (16%) contained more than one species. Only 4 specimens contained yeast organisms, they are indicated in Table 9. Of the 44 samples containing only bacteria, only one SSCP pattern was detected in 32 mixed specimens. Two SSCP patterns were discernable in 10 mixed specimens, whereas two specimens contained two species of a same SSCP pattern. In two specimens, only one species was detected by conventional identification, however two SSCP patterns were discernable.

Table 9 is arranged in a fashion of preferential amplification. Generally, Gram-negative organisms were detected in a mixture of Gram-positive and Gram-negative organisms; *Streptococcus* and *E. faecalis* organisms were detected in a mixture among *Staphylococci* and other Gram-positive organisms.

Table 9. Cases of mixed cultures.

	Laboratory id / 1 blood culture bottle	*n*	SSCP identification
Gram-negative and Gram-positive mixture:			
	P. aeruginosa, S. epidermidis	5	*P. aeruginosa*
	K. pneumoniae, E. coli, C. perfringens x 2	1	*E. cloacae/K. pneumoniae*
	E. cloacae, S. epidermidis	1	*E. cloacae/K. pneumoniae*
	E. coli, E. faecalis	1	*E. coli*
	P. aeruginosa, C. perfringens	1	*P. aeruginosa*
	S. epidermidis, E. amnigenus	1	*E. cloacae/K. pneumoniae*
	S. maltophila, diphtheroids, *Micrococcus sp.*	1	*S. maltophila*
	E. cloacae, E. faecalis, P. aeruginosa, S. haemolyticus	1	*Enterobacter1*
Gram-negative only:			
	E. coli, P. aeruginosa	1	*E. coli*
	*E. cloacae, K. pneumoniae**	1	*E. cloacae/K. pneumoniae*
	E. cloacae, K. pneumoniae	2	*Enterobacter1*
√	*E. cloacae, K. pneumoniae*	1	*Enterobacter1, E. cloacae/K. pneumoniae*
√	*E. coli*	1	*E. coli, E. cloacae/K. pneumoniae*
Gram-positive only:			
	S. epidermidis, S. capitis	2	*S. capitis*
	S. capitis, S. aureus	1	*S. capitis*
√	*S. epidermidis*	2	*S. epidermidis, S. capitis*
	*S. hominis, S. auricularis**	1	*S. aureus* group
	S. warneri, S. hominis	2	*S. warneri*
	S. epidermidis, Pediococcus sp.	1	*S. epidermidis*
	S. epidermidis, S. saprophyticus	1	*S. epidermidis*
	S. epidermidis, Viridans, *S. haemolyticus*	1	*S. pneumoniae/mitis*
	Viridans, CoNS, *Neisseria sp.*	1	*S. pneumoniae/mitis*
	Viridans, *S. hominis*	1	*S. pneumoniae/mitis*
	S. pyogenes, S. aureus	1	*S. pyogenes*
√	*S. aureus*, Viridans	3	*S. aureus* group, *S. pneumoniae/mitis*
√	Diphtheroids, *S. auricularis, S. haemolyticus*	1	*S. capitis, S. aureus* group
	E. faecalis, S. auricularis, diphtheroids, 2xCoNS	2	*E. faecalis*
	S. epidermidis, non-dextrose fermenter	1	*E. faecalis*
	S. epidermidis, E. faecalis	1	*E. faecalis*
	S. auricularis, E. faecalis	1	*E. faecalis*
√	*S. epidermidis, S. hominis*	1	*S. aureus* group, *S. epidermidis*
√	Diphtheroids, *P. assacharolyticus*	1	2 patterns (unknown)
	Micrococcus sp., S. auricularis	1	*M. luteus*
Yeast:			
	C. tropicalis, Corynebacterium sp.	1	*C. tropicalis*
	C. tropicalis, C. albicans	3	*C. tropicalis*

√: Detection of two SSCP patterns

* *E. cloacae* and *K. pneumoniae* have the same SSCP pattern, as well as *S. hominis* and *S. auricularis*.

g. Statistical Analysis of the SSCP Peak Patterns

After having compiled all the SSCP patterns obtained as a result of the blood culture study, the standard deviation in addition to the average of each retention time determined for all peaks pertaining to the five most common SSCP pattern was determined (Table 10). The RW01 and 806R PCR fragments showed less run to run variability than the middle fragments 13B and 515. Whether this is applicable to all species remains to be determined.

Table 10. SSCP pattern variability of the 5 most common patterns

n[a]	Species	RW01(SD)[b]	13B(SD)	515(SD)	806R(SD)
58	*S. epidermidis*	207.4 (0.6)	244.1 (1.5)	253.7 (1.2)	328.0 (0.7)
24	*E. coli*	182.5 (0.3)	219.2 (1.1)	242.6 (1.3)	307.8 (0.6)
70	*S. aureus* group	199.6 (0.3)	238.8 (1.7)	254.3 (0.8)	327.9 (0.5)
21	*S. pneumoniae/ S. mitis*	201.5 (0.3)	211.0 (1.5)	262.0 (0.5)	350-370
18	*E. faecalis*	195.5 (0.3)	202.4 (0.6)	260.3 (0.5)	327.8 (0.5)

[a] *n*: number of strains

[b] SD: standard deviation

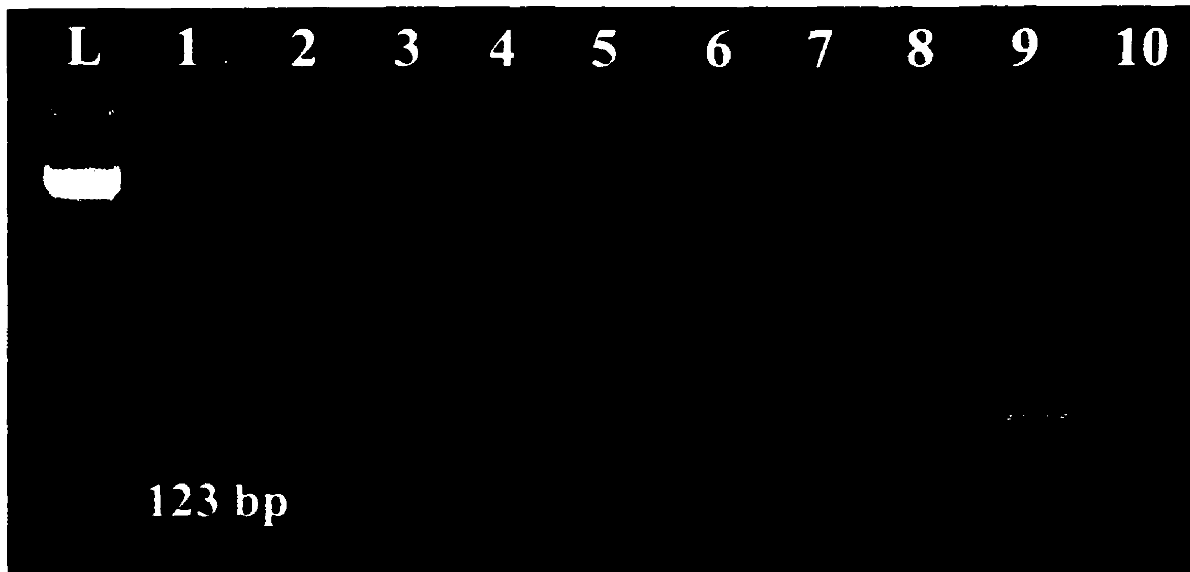
PART II. Fungal Identification Using the ITS2 Genetic Region.

1. PCR Amplification of the ITS2 Region

a. Primer Specificity in Whole Blood

No product of amplification was detected using the universal fungal ITS86 and ITS4 primers against human leukocytes, human whole blood, human liver or against any of the following bacterial organisms: *S. epidermidis*, *E. coli*, *S. aureus*, *P. aeruginosa* and *C. perfringens* (Figure 13).

Figure 13. **Specificity of universal ITS2 primers against bacteria and human genomic DNA.** PCR amplification using the ITS4 and ITS86 primer pair was performed as described in Materials and Methods. The following are the DNA templates used for the PCR. Lane 1: *S. epidermidis* ATCC 12228; lane 2: *E. coli* ATCC 25922; lane 3: *S. aureus* ATCC 25923; lane 4: *P. aeruginosa* ATCC 27853; lane 5: *C. perfringens* ATCC 13124; lane 6: human whole blood; lane 7: human leukocytes; lane 8: human liver; lane 9: *C. albicans* ATCC 10231; and lane 10: H_2O contamination control.



b. Primer Sensitivity in Whole Blood

The sensitivity of detection of *C. albicans* in whole blood using the ITS4 and ITS86 primers was determined to be as low as 1 CFU/ml (Figure 14).

