DEVELOPMENT OF A MOLECULAR GRAM-STAIN ASSAY FOR THE DIAGNOSIS OF BLOOD STREAM INFECTIONS ASSOCIATED WITH SEPSIS

Douglas Bryan Litwin

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DEVELOPMENT OF A MOLECULAR GRAM-STAIN ASSAY FOR THE
DIAGNOSIS OF BLOOD STREAM INFECTIONS
ASSOCIATED WITH SEPSIS

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Presented to the Faculty of 
The University of Texas 
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Graduate School of Biochemical Sciences 
in Partial Fulfillment 
of the Requirements 
for the Degree of 

MASTER OF SCIENCE 

by 
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Houston, TX 
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Development of a Molecular Gram-Stain Assay for the Diagnosis of Blood Stream Infections Associated with Sepsis

Douglas Litwin, BS
Supervisory Professor: Heidi B. Kaplan, PhD

ABSTRACT

Sepsis is a serious medical condition resulting from the severe dysregulation of the immune response that is generally triggered by infection. It affects more than 1.1 million Americans, has an average mortality rate of 30%, and is estimated to cost $24.3 billion annually. Currently, blood culture followed by Gram-stain analysis is the gold standard for diagnosing bacterial infections associated with sepsis. This method generates a high rate of false negative results and, in general, requires 20 to 48 hr to provide results. Both of these problems are related to the requirement that the bacterial pathogens grow under defined laboratory conditions. This delay and lack of accuracy in diagnosis affects the administration of the correct antimicrobial therapy.

I have designed, developed, and begun to validate a rapid, sensitive, and specific DNA-based quantitative PCR (qPCR) assay, designated the Molecular Gran-stain (MGS) assay, to detect bacterial pathogens directly from septic patient blood samples. This assay also differentiates Gram-positive and Gram-negative pathogens. Importantly, results from this assay may be used to remove patients from unnecessary antimicrobial treatment at least 4 days earlier than is currently
possible, because in less than 5 hr it can be determined that no bacterial pathogen is present in a blood sample. The use of this assay may provide for more appropriate antimicrobial administration and decrease antimicrobial resistance and the related costs.

The MGS assay was designed to include internal checks and balances to provide more accurate pathogen detection. The assay utilizes a dual amplicon approach with two probes per amplicon. The presence of any bacterial DNA in a sample is detected by its ability to hybridize to universally conserved regions of the 16S rRNA gene. Hybridization to regions specific to Gram-positive and Gram-negative bacterial DNA serves to differentiate these two groups.

Eighteen clinical blood samples from suspected sepsis patients were analyzed using the MGS assay and compared to results obtained from the Memorial Hermann Hospital Clinical Microbiology Laboratory’s blood culture and Gram-strain analysis. There was 100% agreement between the two positive blood cultures. There was 37% agreement between the 16 culture-negative samples and the MGS assay results. There was 62% non-agreement between the culture-negative samples and the MGS assay results. None of the MGS amplification-negative results were culture positive, supporting the use of this assay as a reliable means to make the call to remove patients from antimicrobial treatment.

It is anticipated that the use of this MGS assay will provide an increase in the standard of septic patient care, resulting in better patient outcomes with more rapid tailored antimicrobial use for those with bone fide infections and removal of antimicrobial therapy from those without infection.
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INTRODUCTION

The severe dysregulation of the immune system resulting from a bacterial, fungal, viral, or parasitic blood stream infection is referred to clinically as sepsis (1-3). Sepsis originates from an isolated infection, which spreads to generate both a systemic infection and an inflammatory response capable of negatively affecting organs and tissues distant from the initial infection site (3). Sepsis can be classified into three types: 1) general sepsis is the presence of a systemic infection and an inflammatory response, 2) severe sepsis includes organ dysfunction or hypofusion (decreased blood flow through an organ), and 3) septic shock includes all of the other symptoms and the persistence of hypotension (low blood pressure) despite resuscitation (1, 4).

Sepsis is consistently among the 15 most common causes of death in the United States (5). More than one million Americans are affected by sepsis each year. The annual health care cost for sepsis treatment increased from $15.4 billion (415,280 cases) in 2003 to $24.3 billion (711,736 cases) in 2007. The number of cases continues to increase annually (6); the number of sepsis-related hospital admissions doubled from 2000 to 2008 (7). Sepsis commonly results in severe morbidity with a high incidence of permanent organ damage (7). Hospital sepsis is the leading cause of death in the intensive care unit (ICU) and morality rates are estimated from 40% to 70% (8). Sepsis is of particular concern as it accounts for approximately 2% of hospital admissions, but comprises 17% of in-hospital deaths. The increasing number of sepsis cases is alarming due to the
increasing treatment costs, and its association with increased antimicrobial resistance and negative patient outcomes.

Prompt identification of the causative organism in sepsis cases is critical, as it allows for tailored-antimicrobial treatment. A recent study revealed that appropriate antimicrobial therapy administration within a patient’s first hour of documented septic symptoms results in a survival rate of 79.9%. However, each hour appropriate antimicrobial therapy is delayed results in a 7% increase in mortality (9). A retrospective study of patient survival rates related to appropriate or inappropriate antimicrobial initiation found a 5-fold reduction (52% to 10.3%) in survival when inappropriate antimicrobial therapy was provided (8). Surprisingly, this study revealed a high rate (20%) of inappropriate antimicrobial administration. This identifies the need for early and accurate identification of sepsis-causing pathogens to directly improve patient outcomes. Here, inappropriate antimicrobial therapy is defined either as the absence of antimicrobials directed at the causative organism or as the administration of an antimicrobial to which the causative organism is resistant (10).

The current standard of care for patients presenting signs of sepsis is to administer broad-spectrum antimicrobials, while waiting for results from the clinical microbiology laboratory. This treatment is critical to decreasing mortality when infection is present (9, 11). However, the administration of broad-spectrum antimicrobials for an extended time presents risks, including secondary infections and antimicrobial resistance. At least two million Americans are affected each year by drug-resistant infections and an estimated 23,000 of these patients die
For example, *Clostridium difficile* infection (CDI) is a common nosocomial infection that can cause severe and recurrent diarrhea and is generally acquired as a result of antimicrobial use. Hospital-administered antimicrobials disrupt the normal gastrointestinal microbiota and allow this multidrug-resistant pathogen to colonize and proliferate in the colon, where it produces the toxin that causes disease. These infections are a serious condition affecting an estimated 250,000 Americans each year and are responsible for at least 14,000 deaths annually (12).

A CDC report estimates that up to 50% of the antimicrobials used are unnecessary and lead to antimicrobial resistance (12). It is expected that rapidly identifying the absence of infection will decrease the inappropriate use of antimicrobials. Under current practices, clinical microbiology laboratories require 5-7 days to provide information concerning a negative blood sample (i.e. to determine that no infectious agent is growing in the culture medium). If infection-negative results were provided 4 to 6 days earlier, patients could be removed from inappropriate antimicrobial therapy, reducing the many negative outcomes associated with inappropriate antimicrobial use. The development of a rapid, accurate, and sensitive method to identify sepsis-associated pathogens would provide more appropriate antimicrobial administration and decrease antimicrobial resistance and its related costs.
Current clinical methods to identify bacterial pathogens

Blood culture and Gram-stain methods

The current blood culturing methods employed by clinical laboratories focus on the detection of viable organisms. This is useful in that live organisms are isolated that can be used for antimicrobial susceptibility testing. However, blood culturing suffers from weaknesses including: 1) ineffective detection of uncultivable or fastidious pathogens, 2) prolonged time to pathogen identification due to the requirement that organisms reach approximately $10^5$ colony forming units (CFU)/ml in culture medium, 3) reduced sensitivity related to the volume of blood cultured, and 4) a loss of sensitivity when blood culture bottles are not placed immediately in the instrument (13-20).

The current process of blood culturing involves drawing two or three blood culture samples at different times and locations, which are directly inoculated into 20 to 30-ml continuous-monitoring blood culture system (CMBCS) bottles, such as the Bactec 9240 system (Becton Dickinson, Franklin Lakes, NJ), the BacT/Alert system (bioMerieux, Durham, NC), and the VersaTREK system (TREK Diagnostic Systems, Independence, OH). The samples are grown and tested both aerobically and anaerobically (21). The CMBCS systems detect bacterial growth by monitoring either CO$_2$ production through fluorescence or colormetric sensors, or by detecting changes in internal bottle pressures resulting from gas production (21-23). These bottles also commonly contain activated carbon (charcoal) or resin to limit the effects of antimicrobial agents present in the blood (24).
In general, a sample is detected as ‘culture positive’ by CMBS systems when the growth reaches between $1 \times 10^5$ cells/ml (25, 26). Optimization of the culture medium and automation of the instrumentation has improved the diagnostic accuracy of blood culturing. However, the previously mentioned limitations still persist and some bacterial and fungal species perform differently in the CMBCS systems (21). Variability has been shown among the CMBCS systems in time to detection (TTD) and the rate of recovery of organisms in the presence of antimicrobials (27, 28). One study noted the TTD of bacteria to ranges from 11.2 to 47.4 hrs depending on the CMBCS system and conditions used, such as preexisting antibiotic concentrations present in the samples and aerobic conditions (28). Furthermore, although it is generally accepted that bacterial concentrations of $10^5$ cells/ml are required for positive identification on the BacT/ALERT, some organisms, such as *Mycoplasma hominis*, can grow to as many as $10^7$ cells/ml in the BacT/ALERT system without being detected as positive (29).