2. Detection of PCR Amplified Fungal DNA

a. Agarose Gel Electrophoresis

The PCR product of all fungal species tested was initially visualized by UV illumination on ethidium bromide stained agarose gel to confirm successful amplification and variability in length of the final product. The yeast family demonstrated the highest level of interspecies variability (Figure 15-A). However, size determination based on agarose gel electrophoresis was not precise enough to definitively confirm species identification. Most other fungi also appeared to have little length variability on agarose gel electrophoresis.

b. Detection of *C. albicans* by Southern Hybridization

We have made use of a *C. albicans* specific probe for the sensitive detection of this organism in clinical samples. The probe, targeting a part of the ITS2 sequence unique to *C. albicans*, also detected *C. tropicalis* and *C. parapsilosis* as well with a 65°C hybridization temperature (Figure 15-B). Lowering the hybridization temperatures improved sensitivity. However, there was a loss in specificity at 57°C, as all *Candida sp* of the same blot, with the exception of *C. krusei*, hybridized with the probe (data not shown).

Figure 14. **Sensitivity of universal fungal ITS2 primers in whole blood.** PCR amplification of variable *C. albicans* ATCC 10231 suspensions in whole blood using the ITS4 and the fluorescent ITS86 universal fungal primers was performed as described in Materials and Methods. L: 123 bp ladder. Lane 1: 10^7 CFU/ml; lane 2: 10^6 CFU/ml; lane 3: 10^5 CFU/ml; lane 4: 10^4 CFU/ml; lane 5: 10^3 CFU/ml; lane 6: 10^2 CFU/ml; lane 7: 10^1 CFU/ml; lane 8: 1 CFU/ml; lane 9: C. albicans (one colony - positive control) and lane 10: H_2O contamination control.

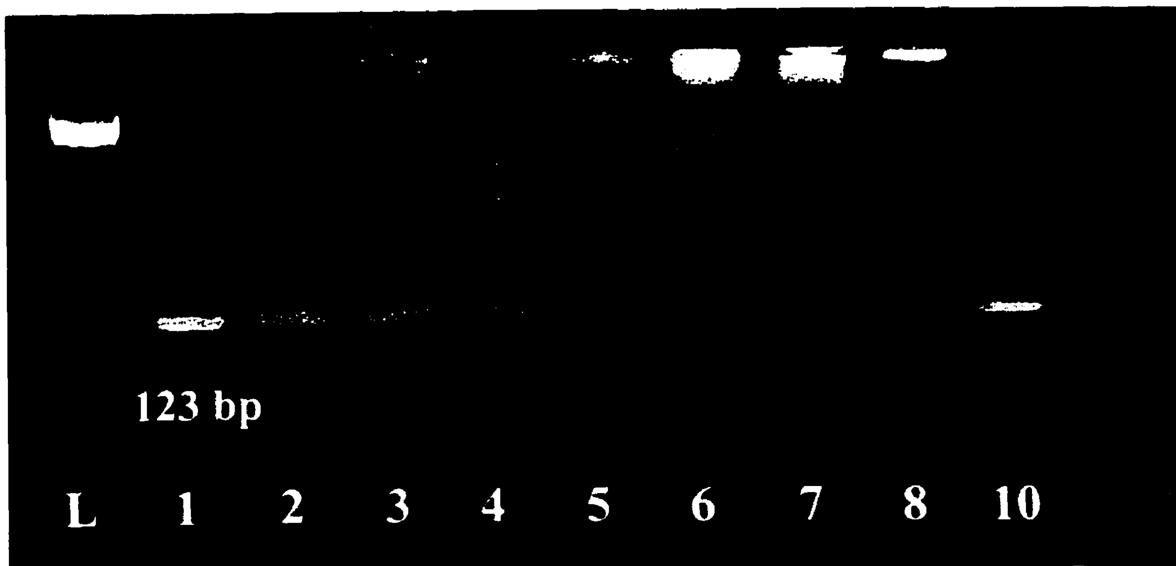
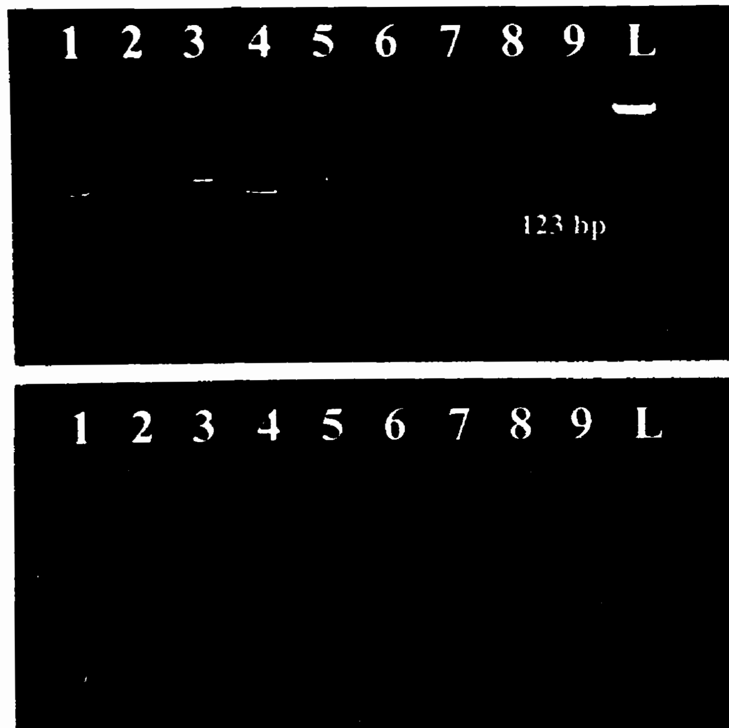


Figure 15. **Detection of fungal species by agarose gel electrophoresis and Southern hybridization.** PCR amplification of variable *Candida* species from culture colonies using the ITS4 and ITS86 universal fungal primers was performed as described in Materials and Methods. DNA transfer onto a nylon membrane and subsequent hybridization was performed as described in Materials and Methods, with a 1 hour hybridization incubation at 65°C. L: 123 bp ladder; lane 1: *C. albicans* control; lane 2: *S. aureus* negative control; lane 3: *T. glabrata*; lane 4: *C. tropicalis*; lane 5: *C. parapsilosis*; lane 6: *C. lusitaniae*; lane 7: *C. krusei*; lane 8: *C. guilliermondii*; and lane 9: *C. albicans*.



3. ITS2 Fragment Length Analysis

The inability to identify fungal organisms accurately by running PCR product containing the fungal ITS2 region on agarose gel electrophoresis compelled us to make use of an automated capillary electrophoresis system such as the ABI PRISM™ 310 Genetic Analyzer for precise fragment length determination.

a. The Determination of the ITS2 Fragment Length of Fungal Blood Culture Isolates.

The ITS2 region was amplified using the ITS4 and ITS86 universal fungal primer pair. Control ATCC, CAP or stock culture collection strains from the Health Sciences Centre, Winnipeg, representing 21 yeasts, 22 molds and 4 dimorphic fungi of clinical significance were used (Table 11). Figure 16 is a representation of ITS2 size differences of various *Candida* species as analyzed by the GeneScan software.

Within 21 species of the yeast group, 18 species had a unique ITS2 length. *C. stellatoides* had the same fragment length as *C. albicans* (279 bp). *C. neoformans*, *C. zeylanoides* and *H. anomala* differs by only one bp (315, 316 and 316 bp respectively).

In addition to the yeasts, 26 other medically important fungi were tested, including opportunistic and dimorphic fungi. The four *Aspergillus* species tested were of close fragment length: *A. fumigatus* (284 bp), *A. flavus* (288), *A. niger* (290 bp) and *A. terreus* (292 bp). Other opportunistic fungi tested included *Fusarium solani* (286 bp), *Absidia corynebifera* (410 bp), and *Cunninghamella bertholletiae* (347 bp). The majority of the other molds, the dimorphic and dematiaceous fungi, had unique ITS2 lengths. However, a few were equal to or one bp apart from other species. These included *Scedosporum prolificans* and *Cladosporum carrionii*, which were both 303 bp; *P.*

marneffei had the same length as *C. albicans*; *E. jeanselmei* and *S. schenckii* were one bp from each other.

Originally, the ITS2 fragment length determination was performed using dUTP in the PCR amplification mixture along with UDG for contamination precautions. Limited use of thermocyclers incited us to combine all fungal and bacterial PCR reaction mixtures to be run in the same instrument. Therefore, the cycling conditions needed to be identical. Preliminary testing showed that the use of UDG impeded upon the successful amplification of both 16S rRNA fragments in the multiplex PCR reaction of blood culture samples containing relatively less DNA: Gram-positives, for example. Consequently, we replaced the dUTP in the fungal amplification procedure with dNTP and omitted the UDG step when we began testing from blood culture isolates (seeded and patient specimens). This resulted in two sets of data. The set of results obtained using dNTP was in general 2-3 pb longer than those obtained using dUTP.

Table 11. The ITS2 length determination of known fungal isolates.

ORGANISM	Strains	Conventional ID	ITS2 length	
Yeasts:			**dUTP**	**dNTP**
Candida albicans	ATCC 10231; SCC[a]	API 20C-AUX	279.3	281.7
C. kefyr	ATCC 4135 ; SCC	API 20C-AUX	372.3	374.8
C. guilliermondii	CAP F1-95; SCC	API 20C-AUX	320.9	323.7
C. tropicalis	ATCC 66029; SCC	API 20C-AUX	268.6	271.0
C. krusei	ATCC 6258; SCC; CT1; K1865; CA37; E175	API 20C-AUX	282.5	283.8
T. glabrata	ATCC 90030; SCC	API 20C-AUX	359.5	362.6
C. zeylanoides	CAP F18-93	API 20C-AUX	316.1	318.6
C. lusitaniae	ATCC 42720	API 20C-AUX	198.7	200.9
C. parapsilosis	ATCC 22019; SCC	API 20C-AUX	251.3	253.1
S. cerevisiae	ATCC 9763	API 20C-AUX	363.9	366.9

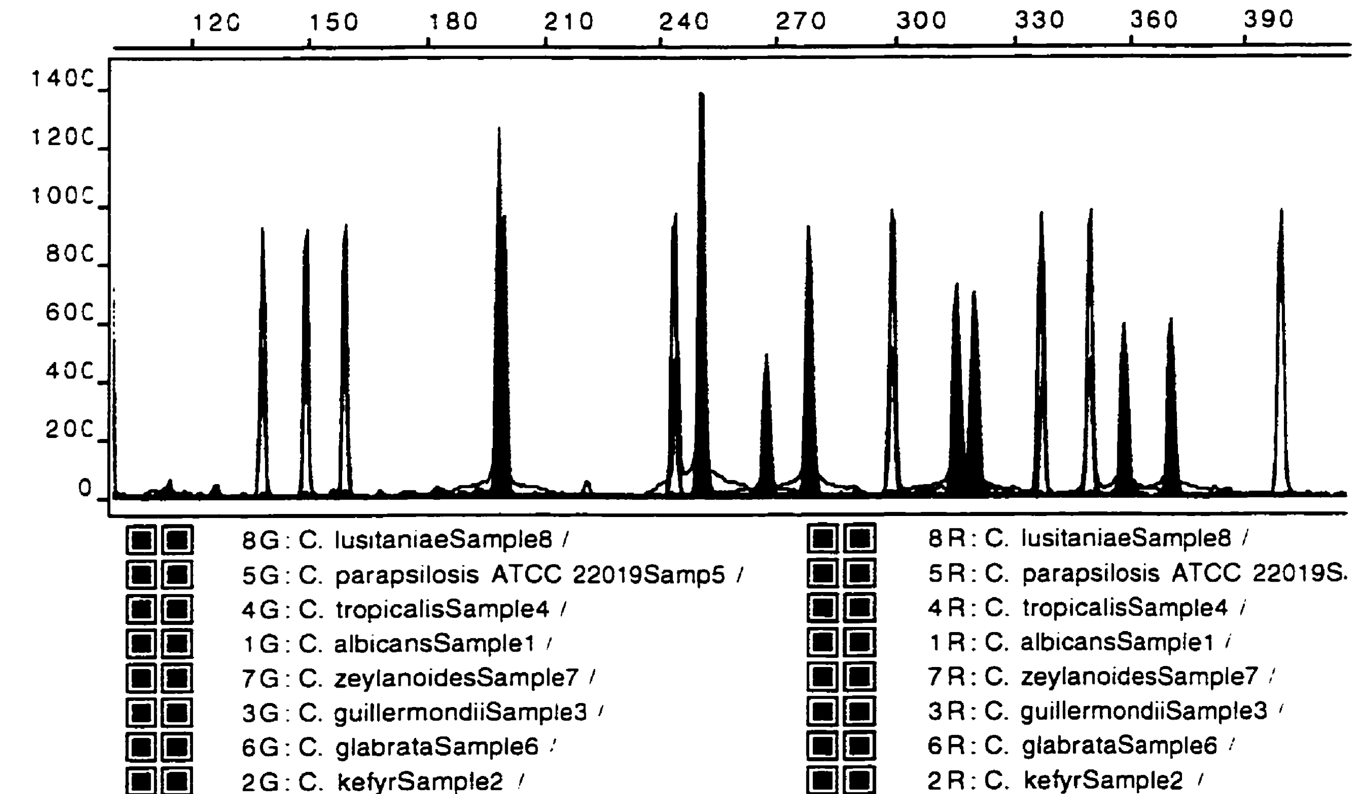
Continued...

Table 11. Continued...

ORGANISM	Strains	Conventional ID	ITS2 length	
Yeasts:			dUTP	dNTP
Trichosporon beigelii	ATCC 28592	API 20C-AUX	295.2	298.1
Geotrichum candidum	ATCC 34614	API 20C-AUX; mannitol	192.2	N/D
Cryptococcus neoformans	ATCC 90112	API 20C-AUX; urease; phenoloxidase	315.0	N/D
Cryptococcus albidus	ATCC 10666	API 20C-AUX	349.8	N/D
Cryptococcus laurentii	ATCC 18803	API 20C-AUX	306.0	N/D
Rhodotorula rubra	ATCC 9449; SCC	API 20C-AUX	347.6	350.1
Blastoschizomyces capitatus	ATCC 10663	API 20C-AUX	248.4	N/D
Hansenula anomala	ATCC 8168	API 20C-AUX	316.4	N/D
C. pseudotropicalis	ATCC 66028	API 20C-AUX	372.1	N/D
C. stellatoidae	ATCC 36232	API 20C-AUX	279.3	N/D
Malassezia furfur	ATCC 14521	API 20C-AUX	493.9	N/D
Other opportunistic fungi:				
Aspergillus fumigatus	CAP F6-91	Morphological	283.6	286.5
Aspergillus flavus	ATCC 10124	Morphological	287.8	291.2
Aspergillus niger	ATCC 16404	Morphological	289.8	292.9
Aspergillus terreus	ATCC 28301	Morphological	292.2	N/D
Alternaria alternata	CAP F13-93	Morphological	284.8	287.8
Penicillium sp.	SCC	Morphological	283.2	N/D
Acremonium strictum	CAP F4-96	Morphological	293.9	N/D
Fusarium solani	CAP F15-95	Morphological	285.9	288.9
Paecilomyces sp.	SCC	Morphological	287.2	N/D
Rhizopus sp.	SCC	Morphological	319.2	N/D
Absidia corymbifera	ATCC 66271	Morphological	410.2	N/D
Mucor sp.	SCC	Morphological	311.7	N/D
Cunninghamella bertholletiae	ATCC 42115	Morphological	347.1	349.3
P. boydii	ATCC 58085	Morphological	321.1	324.0
S. prolificans	SCC	Morphological	303.3	305.8
Phialophora verrucosa	ATCC 28181	Morphological	308.5	N/D
Phialophora richardsiae	ATCC 26465	Morphological	275.9	279.7
Fonsecaea pedrosoi	ATCC 28174	Morphological	319.1	320.9
Cladosporium carrionii	ATCC 32279	Morphological	303.2	306.3
Wangiella dermatitidis	SCC	Morphological	333.5	336.8
Exophiala jeanselmei	ATCC 10224	Morphological	311.3	312.9
Ulocladium (Stemphylium ilicis)	ATCC 18160	Morphological	279.5	383.3
Dimorphic fungi				
Penicillium marneffei	ATCC 18224	Morphological	279.5	N/D
Sporothrix schenckii	ATCC 10212	Morphological; conversion to yeast phase	309.9	N/D
Blastomyces dermatitidis	HSC[b] patient isolate	Nucleic acid probe	314.6	N/D
Histoplasma capsulatum	HSC patient isolate	Nucleic acid probe	299.0	N/D

[a]SCC: stock culture collection from the Health Sciences Centre, Winnipeg.
[b]HSC: patient isolates obtained from the Health Sciences Centre, Winnipeg.

Figure 16. Overlapping electropherograms of 8 Candida species as analyzed by the ABI PRISM™ 310 Genetic Analyzer. All 8 control samples were amplified using ITS4 and fluorescently-labeled ITS86 and dUTP as described in Materials and Methods. Each was run separately on the capillary electrophoresis system using a denaturing polymer along with an internal size standard (GeneScan ROX-500; red peaks) for precise fragment length determination. The actual data obtained from the GeneScan software are also shown with emphasis being placed upon the "size" column.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
8G, 1	16.32	198.67	1267	12360	4451
5G, 11	18.52	251.66	1388	16183	5050
4G, 2	18.30	268.29	502	5546	4989
1G, 2	18.97	279.22	941	10794	5172
7G, 3	19.55	315.85	739	9062	5330
3G, 1	19.77	320.43	710	8707	5392
6G, 1	20.68	358.82	593	7700	5639
2G, 1	21.33	371.51	618	7995	5817

b. Identification of Fungi Obtained from Blood Culture Specimens

α. Seeded Study

Since *Candida* species, the most commonly isolated fungi from blood cultures, constitutes only 5% of all microbial isolates, we included 103 seeded blood culture specimens to be treated as a patient blood culture specimen. When positive, these were identified by molecular technology (ITS2 length determination) and by conventional methodologies. Ten different yeast species were used in the seeded study (Table 12). Two seeded *C. zeylanoides* specimens failed to be detected by the BacT/Alert system. Therefore, they were excluded from our analyses.