**Gram-stain analysis**

After bacterial growth is detected in the CMBCS, the blood culture is subjected to Gram-stain analysis that is generally performed manually. The Gram-stain differentiates bacteria by the ability of their cell wall to retain peptidoglycan-binding crystal violet dye after decolorization with alcohol or acetone. Those bacterial cells whose walls do not retain crystal violet after alcohol decolorization are differentiated by staining with the secondary dye,
safranin. The Gram-positive cells retain crystal violet and appear purple, whereas the Gram-negative cells retain the safranin dye and appear pink (30).

Gram staining is a quick (<15 min) and inexpensive method for general phenotypic characterization. However, certain organisms do not react as predicted to the dyes and thus, give inaccurate results. For example, some Gram-negative bacteria such as Acinetobacter can resist decolorization and appear as Gram-positive cells (31). Other organisms, termed ‘acid-fast’, include mycobacteria that have a high concentration of lipids in their cell walls, which make them impermeable to the Gram-strain dyes and cause variable results (32, 33). Additionally, correct analysis of Gram-stain samples is subjective and requires a high level of experience.

**Species identification and antibiotic susceptibility**

Currently, clinical microbiology laboratories perform species identification and test antimicrobial susceptibility on viable organisms recovered from culture media. Differential media, both liquid and solid, can be used to differentiate bacterial species based on metabolic characteristics. Selective media allows the growth of a specific bacterial species while inhibiting others. Automated bacterial identification and antimicrobial susceptibility determination instruments, such as the Phoenix 100 (Becton Dickinson) and the Vitek 2 system (bioMerieux), combine differential and selective media methods with chromogenic and fluorogenic biochemical tests and broth-dilution antimicrobial susceptibility methods.
Specifically, clinical microbiology laboratories evaluate antimicrobial susceptibility through a variety of methods including broth dilution tests, antimicrobial gradient tests, disk diffusion tests, selective and differential media and automated instrument systems (34). Broth dilutions are performed by using a clinical isolate at ~1X10^6 CFU/ml to inoculate liquid medium containing two-fold dilutions of an antimicrobial and determining the minimal inhibitory concentration (MIC) by visually inspecting the turbidity following overnight growth. The antimicrobial gradient test, the AB BIODISK (bioMerieux), uses printed antimicrobial strips that contain an increasing concentration of the antimicrobial along the length of the strip. The strips are placed onto agar plates that have been inoculated so that a clinical isolate covers the surface. After incubation the MIC is determined by evaluating the changing growth pattern along the length of the antimicrobial strip. Disk diffusion tests are performed by inoculating a clinical isolate at a concentration of ~1x10^8 CFU/ml on a Mueller-Hinton agar plate then placing paper disks containing known concentrations of antimicrobials on the agar surface. After 16 to 24 hr of incubation, the MIC is determined by measuring the area of growth inhibition around the antimicrobial disks and comparing it to standards. Automated instruments are preferred for use in clinical microbiology laboratories due to the reduced manual labor required and their reproducibility. However, in general all of these results are not obtained until about 24 hr after the blood culture is positive due to their requirement for continued growth of the organism (35).
Molecular methods to identify bacterial pathogens

Molecular diagnostic methods offer a solution to the problems associated with pathogen identification requiring the detection of viable organisms. Molecular methods have advantages in comparison to the traditional culture and Gram-stain method in that: 1) they are more rapid, 2) pathogens can be identified and quantitated directly from clinical samples, and 3) they eliminate variability associated with varying growth requirements for different organisms. Many molecular pathogen detection techniques have been developed that rely on: PCR, real-time PCR, mass spectroscopy, microscopy, and nuclear magnetic resonance (NMR) (36-45).

PCR

Many different PCR-based molecular methods for the diagnosis of bloodstream infections have been developed and optimized. LightCycler SeptiFast (Roche Diagnostics, Indianapolis, IN), SepsiTest (Molzym Corp., Bremen, Germany), and VYOO (SIRS-Lab, Jena, Germany) are the three assays most widely used outside of the U.S. No methods are currently approved by the FDA that use molecular methods to directly identify bacteria from blood samples. Other methods in development and testing include PCR coupled with mass spectroscopy (39).
**LightCycler SeptiFast**

The LightCycler SeptiFast (LC-SF) test developed by Roche Diagnostics was the first real-time PCR test designed to detect bacteria and fungi directly from blood samples with no incubation period. This test uses real-time PCR FRET probes to identify the 14 most common bacterial and six most common fungal pathogens. The details of the probes and assay are proprietary; however, it is known that the probes target the internal transcribed regions of the bacterial 16S and 23S rRNA genes and the fungal 18S and 5.6S rRNA genes. Blood samples are extracted using an automated DNA extraction instrument and assayed using a sample capillary real-time PCR instrument. Studies have shown that this method is faster and more accurate than current culturing methods for the identification of fungal and bacterial species (36-38).

**SepsiTest**

The SepsiTest is a standard PCR test using multiplexed primers that amplify regions of either Gram-positive or Gram-negative 16S rRNA genes or regions of fungal 18S rRNA genes. Each primer set produces a band unique to Gram-positive bacteria, Gram-negative bacteria or fungal pathogens. PCR products are then analyzed by gel electrophoresis and distinctive bands are gel extracted and subjected to DNA sequencing. This test is designed to detect 375 pathogenic organisms, most of which are bacteria (40). Studies have shown that this test is rapid and accurate when on-site DNA sequencing is available.
However, it requires a substantial amount of manual labor and requires an experienced technician for PCR and DNA sequence analysis (41).

**VYOO**

The VYOO test developed by SIRS-Lab is a multiplexed standard PCR assay designed to identify bacterial and fungal pathogens and several resistance genes. The assay uses an affinity resin to enrich the pathogen DNA from a clinical sample followed by PCR amplification using proprietary primer sets that are specific to 33 bacteria, five resistance genes, and seven fungal species. The PCR products of different sizes are then analyzed by gel electrophoresis (42). Studies have shown high variability in the sensitivity of this method and it requires significant manual processing time (43).

**MALDI-TOF**

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is the process by which molecules are ionized and then detected after being separated by charge and mass (39). MALDI-TOF MS is most useful for the identification and quantification for proteins and other biomolecules. This technology has been adapted for the identification of biomarkers associated with many medical conditions (44). It has a high rate of accuracy in diagnostic capability in clinical laboratory settings. However, when analyzing complex samples such as blood, sample enrichment steps (pre-culture) are required and this adds additional time to identification of causative
organisms. The cost is also of concern. MALDI-TOF MS instruments typically can cost as much as $180,000 and require a skilled technician (39, 45).

**Microscopy**

Fluorescent *in situ* hybridization (FISH) coupled with automated microscopy has recently been evaluated for identification of seven known bacterial species grown in simulated blood cultures using the BD BACTEC (Becton Dickinson). This method is ‘multiplex compatible’ and has high sensitivity (97 to 99%). However, its ability to replace the downstream analysis of current blood culturing methods is currently undetermined. Thus, this technique has the same problems as the current clinical culturing methods, including time to identification and that a high cell concentration is needed for detection (46).

The use of automated microscopy to identify *Staphylococcus aureus* and *Pseudomonas aeruginosa* from clinical bronchoalveolar lavage (BAL) samples has been successful. This technique uses automated microscopy to analyze bacterial cells immobilized on a microfluidic chip. The bacterial cells are initially given a negative charge through a wash step with electrokinetic buffer (47). The bacterial cells are then inserted into the microfluidic chip and driven toward a poly-cationic poly-L-lysine coating on the opposite side of the chip by an electric charge. The cells stick to the poly-cationic poly-L-lysine coating after the charge is removed. An automated microscopy system then tracks the actively growing cells and analyses their mass, geometry and growth rate (47). This technique is unique and performed well under the limited conditions in which it was tested.
However, it still relies on bacterial growth and has been tested on only a few organisms.

NMR

A recently developed technique utilizing magnetic nanoparticles (MNPs) and miniaturized NMR (µNMR) instrumentation has been developed for pathogen detection in clinical microbiology labs (T2 Biosoftware, Lexington, MA). This technique uses asymmetric reverse transcription PCR targeting the 16 rRNA transcripts to generate signal-stranded DNA that are subsequently bound by bead-capture probes. The DNA-probe complexes have overhangs of non-bound DNA. These overhangs are targeted by MNP-detection probes. Once bound, the MNP-detection probes decrease the transverse relaxation rate of a sample that is measured on a µNMR instrument. This technology has shown promise as a clinical tool due to its high sensitivity and accuracy (48).