Table 12. Results of the identification of the seeded blood cultures with yeast.

Organism	strain	*n*	Length[a] (SD[b])
C. albicans	SCC	42/42	281.7 (0.4)
C. krusei	SCC	6/6	283.8 (0.2)
C. tropicalis	SCC	8/8	271.0 (0.5)
C. guilliermondii	SCC	9/9	323.7 (0.4)
C. glabrata	SCC	7/7	362.6 (0.4)
C. kefyr	SCC	6/6	374.8 (0.2)
C. lusitaniae	CAP 121	8/8	200.9 (0.5)
C. parapsilosis	SCC	7/7	253.1 (0.4)
S. cerevisiae	ATCC 9763	10/10	366.9 (0.3)
C. zeylanoides	CAP 131	failed to grow in BC (2)	N/A

[a]Amplification using dNTP
[b]SD: standard deviation

The standard deviation of the ITS2 fragment length as indicated in table 12 is believed to be similar for run to run variability as well as for species to species variability, as long as there is no length variation among species of a same strain.

β. Blood Cultures from Patients

Of the 304 patient blood culture positive specimens tested, yeast organisms were identified by conventional methodologies in 27 cases. Successful DNA extraction took place in 24 of these, of which the results are indicated in Table 13. Molecular identification corresponded with conventional identification. However, *T. beigelii* appeared to be ~3 bp longer than that determined for the corresponding ATCC strain. Correct molecular identification occurred simply due to the fact that the Gram-stain indicated a yeast organism, and *T. beigelii* was the closest match. Only two specimens were mixed cultures (Table 9): one specimen contained both *C. albicans* and *C. tropicalis* in which *C. tropicalis* was detected, the other contained *C. albicans* (detected) and a *Corynebacterium* species which was not detected by amplification using the 16S rRNA primers.

Table 13. Patient blood culture results.

Organism	*n*	Length[a] - average
C. albicans	5/5	282.2
C. glabrata	3/5[b]	262.4
C. tropicalis	11/12[b]	270.9
C. parapsilosis	2/2	253.1
R. rubra	2/2	350.1
Trichosporon beigelii	1/1	301.1

[a]Amplification with dNTPs
[b]Unsuccessful DNA extraction/amplification occurred for 2 C. glabrata and 1 C. tropicalis

Part III. Time and Cost Analysis of Methodologies

1. Time to Identification from Blood Culture Positivity

a. Conventional Methodology

The time to final identification of blood culture isolates was determined from the time the BacT/Alert system gave a positive signal. This did not include the number of days of incubation prior to the positive signal: 1-7 days (data not shown). The rationale for this was we could not compare this timeline with molecular testing. Molecular analysis would have to be performed on all specimens as they were collected, in which case, only a little over 10% of specimens would potentially be positive. This aspect of the clinical study remains to be investigated.

Table 14. Time to final identification from positive blood culture signal: conventional testing.

	n (%)		
Days to final report	**Bacteria**	**Fungi (patients)**	**Fungi (Seeded*)**
1	10 (3.7%)	0	64 (63.4%)
2	147 (53.8%)	0	18 (17.8%)
3	56 (20.5%)	2 (8.3%)	10 (9.9%)
4	39 (14.3%)	6 (25%)	9 (8.9%)
5	14 (5.1%)	1 (4.2%)	0
6	4 (1.5%)	5 (20.1%)	0
7	1 (0.4%)	3 (12.5%)	0
8	2 (0.8%)	2 (8.3%)	0
9	0	3 (12.5%)	0
10	0	2 (8.3%)	0
Total	273 (100%)	24 (100%)	101 (100%)

* The seeded and patient fungal blood culture specimens results were separated to eliminate any bias, as the seeded specimens were identified much faster than customary.

b. Molecular Identification

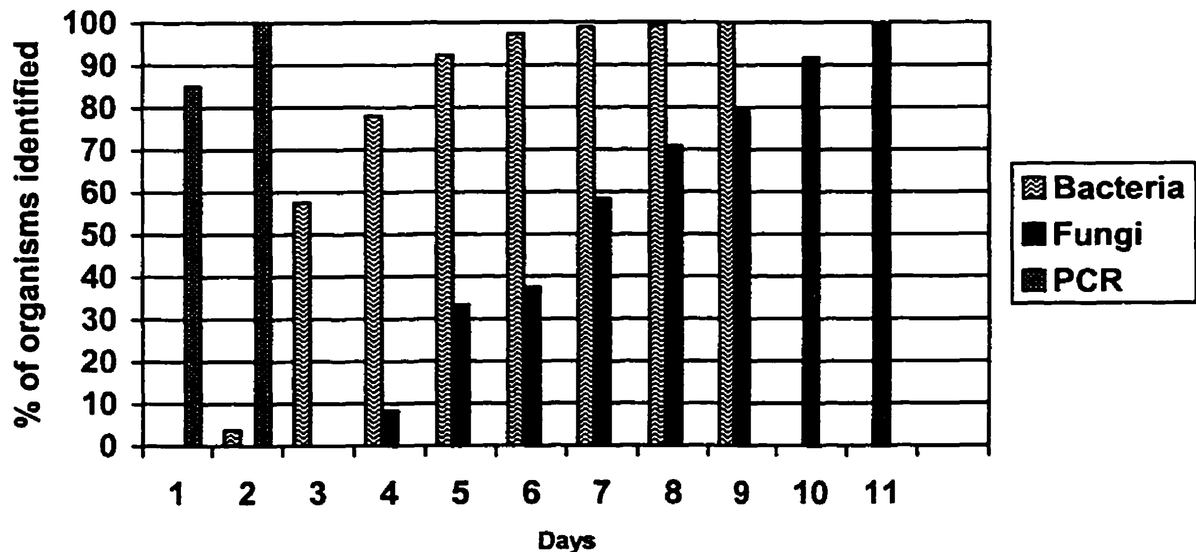
Real time for molecular identification from blood culture positivity was not determined as the work was performed by an individual investigator and efficiency of workload was a priority over rapidity of identification for the purpose of research. In addition, the capillary electrophoresis instrument was shared by other departments and therefore was not available at all times. Consequently, a theoretical approach was used for the determination of the time to identification of an organism using PCR with SSCP or fragment analysis from the time the specimen is made available (Table 15).

Table 15. Time to identification by molecular methodology.

Time to identification using SSCP/Fragment analysis	
Lysate preparation from positive blood culture	2.5 - 3 hours
DNA amplification by PCR (including set up time)	3 hours
SSCP/ Fragment analysis	0.5 hours
Time to identification from positive blood culture:	
Single Capillary, one specimen	**7 hours**
Each additional specimen	0.5 hours (1 run per capillary)
Multiple (ex: 6) Head CE, up to 6 specimens	**7 hours**
Each additional 6 specimens	0.5 hours
Identification of uncommon bacteria isolates by sequencing	
PCR with non-fluorescent primers	3 hours
Sequencing reaction/ product purification	3.25 hours
Sequencing run, single capillary	2.5 hours
BLAST Search on the Internet	15 minutes
Time to sequence identification from SSCP result	**9.0 hours**
Each additional specimen	2.5 hours

The time to identification using GeneScan analysis for SSCP or fragment analysis was from 7 hours to the next day for 1 to 48 specimens. If sequencing was required, a final result could be obtained by the end of the second day for 1 specimen, or the morning of day 3 for up to 7 specimens, or more if run on slab gel. Figure 17 is a schematic comparison of the time to identification of bacteria and fungi between molecular and conventional methodology.

Figure 17. **Time to identification from a positive blood culture signal.** Time to identification for PCR methodology is hypothetical. Time to identification of bacteria and fungi was based on the actual time taken for conventional identification of 273 patient blood cultures positive for bacteria and 24 patient blood cultures positive for yeast.



2. Cost Analysis: Conventional vs. Molecular Methodology

The cost analysis was determined for all reagents and labor for conventional testing and molecular testing (Table 16). The average cost for conventional identification per blood culture isolate ranges from 58$ to 67$. With molecular testing, costing is significantly lower when more than one test is processed at one time. As the number of positive blood cultures obtained in one day is usually lower than 10, we have calculated the cost of running 10 samples for SSCP identification to be $15 per sample. Processing samples for sequencing is also more economical when running more than one sample. However, as the SSCP map becomes more complete, sequencing will be performed only on occasion. The theoretical cost of identification by sequencing of 10 isolates in one group would be 34$ per sample, including prior SSCP testing.

Table 16. Cost comparison of conventional and molecular testing.