The Molecular Gram-stain Assay

We have designed a molecular method, termed the Molecular Gram-stain (MGS) assay, utilizing quantitative real-time PCR for detection of bacterial pathogens directly from blood samples. The pathogens are differentiated as either Gram-positive or Gram-negative. The assay includes internal checks and balances to ensure rapid, sensitive, and quantitative pathogen detection. This assay is unique in that it was designed to detect essentially all bacterial pathogens and to differentiate them based on their Gram status. A
double amplicon approach generates two separate PCR targets within the bacterial 16S rRNA gene with two fluorescent probes targeting each amplicon. The output data determine if the probes target Gram-positive or Gram-negative-specific and/or universal regions. This 16S rRNA gene-based assay should prove critical for the treatment of septic patients, as it will determine if a blood sample is infected with bacteria or not. Rapidly determining the presence or absence of bacteria in a blood sample will inform the decision to maintain antimicrobial therapy or to discontinue its use. The ability of the assay to differentiate between Gram-positive and Gram-negative pathogens will also suggest more specific treatment, as more tailored antimicrobials can then be prescribed. Identifying bacteria universally and by Gram-status a major advantage over the other molecular techniques available that identify pathogens within a predefined group and thus cannot rule out bacterial infection if the causative organism is not one of the target organisms.

A key component of the MGS assay is the use of minor-groove binding (MGB) Taqman fluorescent probes (49). MGB probes are oligonucleotide hydrolysis probes that include a 3’ dihydrocyclopyrroloindole tripeptide (DPI3). When attached to DNA probes, DPI3 peptides bind the minor groove of the complementary target strand upon duplex formation. DPI3 binding increases the melting temperature and the specificity of probe hydrolysis. MGB probes are capable of discriminating single nucleotide mismatches in their target region, which is critical for differentiating regions in highly conserved genes, such as the bacterial 16S rRNA gene (49).
MATERIALS AND METHODS

Determination of relevant organisms

A list of 33 bacterial species isolated from positive blood cultures in the surgical trauma intensive care unit (STICU) from 2009-2012 at Memorial Herman Hospital was obtained from the STICU pharmacy (Table 1). An additional group of 90 bacterial species assembled for an osteomyelitis study performed previously in our laboratory, was used for in silico analysis to test the extent of our probe coverage for general bacterial pathogens.
<table>
<thead>
<tr>
<th>Rank</th>
<th>Species</th>
<th>Frequency</th>
<th>Rank</th>
<th>Species</th>
<th>Frequency</th>
<th>Rank</th>
<th>Species</th>
<th>Frequency</th>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td>5.</td>
<td>Alpha streptococci</td>
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<tr>
<td>5.</td>
<td><em>Serratia marcesens</em></td>
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<td>6.</td>
<td><em>Bacillus sp.</em></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td><em>Eikenella corrodens</em></td>
<td>1</td>
<td>7.</td>
<td><em>Bacillus cereus</em></td>
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<td></td>
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</tr>
<tr>
<td>6.</td>
<td><em>Enterobacter aerogenes</em></td>
<td>1</td>
<td>7.</td>
<td>Cornybacterium</td>
<td>1</td>
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</tr>
<tr>
<td>6.</td>
<td><em>Proteus mirabilis</em></td>
<td>1</td>
<td>7.</td>
<td>Group G streptococci</td>
<td>1</td>
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</tr>
<tr>
<td>6.</td>
<td><em>Salmonella</em></td>
<td>1</td>
<td>7.</td>
<td>Peptostreptococcus</td>
<td>1</td>
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Table 1: List of 33 bacterial species isolated from clinical blood samples in the Memorial-Herman Hospital Surgical Trauma Intensive Care Unit from 2009-2012. Species are grouped by Gram status and listed by the frequency of recovery for the 134 samples analyzed.
EMA and PMA treatment

Pretreatment of solutions containing the appropriate master mix and primers with either ethidium monoazide (EMA) or propidium monoazide (PMA) was required to eliminate contaminating bacterial DNA present in the solutions. EMA and PMA covalently intercalate into double-stranded DNA upon exposure to light. The PCR and qPCR master mixes used were either QuantiFast probe master mix or QuantiFast Sybr green master mix (QIAGEN, Valencia, CA). Stock solutions of EMA and PMA at 239 µM and 20 mM, respectively were stored at -20°C in the dark. In the presence of reduced light, EMA was added to the previously prepared master mix to a final concentration of 9 µM. The solution was mixed briefly by repeat pipetting, incubated at room temperature for 5 min, and exposed to a 500 watt light (Gulo Model A) from a distance of 10 cm for 5 min while on ice. The appropriate probes, (10 µM in EMA-treated H₂O) were added to a final concentration of 0.25 µM while the solution remained on ice. The same procedure was performed for the PMA treatment (20 µM final concentration) of phosphate buffered saline (PBS).

Testing the qPCR assay in artificially inoculated blood samples

Blood was drawn from healthy volunteers into 4.5-ml citrate-containing Vacutainer tubes (Becton Dickinson). Aliquots (1 ml) of the blood were placed into 1.5-ml microcentrifuge tubes. Cultures (10 µl) of Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa and Escherichia coli at concentrations two logs higher than the desired final concentration in the blood sample were added to the 1-ml aliquots to give final concentrations ranging from
10^1-10^5 CFU/ml. Those blood samples were then centrifuged at 13,000 x g for 2 min, the plasma (upper phase) was removed and both fractions (the blood pellets and plasma) were stored at -20°C until analysis. Similar samples were also prepared in 1-ml of 20 µM PMA-treated PBS, by adding 10 µl of the bacterial culture (two logs higher than the desired concentration) and 2 µl of yeast genomic DNA (500 ng/µl) to a sterile 1.5-ml microcentrifuge tube. The samples were stored at -20°C until analysis. The total DNA was later extracted using the Mo-Bio Bacteremia kit (MO BIO Laboratories, Carlsbad, CA). Both the PBS-PMA and blood pellet extracted samples were analyzed using the MGS assay.

**Collection of clinical blood samples**

The clinical blood samples were drawn in sodium citrate-containing Vacutainers by nurses in the Memorial-Herman Hospital’s STICU and Burn Unit based on a physician’s diagnosis of a presumed septic patient. Patients are evaluated for sepsis based on the severe inflammatory response syndrome (SIRS) criteria. The SIRS criteria are: 1) a temperature 1+-37°C, 2) a heart rate > 90 beats per min, 3) a reparatory rate > 20 breaths per min, and 4) an increase or decrease in white blood cell count (1). A patient showing signs of two or more of the SIRS criteria are presumed to be septic. The Vacutainers were maintained at room temperature until they were collected, given a sample number associated with the patient ID number (de-identified), and transferred to the research laboratory by a laboratory manager, generally within 12 hr of the blood draw. Aliquots (1 ml) of the blood from the Vacutainer were transferred into
labeled sterile 1.5-ml microcentrifuge tubes, centrifuged at 13,000 x g for 2 min. The upper phase (plasma) was then removed without disturbing the blood pellet and transferred to a sterile 1.5-ml microcentrifuge tube. The blood pellets, plasma and remaining Vacutainer tubes were then stored at -20°C until analysis.

**Isolation of DNA from blood samples**

DNA was isolated from blood and plasma samples using the Mo-Bio BiOstic® Bacteremia DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer’s recommended protocol, except that all of the reagents were pretreated with EMA. Briefly, the blood pellet was thawed and resuspended in CB1 solution (450 µl), transferred into a 2-ml microbead tube, briefly vortexed and heated to 80°C for 15 min. The tubes were horizontally secured onto a Mo-bio Vortex-Genie® attachment and vortexed at maximum speed for 10 min. The tubes were centrifuged at 10,000 x g for 1 min and the supernatant was transferred to a new 1.5-ml microcentrifuge tube. The CB2 solution (100 µl) was added and the samples were vortexed briefly. The samples were incubated at room temperature for 5 min and then centrifuged at 10,000 x g for 1 min. The supernatant was transferred to a new 1.5-ml microcentrifuge tube. The CB3 solution (1 ml) was added and mixed by repeat pipetting. A portion of the solution (600 µl) was then added to a spin filter column and centrifuged at 10,000 x g for 1 min. The flow-through was discarded. This step was repeated until the entire sample was passed through the column. The column was transferred to a new collection tube and washed twice with CB4 solution (600 µl) and centrifuged
at 10,000 x g for 1 min. The column was centrifuged at 13,000 x g for 3 min to dry the column. The DNA was eluted using CB5 solution or H$_2$O (50 µl). The plasma samples were treated identically, except that twice the volume was used because of their larger volume.