Conventional testing:	Reagent cost ($)	Labour cost ($)	Total ($)
Yeast	17.74	39.84	**57.58**
Anaerobes	21.74	43.75	**65.49**
Gram positive cocci	18.75	44.82	**63.57**
Gram negative bac.	19.59	46.95	**66.54**

Molecular testing:	Reagents ($)		Labour ($)		Total ($)	
	1 test	10 tests	1 test	10 tests	1 test	10 tests
Lysate preparation	3.17	31.70	3.84	38.40	7.01	7.01 ea.
PCR	3.55	18.60	1.60	4.80	5.15	2.34 ea.
SSCP/GeneScan	14.83	33.17	4.80	21.22	19.63	5.44 ea.
Total	21.55	83.47	10.24	64.42	**31.79**	**14.79 ea.**
Sequencing:						
PCR	3.55	18.60	1.60	4.80	5.15	2.34 ea.
Sequencing	17.20	150.14	10.60	19.86	27.80	17.00 ea.
Total (incl. SSCP)	42.30	252.21	22.44	89.08	**64.74**	**34.13 ea.**

DISCUSSION

We have used DNA amplification technology as a more rapid, specific, and cost-effective way to identify bacteremia and fungemia in an attempt to improve upon the impediments associated with blood culture testing. The ITS2 region demonstrates sequence length variability between clinically significant molds and yeasts while the bacterial 16S ribosomal gene demonstrates species-specific sequence variability that results in unique DNA fragment conformation, which can be easily demonstrated. We used the ABI PRISM™ 310 Genetic Analyzer, an automated capillary electrophoresis sequencer, to speciate the fluorescently tagged bacterial and fungal amplicons using the ABI PRISM 310 GeneScan™ Analysis Software. The advantages were promising as this method provided species identification within 7 hours of blood collection and was highly sensitive, specific and cost-effective. This method has yet to be perfected, as obstacles have been encountered such as preferential amplification in mixed cultures, as well as certain species of both bacteria and fungi that possess similar SSCP patterns or ITS2 lengths.

Part I. PCR: From DNA Target Choice to PCR Product Detection

Many factors come into play whenever a new method employing PCR or DNA hybridization is developed for the diagnosis of a bacterial or fungal infection. Decisions need to be made regarding the choice of target DNA and its subsequent detection. If the information to be obtained from a PCR product goes beyond simple detection, the resulting DNA fragment must contain certain characteristics that allow differentiation between the bacterial or fungal species. We used the 16S rRNA gene for rapid bacterial

identification and the ITS2 region for rapid fungal identification. The sequence characteristics of the 16S rRNA gene and the ITS2 region offer a dual advantage: 1) variable regions useful for species identification and 2) conserved regions that permit the potential amplification of all bacteria or fungi.

Many common aspects that apply to most PCR protocols must be investigated such as primer specificity, sensitivity and issues of contamination. To avoid obtaining a false positive PCR reaction as a result of cross-amplification or contamination, we ran a separate negative control with all reactions which underwent the same steps as the test samples, replacing the DNA template with sterile water. We also tested the bacterial and fungal universal primers against various amounts of human DNA, bacteria (for fungal primers) and fungi (for bacterial primers), and no cross-contamination was detected. Due to the highly sensitive nature of PCR technology, all PCR preparations were performed in a designated "sterile" area to prevent possible DNA contamination. The addition of DNA template, the use of the thermocycler and all post-PCR procedures, in which PCR product was manipulated, were performed in separate rooms.

There are many variations of DNA extraction protocols for PCR amplification. An ideal DNA extraction method is one that removes all PCR inhibitors, extracts the DNA from each target cell in the sample and limits the possibility of contamination. Using a phenol-chloroform extraction method generally eliminates possible PCR inhibitors, including hemoglobin, which can be found in blood. We used such a method with both bacteria and fungi from whole blood resulting in successful PCR amplification. The task of extracting DNA from bacteria was performed within 2.5 hours and from yeast organisms, within 1.5 hours. The DNA extraction of some molds, however, was

somewhat lengthy and cumbersome. *A. terreus*, *Penicillium sp*, *Paecilomyces sp.*, *S. schenkii* as well as all dermatophytes (data not included) which were amplified using the universal fungal primers ITS86 and ITS4 required certain additional steps such as NaOH digestion, extended vortexing with glass beads to facilitate cell breakage and extended RNase incubation for successful DNA extraction. Although the great majority of blood isolates are yeasts, the use of the longer extraction method is recommended to detect the presence of all fungi.

Culture broth fluid inoculated with blood is not normally considered an ideal clinical specimen for PCR amplification as the SPS, a common additive to blood cultures that is used to enhance bacterial growth by inactivating complement, is inhibitory to the amplification reaction (Fredricks and Relman, 1997). We were provided with a DNA extraction method for blood cultures which included a benzyl alcohol step that removes SPS from the blood culture aliquots to a level where PCR amplification was successful. This method worked for both bacteria and yeasts in the course of this study. We obtained DNA amplification from 297 of 304 (98%) positive patient blood cultures and from all positive seeded blood cultures (n=101). DNA from 7 of the patient specimens was not successfully amplified. These included a variety of species: 2 *C. glabrata*, 1 *C. tropicalis*, 1 *S. aureus*, 1 *S. pyogenes*, 1 *S. hominis* and 1 *E. cloacae*. At present, no measures have been taken to improve this already excellent extraction method. However, the cause of failure of extraction to be determined. In the context of early diagnosis, we contemplate using whole blood collected in EDTA tubes as our choice specimen, and thus the improvement of the sensitivity of the benzyl alcohol extraction method may not be as important.

For this study, we tested the sensitivity of the 16S rDNA universal primers using whole blood seeded with *E. coli* (10^7 to 10 CFU/ml) collected in EDTA tubes. Amplification was successful at all concentrations. The majority of contaminants (97%) are found at levels of <1 CFU/ml (Henry *et al.*, 1983) and may possibly not be detected. The sensitivity of detection using ITS2 universal primers was determined to be as low as 1 yeast cell/ml. This high sensitivity may be due to the high copy number of ribosomal genes in *C. albicans* (Magee *et al.*, 1987).

DNA template integrity was tested under conditions of prolonged storage of blood samples at 4°C (up to 21 days) and was not affected. Interestingly, while *E. coli* survived well under these storage conditions, storage of *S. epidermidis* in whole blood resulted in the inability to grow these organisms after 10 days. However, since the target DNA was still present in the sample, amplification was not affected. The inhibitory effect of the presence of high and low protein bound antibiotics upon the sensitivity of the PCR reaction was also tested and none was exhibited.

The nature of the 16S rRNA gene is such that a variety of fragments can be amplified using a number of universal primer pair combinations. We used five combinations of 16S rDNA universal primers, each being equally useful for detection of bacteria. These primers can also be used for sequencing and SSCP. However, for these purposes, some are preferred over others. We chose the 515/806R pair and RW01/13B pair for SSCP due to their relatively small fragment size. Sequence-based conformational differences are greater with SSCP if the fragment is short (~200-300 bp). On the other hand, a longer fragment, amplified by the 91E/13B primer pair, was chosen for sequencing because it was long enough to include 3 variable regions (V6-V8) and the

right length for the maximal successful sequencing (<500 bp). The only primer pair we used for amplification of all fungi targeted the two ribosomal genes adjacent to the ITS2 region, resulting in the full amplification of the length-variable intergenic region.

PCR product obtained by using universal primers can be visualized on a standard ethidium bromide stained agarose gel. However, this can only serve as a detection method for the presence of organisms and not as a system for comprehensive species identification. While the use of DIG-labeled probes is very sensitive, it is too costly and time-consuming.

We initially attempted to differentiate Gram-negative organisms from Gram-positive organisms. The ideal hybridization conditions are difficult to attain, yet crucial. Under less than the most stringent conditions, both *E. coli* and *S. aureus* hybridized to the Gram-negative probe. The probe was more specific under more stringent conditions, however, sensitivity was greatly decreased.

We also used a *C. albicans* species-specific probe. However, this probe detected *C. tropicalis* and *C. parapsilosis* in addition to *C. albicans*. This is not surprising, as these three species are extremely close phylogenetically using the 5.8S and ITS2 sequences, particularly in the area targeted by the probe (Lott *et al.*, 1993). In addition, speciation using probes is only possible utilizing species-specific probes and therefore cannot be used for broad-range identification in a practical setting.

Part II. The Molecular Identification of Bacteria from Blood

The advent of capillary electrophoresis allows for the visualization of PCR product amplified with fluorescent primers. The use of the ABI PRISM™ Genetic Analyzer permitted rapid analysis of both ITS2 and 16S rDNA PCR fragments in this study. Advantages in using such a system include speed, where each run is less than half an hour in comparison with an equally precise slab gel system, which runs for approximately 4 hours. Sequencing reactions can now be done within 2.5 hours in comparison with 14 hours using slab gel electrophoresis. Furthermore, minimal labor is required as this "walk away" system is highly automated and can process up to 96 samples in one set-up. The sensitivity of fluorescent detection, along with the rapidity and ease of analysis, renders this system ideal for molecular diagnostics. However, the capillary electrophoresis system is not yet built to run more than one sample at a time. In the near future, multi-head capillary systems will exist to permit the simultaneous detection of multiple samples.