**qPCR of DNA from blood samples**

The qPCR master mix contained QuantiFast Probe PCR +ROX Vial Kit (QIAGEN Valencia, CA), forward and reverse primers (0.5 uM) and H$_2$O. The complete master mix was treated with EMA (at a final concentration of 9 uM). Probes that were previously diluted to 10 uM in EMA-treated H$_2$O were added after the EMA treatment. Aliquots (15 ul) of the completed master mix were placed into each well of a standard 96-well polypropylene qPCR plate (Denville Scientific Inc. Metuchen, NJ). The samples were analyzed in duplicate and standards were used in concentrations representing $10^1$ to $10^6$ cell/ml. The qPCR cycling conditions used were based on the manufacturer's recommendation: 1) initial denaturation step of 95°C for 3 min, 2) denaturation step of 95°C for 10 sec, 3) annealing step of 58°C for 5 sec, and 4) extrusion step of 60°C for 1 min. Steps 2-4 were repeated 40 times.

The qPCR samples were analyzed using the Applied Biosystems 7000 system software (Life Technologies, Grand Island, NY). Quantitation of unknown samples was automatically calculated by standard curve comparison of C$_t$ values generated from *S. aureus* or *E. coli* DNA depending on the Gram-status.
RESULTS

The identification of sepsis-associated infections by molecular methods has great potential for improving patient outcomes (50). The most common method used to identify bacterial pathogens in clinical microbiology laboratories today is blood culture followed by Gram-stain analysis. Although this has clinical utility, it suffers from many shortcomings. The major problems are a lack of sensitivity and prolonged time to detection of the causative organisms (13-18, 20, 29). Recently developed molecular techniques have focused on the identification of pathogens by mass spectroscopy, microscopy, NMR and PCR-based technology (36-48).

We have designed, developed, and begun to validate a quantitative real-time PCR method for rapid, sensitive, and quantitative pathogen detection in blood samples from septic patients. Specifically, this method, termed the Molecular Gram-stain (MGS), can determine the Gram-status of a pathogen in a blood sample within 4.5 hr. The use of minor groove binding probes makes it possible to have high binding fidelity with short target sequences. The MGS assay was designed to couple these highly specific probes with internal checks and balances. Specifically, the MGS assay amplifies two regions of the bacterial 16S rRNA gene, which I term the ‘upstream and downstream amplicons’. Two probes, a universal and a Gram-positive probe, target the upstream amplicon and two probes, a Gram-negative and Gram-positive probe, target the downstream amplicon (Fig. 1). By using this dual amplicon approach, the assay
detects bacterial pathogens and differentiates them by Gram-status with redundancy.

**Figure 1. Schematic of the Molecular Gram-stain assay.** The MGS assay targets the bacterial 16S rRNA gene. The upstream amplicon is comprised of primers and probes: 16S557(19)F, 16S786(23)R, P16S683U, and P16S706P. The downstream target is composed of primers and probes: 16S945(20)F, 16S1222(20)R, P16S1194P, and P16S1194N.

**Primer and probe design**

**Determination of areas of interest within the 16S rRNA gene**

The bacterial 16S rRNA gene was chosen as the target of the MGS assay because it is present in all bacteria in single or multiple copies and is composed of conserved and variable regions that can be used to differentiate genera and species (51, 52). These genes are especially useful for probe-based qPCR assays because the conserved regions function in the amplification the variable regions, which can serve as unique targets for differentiation by DNA probes. To identify the best regions to serve as probe targets, up to three 16S rRNA genes, chosen at random from most of the 33
common pathogens detected in septic patients in the Memorial Hermann Hospital STICU, were aligned using the Geneious software suit’s (Biomatters Limited, San Francisco, CA) CLUSTAL nucleotide alignment algorithm. The nucleotide alignments of the relevant organisms were evaluated for use as probe target regions. Three types of conserved regions were identified (Fig. 2): one universally conserved, two conserved among the Gram-positive species, and one conserved among the Gram-negative species.
Figure 2. Nucleotide alignments of the 16S rRNA genes from the Memorial Hermann Hospital STICU pathogen list. Species are grouped as Gram-positive (upper panel) and Gram-negative (lower panel). The areas identified as ideal targets for Gram-positive identification are shown in blue. The area identified as an ideal target for Gram-negative identification is shown in orange. An area identified as an ideal target for universal bacterial identification is shown in green.
Probe design

To select specific areas highly conserved among the 18 Gram-positive pathogens, the sequences were aligned and compared to same region in the 15 Gram-negative pathogens. The same analysis was performed to select areas highly conserved among the Gram-negative pathogens. Areas unique to either Gram-positive or Gram-negative pathogens were selected and evaluated for probe parameters using the AlleleID software suite (PREMIER Biosoft, Palo Alto, CA). Regions universally conserved (common to both the Gram-positive and Gram-negative pathogens) were also evaluated. One Gram-negative, one universal and two Gram-positive probes listed below (Table 2) were identified that met our criteria. These criteria included: 1) the region spanned at least 13 nucleotides that maintained 90-100% homology among all of the Gram-positive, Gram-negative, or all of the species in the alignments, 2) the sequence had more cytosines than guanines and no 5’ guanine, and 3) at least one discriminatory mismatch was located near the 3’ terminus. The number of guanines is critical because they can act as a fluorescence quencher, which suppresses the fluorescent signal available for detection in the qPCR reaction, and guanine-guanine interactions can interfere with probe annealing.

One region approximately 100% conserved among all the species and one region slightly downstream, which is 100% conserved among the Gram-positive species were identified (Table 2 and Fig. 3, 4). The region conserved among Gram-positive species is of particular interest in that it had two or more
<table>
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<th>Upstream Probes</th>
<th>Probe Sequence (5’-3’)</th>
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<tr>
<td>P16S683U</td>
<td>6FAM-TTTCAACGCTACAC-MGBNFQ</td>
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<tr>
<td>P16S706P</td>
<td>NED-ATATGGAGGAACACC-MGBNFQ</td>
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<table>
<thead>
<tr>
<th>Downstream Probes</th>
<th>Probe Sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>P16S1194N</td>
<td>6FAM-TCAAGCTCATCATGG-MGBNFQ</td>
</tr>
<tr>
<td>P16S1194P</td>
<td>NED-TCAATCATCATGC-MGBNFQ</td>
</tr>
</tbody>
</table>

**Table 2: Molecular Gram-stain assay probe sequences.** Probe sequences are listed in pairs for the upstream amplicon and downstream amplicons. P16S683U is a universal probe that should detect all bacterial 16S rRNA genes. P16S706P and P16S1194P are Gram-positive-specific probes. P16S1194N is a Gram-negative-specific probe. All probes have a 5’ fluorophore (6FAM or NED) and a 3’ minor-groove binding motif and non-fluorescent quencher (MGBNFQ).
Figure 3. Alignments of the universal probe P16S683U. (A) The P16S683U probe aligned with Gram-negative species. (B) The P16S683U probe aligned with Gram-positive species. Nucleotide mismatches with the probe sequence are shown in bold in the alignment.
Figure 4. Alignments of the Gram-positive probe P16S706P. (A) The P16S706P probe aligned against Gram-negative species. (B) The P16S706P probe aligned against Gram-positive species. Nucleotide mismatches with the probe sequence are shown in bold in the alignment.
nucleotide mismatches with all of the Gram-negative species (Table 2 and Fig. 4). After verifying that they fit our criteria for probe design, both regions were aligned with the 16S rRNA gene from *E. coli* O104:H4 and named according to the *E. coli* nucleotide number at which the most 5’ end of the probe aligned: universal probe P16S683U and Gram-positive probe P16S706P.

The next region identified was of particular interest because it maintained a high degree of homology among all of the species, except that there were two nucleotide mismatches between all of the Gram-positive and all Gram-negative species, one of which was located at the 3’ end. Mismatches on the 3’ end have been shown to have the greatest discriminatory capabilities (49). This region was aligned on the 16S rRNA gene from *E. coli* O104:H4 and the probe with close to 100% homology to the Gram-negative species was designated P161194N. The probe with 100% homology to the Gram-positive species was designated P161194P (Table 2 and Fig. 5, 6).
Figure 5. Alignments of the Gram-negative probe P16S1194N. (A) The P16S1194N probe aligned against Gram-negative species. (B) The P16S1194N probe aligned against the Gram-positive species. Nucleotide mismatches with the probe sequence are shown in bold in the alignment.
Figure 6. Alignments of the Gram-positive probe P16S1194P. (A) The P16S1194P probe aligned against Gram-negative species. (B) The P16S1194P probe aligned against the Gram-positive species. Nucleotide mismatches with the probe sequence are shown in bold in the alignment.
Primer design

The 100 bp regions flanking the probe target regions were evaluated for primer selection. The criteria for suitable primers were: 1) sequences of 18-24 bp, with a $T_m$ near 60°C that produced an amplicon 100 to 250 bp, 2) acceptable 3’ stability, and 3) minimal nucleotide repeats and single nucleotide repetitive runs. Two potential forward and reverse primers were identified for the upstream probes (Table 3). The four primers designed for the upstream amplicon were tested in all possible configurations and analyzed by gel electrophoresis for amplification (Fig. 7). Three potential forward and four potential reverse primers were identified for the downstream probes. The five primers designed for the downstream amplicon were tested in all possible configurations and analyzed on gel electrophoresis for amplification (data not shown). The primers 15S557(19)F and 16S786(23)R had the highest PCR product yield and were selected for amplification of upstream amplicon. The primers 16S945(20)F and 16S1222(20)R had the highest PCR product yield and were selected for amplification of downstream amplicon.
### Table 3. Primers tested for the amplification efficiency for Molecular Gram-stain assay.

The primers are listed according to the upstream and downstream targets. The primers in bold and marked with an asterisks were selected for use in the Molecular Gram-stain assay.

<table>
<thead>
<tr>
<th>Upstream Primer</th>
<th>Primer Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>16S557(19)F *</td>
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<tr>
<td>16S560(18)F</td>
<td>TTTATGGGCGTAAAGCG</td>
</tr>
<tr>
<td>16S786(18)R</td>
<td>CTACCAGGGTATCTAATC</td>
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<tr>
<td>16S786(23)R *</td>
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<tr>
<th>Downstream Primer</th>
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<td>16S945(20)F *</td>
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<td>16S946(18)F</td>
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<td>16S1210(18)R</td>
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<tr>
<td>16S1222(20)R *</td>
<td>CATTGTAGCAGGTGTAGC</td>
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Figure 7. An electrophoresis gel analysis of different primer pair efficiencies. Each primer pair set was used to amplify *E. coli* genomic DNA and the PCR products were electrophoresised through a 1% agarose gel, stained with ethidium bromide, and photographed. Lanes: 1) primers 16S560(18)F and 16S786(18)R, 2) primers 16S560(18)F and 16S786(23)R, 3) primers 16S557(19)F and 16S786(18)R, 4) primers 16S557(19)F and 16S786(23)R.
Primer and probe optimization

Compatible primer pairs were tested in conventional PCR analysis using Qiagen QuantiFast SYBR Green PCR Kit (QIAGEN) master mix and the amount and specificity of amplification was compared by electrophoresis through agarose. The primer pairs showing the highest product yields were tested subsequently by qPCR. Both of these analyzes were performed with the same standard template DNA: (S. aureus DNA [2.85 ng/ml], E. faecalis DNA [2.81 ng/ml], P. aeruginosa DNA [4.96 ng/ml] and E. coli DNA [5.59 ng/ml]). First, the primers were tested to determine that they universally anneal to bacterial species and amplified the target regions efficiently. Subsequently, the primer pair 15S557(19)F/16S786(23)R was used to test the upstream probe set and primer pair R16S945(20)F/16S1222(20)R was used to test the downstream probe set. For all of these tests the standard template DNA solutions were prepared by making four 1:10 dilutions. For example, the S. aureus template DNA was used at 2.8 ng/ml, 0.28 ng/ml, 0.028 ng/ml, and 0.0028 ng/ml. Each assay was then tested for efficiency after EMA pretreatment.

SYBR Green qPCR analysis of primers

The primers that produced the best yields using standard PCR with electrophoresis analysis were tested for amplification efficiency using SYBR Green qPCR analysis. The Ct values were plotted against the log dilutions of DNA concentrations to create standard curves. Melting curve analysis was also performed to test for the amplification of non-specific products. The slope of the trend line that is generated from the standard curve can determine the
amplification efficiency. A trend line slope of 3.33 is indicative of 100% efficiency in the amplification cycles, which represents a 2-fold increase in detection between C_t values. Trend lines with slopes less than 3.33 are indicative of greater than 100% efficiency and trend lines with slopes greater than 3.33 are indicative of lower than 100% efficiency.

For the upstream primer set, 15S557(19)F and 16S786(23)R, a standard curve slope of 2.3 was produced on *E. faecalis*, 2.7 for *S. aureus*, 2.8 for *P. aeruginosa*, and 2.9 for *E. coli* (Fig. 8 A and B). Each reaction produced trend lines indicative of a greater than 100% efficiency, which is most likely the result of high primer concentration and slight amplification of the bacterial DNA present in the qPCR master mix and reagents. This leads to a slight plateau at the higher C_t values. The melting curve analysis for the upstream primers detected no amplification of non-specific products.

The downstream primer set, 16S945(20)F and 16S1222(20)R, produced standard curve slopes of 3.0 for *E. faecalis*, 3.2 for *S. aureus*, 2.8 for *P. aeruginosa* and for *E. coli* (Fig. 9 A and B). Trend lines indicative of amplification efficiencies of greater than 100% were again produced and were likely to be a result of primer concentration and contaminant amplification. Melting curve analysis showed that there were no non-specific products amplified in the reaction.

As all four of these primers resulted in high amplification efficiency and no non-specific amplification, they were all chosen for assay development. No
Figure 8. Standard curve analysis of upstream primers 16S557(19)F and 16S786(23)R. (A) Primers 16S557(19)F and 16S786(23)R amplifying Gram-positive species S. aureus and E. faecalis. (B) Primers 16S557(19)F and 16S786(23)R amplifying Gram-negative species E. coli and P. aeruginosa.
Figure 9. Standard curve analysis of upstream primers 16S945(20)F and 16S1222(20)R. (A) Primers 16S945(20)F and 16S1222(20)R amplifying Gram-positive species *S. aureus* and *E. faecalis*. (B) Primers 16S945(20)F and 16S1222(20)R amplifying Gram-negative species *E. coli* and *P. aeruginosa*.
further SYBR Green optimization was performed. Additional optimization was required for the qPCR assay with the Taqman probes.

**Testing probes on Gram-positive and Gram-negative genomic DNA**

The specificity and efficiency of the upstream and downstream primer and probe sets were determined using a range of four orders of magnitude of genomic DNA equivalent to $10^2$ to $10^5$ cells/ml of *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* DNA as template. The upstream universal probe P16S683U was tested using primers 16S557(19)F and 16S786(23)R. Probe P16S683U detected all bacterial DNA tested with amplification efficiencies of approximately 75% (Fig. 10 A, B). The Gram-positive upstream probe P16S706P was tested using the same primers (16S557(19)F and 16S786(23)R). Probe P16S706P detected only Gram-positive DNA with amplification efficiencies of approximately 86% (Fig. 11 A, B).

The downstream Gram-positive probe P16S1194P was tested using primers 16S945(20)F and 16S1222(20)R. Probe P16S1194P amplified the only Gram-positive bacterial DNA with approximately 89% efficiency (Fig. 12 A, B). The downstream Gram-negative probe P16S1194N was testing using the same primers (16S945(20)F and 16S1222(20)R). Probe P16S1194N amplified only the Gram-negative bacterial DNA with approximately 70% efficiency (Fig. 13 A, B).

The downstream probes, P16S1194P and P16S1194N, share the same probe target region, which contains two nucleotide mismatches. This could result in competition for the binding sites. An analysis of competitive binding was
Figure 10. Fluorescent detection and standard curve plots for the upstream Probe 16S683U. A) Amplification of probe 16S683U analyzing E. coli, P. aeruginosa, S. aureus and E. faecalis DNA. B) Standard curve for probe 16S683U analyzing E. coli, P. aeruginosa, S. aureus and E. faecalis DNA.
Figure 11. Fluorescent detection and standard curve plots for the upstream probe P16S706P. A) Amplification of probe P16S706P analyzing E. coli, P. aeruginosa, S. aureus and E. faecalis DNA. B) Standard curve for probe P16S706P analyzing E. coli, P. aeruginosa, S. aureus and E. faecalis DNA.
A)  

\[ \Delta Rn \]

\[ y = 3.593x + 17.72 \]

\[ y = 3.7895x + 18.735 \]

B)  

\[ y = 3.7895x + 18.735 \]

\[ y = 3.593x + 17.72 \]
Figure 12. Fluorescent detection and standard curve plots for the downstream probe P16S1194P. A) Amplification of probe P16S1194P analyzing *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* DNA. B) Standard curve for probe P16S1194N analyzing *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* DNA.
A)

\[ \Delta R_n \]

Cycle #

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B)

\[ y = 4.6586x + 18.385 \]

\[ y = 4.5122x + 18.208 \]

Cell Equivalent

EC

PA

Linear (EC)

Linear (PA)
Figure 13. Fluorescent detection and standard curve plots for the downstream probe P16S1194N. A) Amplification of probe P16S1194N analyzing *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* DNA. B) Standard curve for probe P16S1194N analyzing *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* DNA.

performed using probes P16S1194P and P16S1194N in singleplex and duplex reactions. The downstream Gram-positive probe P16S1194P was tested by using as template a four order of magnitude range of genomic DNA equivalent to $10^2$ to $10^5$ cells/ml from *E. faecalis*. There was no significant difference between the specificity and efficiency in the singleplex reactions and the duplex reactions in which it was coupled with probe P16S1194N (Fig. 14A). In converse, the downstream, Gram-negative probe P16S1194N was tested by using as template a four order of magnitude range of genomic DNA equivalent to $10^2$ to $10^5$ cells/ml from *E. coli*. There was no significant difference in the specificity and efficiency between the singleplex reactions and the duplexed reactions in which it was coupled with probe P16S1194P (Fig. 14B).
Figure 14. Analysis of competitive binding for downstream probes. A) Probe P16S1194P is analyzed using *E. faecalis*.DNA as a template in singleplex reactions. In the duplex reactions the P16S1194N probe is included. *E. coli* and *P. aeruginosa* templates are added in two of the duplex reactions as negative controls. B) Probe P16S1194N is analyzed using *E. coli* DNA as a template in singleplex reactions. In the duplex reactions the P16S1194P probe is included. *S. aureus* and *E. faecalis* DNA are added in two of the in duplex reactions as negative controls.
EMA decontamination treatment