1. SSCP: The Identification of Species Based on Unique Patterns

The PCR-SSCP patterns of the most commonly isolated bacterial species from blood cultures (comprising of approximately 87% of isolates) has been determined using, for most species, a control ATCC strain and a previously identified blood culture isolate of the same species. There was no discrepancy in the SSCP pattern within any one species. An SSCP pattern map was then created and served as reference for the identification of all blood culture isolates that became positive from December 1997 to March 1998, with a final total of 304 blood culture specimens: 277 bacteria and 27 yeast. The ultimate objective is to use the methodology developed here for the identification of

bacteremia and fungemia directly from patient blood. However, in this study, we have tested the method from positive blood cultures to ensure we had an adequate sample size to determine the specificity of organism identification. This was a double-blinded study in which the only information given to this researcher was whether the blood culture contained bacteria or fungi, as determined by a Gram-stain routinely performed with positive blood cultures. The comparison of PCR-SSCP identification and conventional blood culture testing results of the 304 blood culture positive specimens was performed at the end of the study.

Molecular identification of bacteria found in the blood culture samples was determined by comparing the SSCP result obtained with the map containing the previously determined SSCP pattern from known species. If a new SSCP pattern belonging to a less common organism was observed, identification was determined by sequencing part of the 16S rRNA gene from the corresponding blood culture lysates with non-fluorescent primers 91E and 13B. These primers were very successful for sequencing, as they amplified a longer fragment containing regions V6 to V8. The newly discovered SSCP patterns were then added onto the formerly created map, making it more and more complete for future referencing (Table 17).

A total of 252/273 (92%) specimens containing only bacteria resulted in the detection of organisms presenting a known SSCP pattern that could be referenced by the original SSCP pattern map created using control strains. All isolates identified conventionally as a species of which the SSCP pattern had previously been determined (*n*=245) presented a correct SSCP pattern (on the species level, as a CoNS, or as viridans) upon detection, resulting in a specificity of 100%. Six specimens presented a known

Table 17. Map of the SSCP peak patterns acquired to date. The following include a combination of the SSCP pattern of those originally determined using control strains and of those acquired and sequenced in the course of this study.

Species	RWO1	13B	515	806R	Similar pattern
S. epidermidis	**207**	**243**	254	328	
S. aureus	**199**	**238**	254	328	*S. hominis* *S. haemolyticus* *S. auricularis* *S. simulans*
S. capitis	**197**	238	254	328	
S. warneri	**202**	**232**	254	328	
S. cohnii	**204**	**235**	254	328	
S. pneumoniae	**201**	**211**	**262**	**350-370**	*S. mitis*
S. salivarius	197	**221**	258	328	
S. mutans	196	**214**	258	328	
Streptococcus A	199	214	259	**326**	
Streptococcus B	199	214	262	**322**	
E. faecalis	195	**202**	260	328	
E. faecium	192	**211**	260	328	
Micrococcus sp.	**205**	235	242	**320**	
P. acnes group	**182**	**226**	-	-	
Clostridium sp.	209	235	250/ 256[a]	336	
Corynebacterium-1	185	222	242	**315**	
Corynebacterium-2	185	222	242	**325**	
Corynebacterium-3	**192**	**225**	**233**	326	
L. monocytogenes	195	220	258	313	
Actinomyces sp.	209	241	-	-	
Peptostreptococcus sp.	216	243	278	330	
E. coli	182	219	243	308	
K. pneumoniae	187	227	235	318	*E. cloacae*
Enterobacter-1	187	**217**	235	318	
Enterobacter-2	**182**	227	235	318	
P. aeruginosa	183	230	253	313	
S. maltophila	194	227	250	330	
K. oxytoca	195	216	234	318	
B. fragilis	186	215	249	318	
C. koseri	189	217	240	307	
H. influenzae	190	232	267	325	
N. gonorrhoeae	196	223	256	315	

[a]Two peaks detected. Perhaps two related clostridium species present.

SSCP pattern but were identified conventionally as a new species. These were sequenced to confirm identity and included: *E. amnigenus* (*E. cloacae* pattern); *Gemella sp.* and *Stomatococcus sp.* (*M. luteus* pattern); *Propionibacterium sp.* and 2 diphtheroids (*P. acnes* pattern). This information indicates that closely related species can demonstrate an identical SSCP pattern. One specimen presented an SSCP pattern unique to *E. faecalis* and was sequenced as such, however *S. epidermidis* and a non-dextrose fermenter was isolated upon culture. A possible explanation would be that *E. faecalis* was present in the sample, but was not isolated by conventional culture techniques. This was perhaps due to the overgrowth of the other two organisms.

A total of 21/273 (8%) specimens contained organisms presenting a new SSCP pattern. The species identification by sequencing corresponded to 14/21 (67%) specimen results from conventional testing. Questions raised with the conventional and sequencing identification of the remaining 7 specimens are discussed in section D. Part II. 2.

The standard deviation (SD) was determined for the retention time of each SSCP peak obtained from the five most commonly detected organisms. In general, the first (RW01) and last (806R) peaks showed a constant value from run to run and from strain to strain (SD: 0.3–0.6), whereas the center peaks (13B and 515) spanned a slightly wider range of data points (SD: 0.6–1.7) between runs. It is highly probable that the determined SDs of these peaks apply to most SSCP patterns belonging to other species.

2. Issues of Mixed Cultures, BLAST Analysis, and Other Concerns

In theory, all organisms present, even in mixed cultures, are subject to amplification. Although the analysis of additional peaks for 2 organisms may become

difficult, it was in actual fact quite easy to interpret. However, we did encounter selective amplification of a single organism in 32/44 (73%) of mixed cultures containing only bacteria. In all cases with mixed Gram-positive and Gram-negative organisms (*n*=12), Gram-negative organisms amplified preferentially over Gram-positive organisms. This may have been due to the ease with which DNA can be extracted from Gram-negatives, which lack a cell wall. Another possible reason for preferential amplification is primer-template mismatches (Edwards and Gibbs, 1995). Although universal primers can amplify all microorganisms in general, there can be sequence variation present within a primer region that may result in preferential amplification of an amplicon with a better match. Other causes include the variable concentration of the organisms. Unfortunately, we unable to perform quantitative cultures to correlate quantitation with amplification due to experiment design.

Preferential amplification occurred in cultures containing exclusively Gram-positive bacteria as well. *Streptococcus* species alone were detected when mixed with *Staphylococcus* species (*n*=4), with the exception of three samples in which both were detectable. *E. faecalis* alone was detected when mixed with other Gram-positive organisms (*n*=4). Out of a total of 40 isolates undetected in 32 specimens, 2/3 of them were considered contaminants. Perhaps these have not successfully amplified due to their low concentration in the specimen itself. Blood culture positivity may have resulted exclusively from the responsible pathogen in some cultures and the contaminants, isolated upon culture. Preferential amplification in mixed blood specimens must be studied further to determine if optimizing amplification conditions can circumvent this issue.

Of the 44 mixed specimens, 10 had two identifiable peak patterns and, of those, 2 resulted in 8 peaks. One of them had a pattern corresponding to *E. coli* and to the *E. cloacae/K. pneumoniae* group. Interestingly, conventional culture methods yielded only the *E. coli*. Perhaps the *E. coli* was by far the predominant organism, having overgrown the other Gram-negative organism on culture and was therefore the only organism biochemically tested for identification. In the other specimen, two new patterns appeared, which were identified by sequencing as *Actinomyces neuii* and *Peptostreptococcus hareii*.

Other blood cultures revealing two SSCP patterns included mixed species that were different from each other by only one or two bands, therefore no more than 5 or 6 bands would be detected altogether. These included 3 specimens that contained 2 *Staphylococcus* species and 1 specimen conventionally identified as *E. cloacae* and *K. pneumoniae* of which the SSCP patterns observed were *E. cloacae/K. pneumoniae* and *Enterobacter*-1. In three samples from the same patient containing both *S. aureus* and Viridans streptococci, all 4 peaks were detected for *S. aureus*, while 2 peaks belonging to the primer pair yielding the shorter amplicon (RW01 and 13B) of the *S. pneumoniae/S. mitis* pattern was detected. It has been suggested that preferential amplification can occur for the shorter amplicon in a multiplex reaction (Edwards and Gibbs, 1995). Be that as it may, that primer pair alone was sufficient to discriminate between most organisms, allowing identification of both species.

Issues of minor discordance between conventional testing and PCR-SSCP/sequencing were re-examined by conventional methodologies. Among these, 7 were CoNS that were identified as one species using conventional methodology, while

the SSCP pattern showed they could not be of that CoNS species. Their biochemical re-evaluation using MicroScan panels indicated CoNS species of low probability (~40-73% probability), signifying that the organism was CoNS, but with an incorrect species identification. Previous studies have shown that MicroScan panels accurately speciate CoNS only 50-90% of the time (Kloos and George, 1991). Since the clinical importance of reporting the specific species of these organisms is limited, it does not warrant further testing.

In 2 cases, a new SSCP pattern, which warranted subsequent sequencing, resulted in unsuccessful positive identification. One of these identified as *S. kloosii* (low probability) by conventional methodology was most closely matched with *Brachybacterium conglomerans* upon sequencing, although 4 true mismatches out of 285 bp entered in BLAST Search were detected. All matches following up to 15 mismatches also belonged to that genus, with the exception of *Micrococcus sedentarius*, with 11 mismatches. The Gram-stain indicated that this organism was a Gram-positive coccus; however, *Brachybacterium* are Gram-positive bacilli. A possible explanation could be that this organism that was not accurately identified by the MicroScan panel. As well, its sequence may not have been determined and thus did not appear in Genbank. Similar problems were encountered with another organism with a unique SSCP pattern. The resulting sequence came to a closest match of 5 mismatches to an EMBL sequence identified only as "Bacterium unidentified Gram + rod". Next on the match list was *Arthrobacter sp* followed by *Brevibacterium casei*. The conventional methodologies were unable to identify this organism other than its being a Gram-positive bacillus.

We have not been able to determine why different patient strains, identified by conventional testing as *E. cloacae*, demonstrated 3 different patterns. Sequencing of *Enterobacter* species is questionable as there is only one 16S sequence of *E. cloacae* in all sequence databanks and it does not perfectly match the sequence we have ourselves obtained using the ATCC strain. In fact, the ATCC strain, as well as strains having the *Enterobacter*-1 pattern, are a 100% match with *E. asburiae* or *Pantoea sp.* as determined from the BLAST Search. Biochemical testing confirmed that these isolates were motile like *E. cloacae*, contrary to *E. asburiae* and *Pantoea* species. Whether these strains represent a non-typical *E. cloacae* SSCP pattern or in fact another *Enterobacter* species remains to be determined. Another possibility may be that some fragments may adopt two conformations. However, the pattern obtained from each strain was consistent. Perhaps sequencing the multiplex PCR fragments (separately) and examining the alignment of the obtained sequences would help to determine whether these organisms are in fact the same species. Extensive biochemical testing would then be useful in determining the true identity of these strains if sequencing indicated different species.

The major drawback of the BLAST Search site is that the percentage similarity (indication of the number of mismatches or N's) is affected by the quality of the sequence obtained and by the accuracy of the database sequence. As sequence submission to GenBank or other databases are not peer reviewed, there is no indication of the quality of the sequence, or how the sequencing was performed. For many organisms, particularly commonly referenced organisms such as *E. coli* and *S. aureus*, there may be 10, 20 or even 50 submitted sequences belonging to the 16 S rRNA gene alone. Some of these, coming from different sources, may have slight variations. However, within our

laboratory, 16S rDNA sequencing was shown to be consistent within a species (Turenne *et al.*, 1998).

Part III. The Molecular Identification of Fungi from Blood: The Identification of Species Based on Unique ITS2 Lengths

Previous studies have demonstrated the potential of the ITS2 region for species-specific identification of common *Candida* pathogenic species as well as for other pathogenic fungi. Unlike SSCP, which is dependent upon sequence variability, fragment analysis using a denaturing gel (such as one containing urea) detects variations in the ITS2 fragment length found among fungal species. In this study, using fluorescent primers, we have amplified the ITS2 regions of most pathogenic fungi, allowing the PCR product to be rapidly and accurately sized using the ABI PRISM™ 310 Genetic Analyzer. The use of rDNA genes for identification of fungal species was based on specific detection of conserved sequences in the 5.8S rDNA and 28S rDNA that exist in fungi, enabling amplification of the ITS2 region in all fungi.

The PCR product length containing the ITS2 region of 47 various fungal species, including the most clinically significant yeast and molds, was determined using ATCC strains (Table 18). Reference strains were used for the majority of the specimens, as well as some clinical strains, to ascertain intraspecies parallelism for example with *C. krusei*, whose ITS2 length is close to that of *C. albicans*. This map served as a reference for the identification of fungi present in seeded and patient blood culture specimens.

Table 18. Map of ITS2 fragment length of clinically significant fungi (with dUTP).

Yeast	ITS2	Dimorphic fungi	ITS2	Molds	ITS2
G. candidum	192.2				
C. lusitaniae	198.7				
B. capitatus	248.4				
C. parapsilosis	251.3				
C. tropicalis	268.6				
				P. richardsiae	275.9
C. albicans	279.3	*P. marneffei*	279.5	*S. ilicis*	279.5
C. stellatoides	279.3				
C. krusei	282.5			*Penicillium sp.*	283.2
				A. fumigatus	283.6
				A. alternata	284.8
				F. solani	285.9
				Paecilomyces sp.	287.2
				A. flavus	287.8
				A. niger	289.8
				A. terreus	292.2
				A. strictum	293.9
T. beigelii	295.2				
		H. capsulatum	299.0		
				C. carionii	303.2
				S. prolificans	303.3
C. laurentii	306.0				
				P. verrucosa	308.5
		S. schenckii	309.9		
				E. jeanselmei	311.3
				Mucor sp.	311.7
C. neoformans	315.0	*B. dermatitidis*	314.6		
C. zeylanoides	316.1				
H. anomala	316.4				
				F. pedrosoi	319.1
				Rhizopus sp.	319.2
C. guilliermondii	320.9			*P. boydii*	321.1
				W. dermatitidis	333.5
R. rubra	347.6			*C. bertholletiae*	347.1
C. albidus	349.8				
T. glabrata	359.5				
S. cerevisiae	363.9				
C. pseudotropicalis	372.1				
C. kefyr	372.3				
				A. corymbifera	410.2
M. furfur	493.9				

A total of 103 seeded blood culture specimens with various yeasts were included, in addition to the 304 patient blood culture specimens, as the prevalence of fungemia was relatively low compared to bacteremia. All seeded blood culture specimens which became positive (n=101) were correctly identified by ITS2 analysis. Interestingly, 2 seeded *C. zeylanoides* specimens failed to be detected on the BacT/Alert system, suggesting that perhaps growth of this organism does not occur in this type of media. Out of 304 patient blood cultures, 27 of them contained yeast, of which 24 were successfully amplified for ITS2 analysis. As the number of species usually isolated from blood is quite limited, the ITS2 map created contained all species that we had isolated.

All patient fungal isolates were identified correctly by fragment analysis, resulting in a specificity of 100%. However, one *T. beigelii* isolate was found to have an ITS2 length 3 bp longer than that determined from the ATCC strain. Kemker *et al.* have indicated, using restriction fragment length polymorphism of a segment of the ribosomal genes including the ITS2 region, that isolates identified as *T. beigelii* are genetically distinct whether they are causative of invasive disease (isolated from blood), superficial infection or if they are nonclinical (ATCC) or environmental isolates. It is suggested that the name *T. beigelii* may represent several distinct entities and that, for the purpose of molecular diagnostic research, isolates from invasive disease must be used as opposed to culture collections. Similar differences between reference strains and patient strains were not observed among *Candida* species to date.

To ensure the accuracy of fragment analysis, the standard deviation of the bp length determinations as analyzed by the ABI PRISM™ 310 was determined using the yeast species detected from the seeded blood cultures. The run-to-run or lane-to-lane

variation resulted in a standard deviation equal to or less than 0.5 bp. Although the variation between different strains of a species was not extensively tested, it was comparable to the run-to-run variation of a single strain, indicating that the different strains tested had identical results. Results differ if one uses dUTP or dNTP as nucleotides in the PCR reaction. While the use of dUTP with UDG serves as a decontamination step, the UDG weakens the bacterial 16S multiplex reaction. This is important, as we may choose to amplify both bacteria and fungi in the same reaction in the future, and therefore the conditions need to be identical. On average, the ITS2 fragment length was determined to be 2-3 bp larger when amplified with dNTP instead of dUTP.

While many fungi have a unique ITS2 length, some can only be identified to a certain small group of species. Of those which are likely to be isolated in blood specimens, *C. albicans*, *C. tropicalis*, *T. (C.) glabrata*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae* and *C. pseudotropicalis* (*C. kefyr*), all have a unique ITS2 length. The ability to differentiate between these species is of clinical relevance. While fluconazole has become a drug of choice due to its lower level of toxicity in contrast to amphotericin B, non-albicans *Candida* species, particularly *C. krusei*, *C. glabrata*, and *C. parapsilosis* (Rex *et al.*, 1995; Nguyen *et al.*, 1996) have demonstrated fluconazole resistance and are more likely to occur in breakthrough fungemia during antifungal therapy. The ITS2 length similarity of *C. stellatoidea* and *C. albicans* is not surprising since the genetically distinct *C. stellatoidea* Type II has been classified as a sucrose-negative variant of *C. albicans* (Kwon-Chung *et al.*, 1990) and is normally reported as *C. albicans*. We were not able to evaluate extensively the possibility of detection in mixed cultures. In three

specimens from the same patient, which grew both *C. albicans* and *C. tropicalis* upon culture, only *C. tropicalis* was detectable by fragment analysis. However, one blood culture seeded with the same two yeasts (data not shown) resulted in the detection and identification of both species by PCR and fragment analysis.

It is important to keep in mind the clinical importance of the organisms that need to be identified. The ITS2 length in association with the clinical picture can be an effective method for early diagnosis of fungal infection. Using the same method, we could potentially identify fungal organisms in paraffin embedded tissues.