Bacterial DNA is commonly found as a contaminant in commercially available PCR and qPCR solutions (53) and can be detected with primers and probes specific for the 16S rRNA genes. To eliminate this background contamination from the reagents, I first treated them with ethidium monoazide (EMA), which covalently intercalates into the double-stranded DNA and eliminates it from further denaturation, so that it cannot serve as a template in PCR reactions (54, 55). As a result, the optimization of the assay included testing pretreatment of the reagents with various concentrations of EMA (4 µM to 15 µM). At a concentration of 9 µM no inhibition of the qPCR reaction was observed and the background amplification was removed (Fig. 15). In this test reaction S. aureus genomic DNA was used at concentrations corresponding to $10^3$, $10^4$ and $10^5$ chromosomal equivalents. Samples were either treated or not treated with EMA (Fig, 15). Elimination of the high Ct value background amplification was observed with a 9 µM EMA pretreatment.
Figure 15. Effects of EMA treatment on qPCR reaction. Amplification plots are shown of three log dilutions of *S. aureus* DNA with and without EMA (9 µM) pretreatment of qPCR mastermix. Samples with EMA pretreatment are shown in blue. Samples without EMA pretreatment are shown in green. The reagents alone without EMA treatment is shown in orange. The reagents alone with EMA treatment is shown in red.
Synthesis and testing of phosphorothioate blocking oligonucleotides

There is concern that in a multiplex reaction in which all four probes were present there would be interference of the amplification of the desired products by amplification from outlying primers that occur because of the close proximity of the primer target sites (Fig. 16). To resolve this issue I developed phosphorothioate blocking oligonucleotides to prevent the generation of large PCR amplicons that arise from the amplification of products from outlying primers that occur in multiplex PCR reactions with targets in close proximity (Fig. 16).

Oligonucleotides in which a sulfur atom replaces one of the non-bridging oxygen's on the phosphate linkage are termed phosphorothioates (Fig. 17). This modification is known to provide greater resistance to enzymatic hydrolysis including to the exonucleolytic activity of DNA polymerase (56, 57). Phosphorothioates have been used in various applications including mutagenesis, antisense gene regulation, and single nucleotide polymorphism (SNP) genotype analysis (58-60).

I designed a functional phosphorothioate-containing oligonucleotide that anneals to the template DNA between the reverse primer of an upstream target (UpRp) and the forward primer of the downstream product (DnFp). This ‘blocking oligo’ prevents the amplification of the entire undesired amplicon (UpFp to DnRp) (Fig. 18). Specifically, I designed a 23-mer ‘blocking oligo’ that contains five phosphorothioate-modified linkages at the 5’ end that are directly followed by a nine-nucleotide region enriched in guanine and cytosine nucleotides, termed a ‘GC clamp’, and includes four adenines at the 3’
Figure 16. Schematic diagram of the use of phosphorothioate blocking oligonucleotides. The phosphorothioate blocking oligonucleotides prevent the generation of large PCR amplicons that arise from the amplification of products from outlying primers that occur in multiplex PCR reactions with primer targets in close proximity. (A) Two PCR targets in close proximity facilitate the generation of a large PCR amplicon by amplification initiating from the outlying primers (UpFp and DnRp). (B) The phosphorothioate blocking oligonucleotide anneals to the template downstream of the upstream forward primer and terminates extension.
Figure 17. 5' to 3' Phosphodiester linkage and 5' to 3' phosphorothioate linkage. (A) A phosphodiester linkage contains two non-bridging oxygens (one is shown in red). (B) A phosphorothioate linkage contains a non-bridging oxygen and a non-bridging sulfur shown in red.
Figure 18. A model of the phosphorothioate barrier to PCR amplification. (A) The phosphorothioate blocking oligonucleotide anneals to the template downstream of the upstream forward primer. The DNA polymerase extends from the upstream forward primer toward the blocking oligo. (B) and (C) The polymerase encounters the blocking oligo and is unable to hydrolyze the phosphorothioate-modified nucleotides. The extension is terminated and the polymerase detaches from the DNA. (D) If the blocking oligo lacking the phosphorothioate modifications anneals to the template, the 5′ to 3′ exonuclease activity of polymerase removes the nucleotides and extension is completed. Note: The phosphorothioate blocking oligo can also be designed to anneal downstream of the reverse primer.
end. The phosphorothioate modifications block the 5’ to 3’ exonuclease activity of the DNA polymerase and terminate extension. The ‘GC clamp’ ensures specific and strong annealing. The four 3’ adenines are designed to be mismatches and prevent possible extension from the blocking oligonucleotide. These specific design elements will be different for each target, but will always include four to five phosphorothioate-modified linkages at the 5’ end, followed by a strong ‘GC clamp’, and have four to five mismatches at the 3’ end.

I have demonstrated that phosphorothioates can be used as a barrier to PCR amplification. Experimental results show that an oligonucleotide containing all of our blocking oligo parameters except phosphorothioate modifications is not capable of blocking flanking amplification; however, this same nucleotide sequence with the phosphorothioate modifications is able to block flanking amplification (Fig. 19). It is anticipated that these ‘blocking oligos’ will have important applications.
Figure 19. The use of phosphorothioate blocking oligonucleotides to prevent the generation of large PCR amplicons. The large PCR amplicons arise from the amplification of products from outlying primers that occur in multiplex PCR reactions with targets in close proximity. (A) An agarose gel shows the presence of the large flanking amplicon resulting from amplification from the outlying primers in the reaction in which the blocking oligonucleotide lacked the phosphorothioate linkage modifications (lane 3) and elimination of the large amplicon in the reaction with the phosphorothioate blocking oligo (lane 4).

Testing S. aureus viability with EMA and PMA exposure

Initially when performing the artificial blood contamination experiments (see below) I found that S. aureus DNA was detected at one order of magnitude below the concentration of cells added to the blood samples. After reviewing the literature and performing some experiments, I hypothesized that the EMA-treated PBS used for the washing and dilution steps was toxic to the S. aureus cells (61, 62). To test this idea, I performed an experiment in which I exposed S. aureus cells to EMA-treated PBS for 10 and 30 min and to with non-EMA treated PBS as a control. I found that after a 10-min incubation with EMA-PBS there was
approximately a one-log loss in cell viability when compared to the non-EMA exposed cells. After exposure to EMA-PBS for 30 min no viable cells were recovered. I performed the same experiments in which EMA was replaced with PMA and found that there was no significant difference between the PMA-treated PBS and the non-treated PBS. Therefore, I replaced the EMA with PMA only in the reagent pretreatment protocol for the PBS solution used to suspend and dilute the live cells before DNA extraction. These samples served as controls for the experiments that tested the efficiency of extracting DNA from blood samples.

**Testing the qPCR assay in artificial blood samples**

**Detection of *S. aureus* DNA in Blood and PBS**

To evaluate the sensitivity and reproducibility of the MGS assay, artificial blood samples were prepared containing each of four laboratory strains of representative Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria at concentrations ranging from $10^1$ to $10^5$ cell/ml. As an additional control, similar samples were prepared in PBS. Yeast genomic DNA (200 ng) was added as carrier DNA to the PBS samples. Each of these eight samples was treated using the standard MGS assay protocol. The DNA was extracted, quantified, and a qPCR reaction was performed and analyzed. The efficiency of the whole protocol from DNA extraction through analysis was compared to the input cell concentration that was based on colony counts.

The *S. aureus* DNA detected in the blood ranged from 34 to 83 percent of the number of input cells from which it was extracted ($2.3 \times 10^2$ to $2.3 \times 10^5$).
cells/ml). The limit of detection for *S. aureus* cells was estimated at 100 cells/ml, as the DNA was not generally detected in blood at input cell concentrations less than $10^2$ cells/ml. Interestingly, the DNA detected from the cells resuspended in PBS had a poor correlation with the input cell concentrations. The *S. aureus* DNA detected in the PBS ranged from 0 to 20 percent of the numbers of input cells from which it was extracted. This poor recovery was observed with all of the different bacterial cells suspended in PBS (see above). Furthermore, the limit of detection for the PBS samples was approximately 1000 cells/ml. As expected the universal and Gram-positive probes amplified all of the *S. aureus* DNA samples and no signal was detected from the Gram-negative probe (Table 4).
<table>
<thead>
<tr>
<th>CFU per ml added to blood (determined by plate count)</th>
<th>Blood</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Average value</th>
<th>% Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Universal</td>
<td>Gram +</td>
<td>Gram +</td>
<td>Gram -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.3 x 10^3</td>
<td>103840 (45%)</td>
<td>64620 (28%)</td>
<td>68260 (29%)</td>
<td>-</td>
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<td></td>
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<td>13870 (60%)</td>
<td>15720 (68%)</td>
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<td>61</td>
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<tr>
<td>2.3 x 10^7</td>
<td>60 (260%)</td>
<td>-</td>
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<td></td>
</tr>
</tbody>
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<th></th>
<th></th>
<th></th>
<th>Average value</th>
<th>% Detection</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Gram +</td>
<td>Gram +</td>
<td>Gram -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.3 x 10^3</td>
<td>6180 (3%)</td>
<td>4370 (2%)</td>
<td>18230 (8%)</td>
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<td>570 (2%)</td>
<td>340 (1%)</td>
<td>3730 (16%)</td>
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<td>2.3 x 10^5</td>
<td>20 (1%)</td>
<td>-</td>
<td>260 (11%)</td>
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<tr>
<td>2.3 x 10^6</td>
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<tr>
<td>2.3 x 10^7</td>
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</tr>
</tbody>
</table>

Table 4. Detection of *S. aureus* DNA from inoculated blood and PBS samples.

**Detection of *E. faecalis* DNA in Blood and PBS**

The *E. faecalis* DNA detected in the blood ranged from 34 to 81 percent of the number of input cells from which it was extracted. The limit of detection of *E. faecalis* cells, similar to *S. aureus*, was estimated at 100 cells/ml, as no DNA was detected in the blood at concentrations less than 10^2 cells/ml. In this case, the DNA extracted from cells resuspended in PBS has more correlation with the number of input cells. The *E. faecalis* DNA detected in PBS ranged from 60 to
132 percent of the numbers of input cells. The limit of detection of *E. faecalis* cells in PBS was again estimated at 100 cells/ml. The probes amplified as expected for Gram-positive samples in all of the *E. faecalis* assays (Table 5).

<table>
<thead>
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<th>CFU per ml added to blood (determined by plate count)</th>
<th>Blood</th>
<th>PBS</th>
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</thead>
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<td>Downstream</td>
</tr>
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<td>Gram +</td>
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<td>3.6 x 10^4</td>
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<td></td>
</tr>
<tr>
<td>3.6 x 10^1</td>
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<td></td>
</tr>
</tbody>
</table>

**Table 5. Detection of *E. faecalis* DNA from inoculated blood and PBS samples.**
Detection of *E. coli* DNA in Blood and PBS

The *E. coli* DNA detected in the blood ranged from 66 to 169 percent of the numbers of input cells from which it was extracted. The limit of detection of *E. coli* cells in blood was approximately 10 cells/ml with the upstream universal probe, as DNA was detected at all the DNA concentrations. The limit of detection of was approximately 100 cells/ml with the downstream Gram-negative probe, as DNA was not generally detected in the blood at cell concentrations less than $10^2$ cells/ml. The *E. coli* DNA detected in PBS ranged from 70 to 198 percent of the number of input cells. The limit of detection of *E. coli* cells in PBS was approximately 100 cells/ml, as DNA was not detected in PBS at cell concentrations less than the $10^2$ cells/ml. As expected, the universal and Gram-negative probe amplified all the DNA and no signal was detected using the Gram-positive probes (Table 6).
Detection of *P. aeruginosa* DNA in Blood and PBS

The *P. aeruginosa* DNA detected in the blood ranged from 84 to 225 percent of the number of input cells. The limit of detection of *P. aeruginosa* cells was approximately 10 cells/ml with the upstream universal probe, as DNA was detected in samples at all DNA concentrations. The limit of detection of *P.
aeruginosa cells was approximately 100 cells/ml with the downstream Gram-negative probe, as DNA was not generally detected in the samples at cell concentrations less than $10^2$ cells/ml. The P. aeruginosa DNA detected in PBS ranged from 74 to 140 percent of the number of input cells. The limit of detection for the PBS was approximately 10 cells/ml. As expected, the universal and Gram-negative probe amplified all DNA and no signal was detected from the Gram-positive probes (Table 7).
<table>
<thead>
<tr>
<th>CFU per ml added to blood (determined by plate count)</th>
<th>Blood</th>
<th></th>
<th></th>
<th>Average value</th>
<th>% Detection</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Universal</td>
<td>Gram +</td>
<td>Gram +</td>
<td>Gram -</td>
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<tr>
<td>4.6 x 10^2</td>
<td>562840 (123%)</td>
<td>-</td>
<td>-</td>
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<td>4.6 x 10^4</td>
<td>77370 (168%)</td>
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<td>-</td>
<td>23630 (51%)</td>
<td>5 x 10^4</td>
</tr>
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<td>4.6 x 10^1</td>
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<td>-</td>
<td>-</td>
<td>7890 (171%)</td>
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<td>4.6 x 10^2</td>
<td>1070 (232%)</td>
<td>-</td>
<td>-</td>
<td>1016 (220%)</td>
<td>1 x 10^2</td>
</tr>
<tr>
<td>4.6 x 10^1</td>
<td>60 (130%)</td>
<td>-</td>
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</table>

<table>
<thead>
<tr>
<th>CFU per ml added to blood (determined by plate count)</th>
<th>PBS</th>
<th></th>
<th></th>
<th>Average value</th>
<th>% Detection</th>
</tr>
</thead>
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<td><strong>Downstream</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Universal</td>
<td>Gram +</td>
<td>Gram +</td>
<td>Gram -</td>
<td></td>
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<tr>
<td>4.6 x 10^2</td>
<td>413170 (89%)</td>
<td>-</td>
<td>-</td>
<td>297490 (114%)</td>
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<tr>
<td>4.6 x 10^4</td>
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<td>-</td>
<td>84550 (183%)</td>
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<td>-</td>
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<td>4.6 x 10^2</td>
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<td>-</td>
<td>264 (57%)</td>
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<tr>
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<td>14 (30%)</td>
<td>-</td>
<td>-</td>
<td>70 (152%)</td>
<td>4.2 x 10^1</td>
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Table 7. Detection of *P. aeruginosa* DNA from inoculated blood and PBS samples.

**Analysis of clinical samples**

Eighteen clinical samples were analyzed using the MGS assay and compared to results obtained by the Memorial Hermann Hospital Clinical Microbiology Laboratory. The Clinical Microbiology Laboratory, using culture and Gram-staining classified two of the 18 clinical samples (11%) as positive and 16 (89%) as negative (Table 8). Of the two positive samples, both were identified as Gram-negative bacteria by both the Clinical Microbiology Laboratory and the
<table>
<thead>
<tr>
<th>Sample</th>
<th>Patient</th>
<th>Upstream</th>
<th>Downstream</th>
<th>Blood Culture Result</th>
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<td>Gram +</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S3</td>
<td>Pt3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S2</td>
<td>Pt2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>Pt4</td>
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</tr>
<tr>
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<td>Pt11</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S10</td>
<td>Pt3</td>
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<td>0</td>
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<td>Pt13</td>
<td>320</td>
<td>0</td>
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</tr>
</tbody>
</table>

**Table 8. Analysis of clinical blood sample pellets with the Molecular Gram-stain assay.** Samples highlighted in yellow are positive blood samples with 100% congruence with the Molecular Gram-stain assay results. Samples highlighted in green are negative blood samples. Data highlighted in orange are negative blood samples that have 62% congruence with the Molecular Gram-stain assay results. <sup>a</sup> 22 hr to blood culture results. <sup>b</sup> 18 hr to blood culture results. An asterisk represents samples for which the values are below limit of detection. The values (cell/ml) are calculated based on *E. coli* standards for the samples that were detected by the universal and Gram-negative probes. S = sample, Pt = Patient.
MGS assay. This represents 100% congruence between the samples that were measured as positive by the ‘gold standard’ of blood culture and Gram-stain.

The Clinical Microbiology Laboratory after further analysis determined that these samples were *E. coli* (sample #5) and *Serratia marcescens* (sample #15).

Of the 16 culture-negative samples, six were also determined by the MGS assay to lack amplifiable bacterial DNA above the threshold equivalent to 100 cells/ml. This represents 37% congruence between the culture-negative samples and the MGS assay negative samples. Ten of the 16 culture-negative samples resulted in amplification of bacterial DNA above the 100-cell/ml threshold. This represents 62% non-congruence between the culture-negative samples and the MGS assay.

The average time to determination (TTD) of a positive blood culture result was 16.8 hr for the clinical laboratory (Table 8). The TTD for the MGS assay was estimated to be 4.5 hr by calculating the times required for sample processing, DNA extraction, and qPCR analysis.