Part IV. The Potential of Using the 16S rRNA Gene and the ITS2 Genetic Region for the Rapid and Cost-Effective Identification of Bacteria and Fungi from Clinical Specimens.

The question to be answered is how the molecular methods established here are of advantage in contrast to conventional technology. First, molecular identification is impartial to phenotypic characteristics. Conventional biochemical testing may successfully identify the majority of the pathogenic bacteria. Nevertheless, speciation problems are often encountered with organisms such as CoNS and Gram-positive bacilli when using MicroScan panels and other automated biochemical systems. As of yet, the incongruities found between conventional testing and molecular testing would not have resulted in a change in the clinical diagnosis.

The advantage of using PCR to identify fungi is evident, as time is a crucial factor in the success of fungemia recovery. Presently, the detection of fungi from blood cultures requires extended incubation (3-10 days in this study) and may too often go

undetected. This method can positively and rapidly, within 3.5 hours after DNA extraction, indicate the presence of fungi in blood or tissues due to the generic nature of the ITS4 and ITS86 primers. We have shown that *C. zeylanoides* does not grow in our blood culture system. In fact, we have never isolated *C. zeylanoides* from blood and this may have been due to the blood culture medium being inappropriate for growth of this organism. Molecular techniques do not discriminate between organisms based on their growth needs or even growth itself.

We have found SSCP to be a highly successful method of identification, in that once a pattern is determined for an organism, that organism will always be recognizable by this pattern. We can further improve SSCP as an identification system by adding an extra set of primers. The study made by Widjojoatmodjo *et al.* made use of three primer sets. However, as we want to be able to use this method to identify blood pathogens, the number of peaks may be too overwhelming to identify species in a mixed culture.

Most bacterial species have a unique SSCP pattern. Unfortunately, two important pathogens, *S. aureus* and *S. pneumoniae*, cannot be differentiated from others of the same genus (CoNS and *S. mitis*, respectively). If a third primer pair cannot discriminate between these, we may have to explore differences found elsewhere among the ribosomal genes, such as the 5S, 23S or intergenic regions, or perhaps among completely different genes known to have interspecies variability such as the HSP60 (Goh *et al.*, 1996) or recA (Kullen *et al.*, 1997) genes.

In cases where the ITS2 lengths may be similar, it is highly probable that their sequences differ and may potentially be differentiated by single-stranded conformation polymorphism (SSCP). However, SSCP may be too sensitive to pattern variation

considering that it is more likely to detect intraspecies variations in the sequence content than fragment length determination. Another possibility may be to include the amplification of the ITS1 fragment in a multiplex PCR reaction, although this also has an increased chance of resulting in intraspecies length differences. More work needs to be done in a clinical setting to determine the extent to which our method is beneficial. Furthermore, the ability to amplify DNA from fresh tissue, paraffin embedded or in formalin, will have a great impact in diagnosis. The sensitivity of the ITS2 primers in these specimen types remains to be determined. We are in the process of improving our fungal DNA extraction method from such tissues received in our laboratory.

The greatest impact these methods may have in the clinical laboratory is upon the time factor from sample collection to a final diagnosis. The next step involves applying this method to clinical specimens as they are collected directly from patients as opposed to waiting for a blood culture positive signal. The time to identification from specimen collection can be as little as 6.5 hours. This contrasts with conventional blood culture testing, which take on average 2-5 days before positive fungal or bacterial isolation. Timing is crucial given the importance of early diagnosis from a clinical point of view. The isolates that took longest to final identification using conventional methodology were the yeasts, followed by mixed cultures, CoNS, *Micrococcus*, and the Viridans streptococci amongst a few others. Those that normally took only 1 to 2 days from a blood culture positive signal were mainly *E. cloacae, E. coli, E. faecalis, E. faecium, S. aureus*, and *S. pneumoniae*.

The cost analysis demonstrated significant cost savings using SSCP identification: $14 per specimen compared to $60 per blood culture. Furthermore, its cost effectiveness

not only pertains to materials and labor, but also can be seen as a result of early administration of appropriate treatment when a patient is bacteremic, thus reducing the mortality and morbidity rates, or the reduction of empiric therapy in patients who have no systemic infections.

DNA amplification techniques are independent of (i) organism viability and growth, (ii) the presence of antibiotics, (iii) appropriate media, and (iv) biochemical and morphological phenotypes. These techniques require only minimal technological expertise, in contrast with more subjective interpretations as in the morphological analysis of molds. Smaller sample volumes can also be used for successful detection. However, DNA amplification techniques do not establish antifungal or antibacterial susceptibilities, which must still be determined by conventional methodology necessitating organism growth. We believe this problem will be overcome in the future, as more and more antibiotic-resistance markers are discovered and identified by PCR methodology.

The ultimate goal of using molecular technology for diagnosis of infection is for the early detection of pathogens. Patient outcome is greatly improved due to the early administration of appropriate antibiotic therapy. Empiric therapy is then reduced, helping lead to the attenuation of the emergence of antibiotic-resistant organisms.

In summary, we have developed molecular methods for comprehensive identification of bacterial and fungal infections. These techniques are sensitive, specific, rapid, and cost-effective. The determined SSCP patterns and ITS2 lengths have been shown to be 100% specific, in the sense that a species will always demonstrate the same pattern, as the sequences of the ribosomal genes are considered a stable property.

Utilizing this information together with a rapid detection system performing fluorescence-based PCR-SSCP and fragment analysis has the potential to overcome all the present limitations mentioned, leading to the eventual use of these methods in clinical laboratories.

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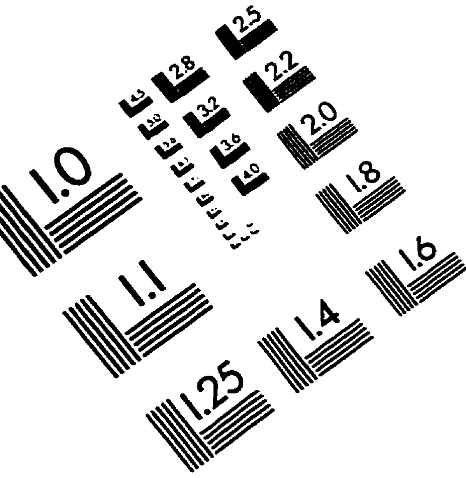
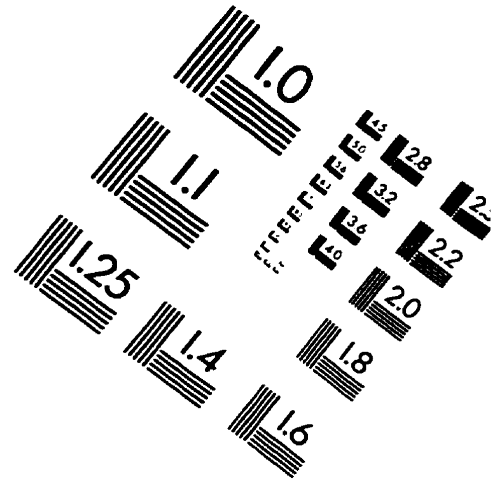
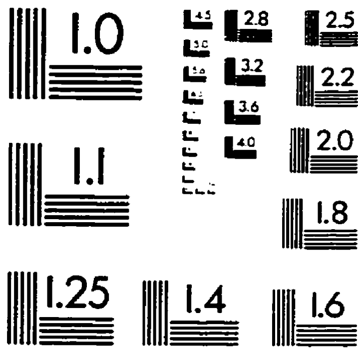
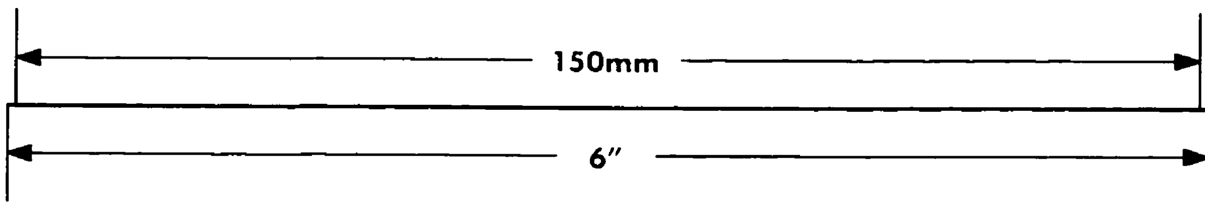
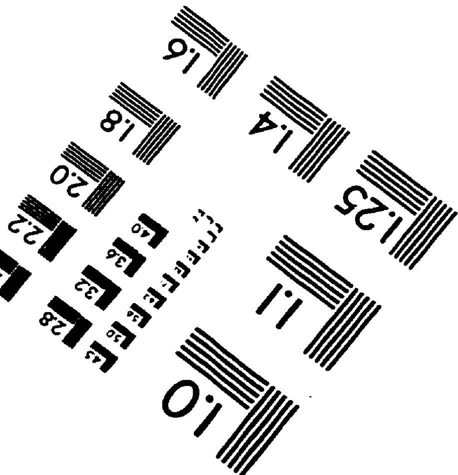
IMAGE EVALUATION TEST TARGET (QA-3)











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