I hypothesized that live and dead bacteria in the clinical blood samples could be differentiated by detection of DNA in the samples after centrifugation; the DNA from live cells would be associated with the pellet and the DNA of dead (lysed) cells would be in the plasma (supernatant). To test this hypothesis, I centrifuged all of the blood samples after collection and stored the pellet and supernatant separately at until analysis. The plasma DNA was extracted for samples #8 though #19 and used as template for the MGS assay (Table 9). The
<table>
<thead>
<tr>
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<th>P16S706P Cells/ml</th>
<th>P16S1194N Cells/ml</th>
<th>P16S1194P Cells/ml</th>
<th>Blood Culture Result</th>
</tr>
</thead>
<tbody>
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<td>-</td>
</tr>
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<td>10*</td>
<td>20*</td>
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<td>880 (18%)</td>
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</tr>
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<td>6900</td>
<td>0</td>
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<td>S14P</td>
<td>Pt9</td>
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<td>0</td>
<td>3830 (55%)</td>
<td>0</td>
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<td>0</td>
<td>7050</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>S10P</td>
<td>Pt3</td>
<td>0</td>
<td>0</td>
<td>6450 (91%)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>S18</td>
<td>Pt12</td>
<td>1910</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>S18P</td>
<td>Pt12</td>
<td>510 (27%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>S15</td>
<td>Pt10</td>
<td>210</td>
<td>0</td>
<td>50 (0.12)*</td>
<td>0</td>
<td>+(Gram-negative)</td>
</tr>
<tr>
<td>S15P</td>
<td>Pt10</td>
<td>0</td>
<td>0</td>
<td>39190</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 9. Analysis of clinical blood sample pellet compared with plasma.**
Samples highlighted in yellow are classified as positive blood samples and the plasma amplification value is higher than the pellet. Samples that are not highlighted are amplification-negative. Samples highlighted in blue are plasma amplification-negative and pellet positive. The samples highlighted in green are amplification-positive for both plasma and pellet. The samples highlighted in orange are universal probe positives. An asterisk represents samples for which the values are below limit of detection. S = sample, P = plasma, Pt = patient.
the plasma was not preserved for samples #2 through #7. For two of these samples (#8 and #16) there was no amplification above background detected for the plasma or the pelleted samples. For five of the samples (#11, #12, #13, #17, and #19) the plasma values were negligible, whereas the pellet samples were above background. For three of these samples (#9, #10, #14) the plasma values were above background and ranged from 18-91% of the pelleted value, but were always less than the pellet levels. For all of the plasma samples, except #18, the values for the universal primer were negligible. However, #18 was only amplified by the universal primer for both the plasma and pellet samples and the plasma amplification was 20% of that of the pellet. For only one sample (#15) that was positive by both culture and Gram-strain and MGS assay, was the value of the DNA in the plasma greater than the pellet. In this case the amplification in the plasma indicated that there was greater than 100 times more DNA in the plasma than in the pellet. This patient was released from the hospital, so presumably the antimicrobial treatment was effective and caused the DNA from many lysed cells or extruded DNA to be present in the plasma.
DISCUSSION

Sepsis is a severe and lethal health problem that is a growing concern with respect to patient outcomes and financial considerations. The current clinical ‘gold standard’ technique of blood culture and Gram-stain analysis used to diagnose sepsis has many shortcomings. Many molecular methods for the identification of pathogens associated with sepsis are being developed. These methods generally focus on rapid and accurate pathogen identification and include techniques such as PCR, microarrays, mass spectroscopy, NMR, and microscopy.

I have developed a novel quantitative real-time PCR assay to detect and classify the Gram-status of essentially all bacterial pathogens that is based on 33 bacterial pathogens commonly recovered from sepsis patients in the Memorial Hermann Hospital Surgical Trauma Intensive Care Unit. The MGS assay was designed to include internal checks and balances to provide an accurate detection and differentiation method. The assay utilizes a dual amplicon approach with two probes per amplicon to detect essentially all bacterial DNA by hybridization to a universally conserved region and to differentiate Gram-positive and Gram-negative bacterial DNA by hybridization to regions specific to these two types of bacteria.

The MGS assay was initially optimized using purified bacterial DNA. Subsequently, the entire protocol from DNA extraction through analysis was tested and optimized in samples in which each one of the four representative
bacterial species were added to previously uninfected human blood samples and PBS independently in a range of five orders of magnitude concentrations (10^1 to 10^5). Finally, 18 clinical blood samples from patients suspected to have sepsis were analyzed by the MGS assay and the Memorial Hermann Hospital Clinical Microbiology Laboratory and the results were compared.

I have determined that the assay is capable of quantitatively measuring the concentration of bacterial DNA of *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* extracted from blood and PBS with amplification efficiencies from 70 to 90%. The universal probe P16S683U detected DNA of all four bacterial species tested and was determined by *in silico* analysis to identify many other genera and species. The two Gram-positive probes, P16S706P and P16S1194P, detected both of the Gram-positive species without detecting the two Gram-negative species. In addition, *in silico* analysis determined that the probes could detect many other Gram-positive genera and species, but not Gram-negative species. The Gram-negative probe P16S1194N detected both of the Gram-negative species without detecting the two Gram-positive species and *in silico* analysis determined that it detected many other Gram-negative genera and species, but not Gram-positive species.

The MGS assay analysis of the 18 clinical blood samples was compared to results obtained by the Memorial Hermann Hospital Clinical Microbiology Laboratory. There was 100% congruence between the two positive blood culture results and the MGS assay results. In both cases, the pathogens identified were Gram-negative bacteria. There was 37% congruence between the 16 culture-
negative samples and the MGS assay results, as six of these samples were also
detected as amplification negative by the MGS assay or the values detected
were below the 100-cell/ml threshold limit of detection. There was 62% non-
congruence between the culture-negative samples and the MGS assay results,
as ten of the 16 culture-negative samples were amplification positive by the MGS
assay.

One of the advantages of the MGS method over other molecular detection
methods is that this method can 'rule out' infection as the cause of patient's
symptoms. A 'rule out' is possible due to the inclusion in the assay of the
universal probe that will bind to essentially any bacterial DNA in the sample. All
other assays developed to date are a 'rule in' type, in which the method is used
to determine if the sample contains a pathogen included in the list of organisms
that can be detected by the method. As a result, if a bacterium not within the
group is the causative organism it will not be detected and thus, cannot be ruled
out. This leaves physicians to depend on the blood culture results. However, it
is clear that blood culture is not a definitive assay, as many bacteria will not grow
well enough to reach the approximately $10^5$-cell/ml requirement for detection.
Furthermore, the high number (62%) of samples here that were negative by
culture, but resulted in a positive amplification supports this concept.

The MGS assay had a faster TTD, which was estimated as 4.5 hr from
sample arrival in the laboratory through analysis. The positive blood culture and
Gram-stain analysis required an average of 16.8 hr until the information was
transmitted to the physician. It is important to note that when the blood culture
analysis was negative the ‘TTD’ was 5-7 days. Although blood culturing is the current ‘gold standard’ in pathogen detection in blood samples, it appears that it should not be used as an benchmark for molecular methods of diagnosis.

One concern with using DNA-based molecular methods to detect pathogen DNA directly from blood is that these assays can detect DNA from both live and dead bacteria and cannot differentiate between them. This is of concern because only the live bacteria are considered ‘clinically relevant’. I hypothesized that the DNA in live cells would be associated with the pellet after centrifugation of a blood sample, whereas the DNA from dead (lysed) cells would remain in the plasma or supernatant fraction. The results showed that in all but one case there was no additional information provided by the analysis of the plasma samples. However, in one sample, which was determined by both the blood culture and Gram-strain analysis and the MGS assay to contain a Gram-negative bacterial pathogen, there was about three orders of magnitude more DNA in the plasma sample than the blood pellet. These data suggest that this pathogen primarily existed in the blood as lysed cells. This would suggest that the antimicrobial treatment was working to control the infection; the outcome for this patient was positive as he/she was released from the hospital. These experiments indicate that my hypothesis was correct and that determining the DNA of both the plasma and pellet fractions can have clinical relevance.

An important concern associated with using the 16S rRNA gene as an assay target is that the assay is so sensitive that it can detect any bacterial DNA that is present, including DNA contaminating the PCR and DNA extraction.
solutions. I addressed this issue by pretreating my qPCR mixes and DNA extraction solutions with EMA. However, problems arose with the qPCR amplification efficiency when EMA treatment was performed on the qPCR master mix containing the probes. The reason for the EMS-sensitivity of the probes is unclear. It was noted that the in the reactions the EMA-treated probes fluoresced much less than the non-EMA treated probes. This issue was resolved by using EMA-treated H\textsubscript{2}O to make the dilutions of the probes from the 100 uM and the 10 uM-working stock solutions. The probes were then added to the master mix after the EMA treatment of the qPCR master mix. This eliminated the exposure of the probes to EMA crosslinking and prevented the detection of contaminant DNA.

One surprising advantage to the extraction of small quantities of bacterial DNA from blood samples is that the concurrent extraction of human DNA serves as carrier DNA that increases the yield of the bacterial DNA. This became obvious when we noted the much greater yield of DNA from blood compared to PBS when each of the four control bacterial species were tested in blood samples and PBS. The results from the DNA extracted from blood samples were typically within one log of the input cell concentration used, whereas the DNA extracted from PBS were consistently 2-logs lower than the input cell concentration. The carrier DNA can also serve to dilute the bacterial DNA during the DNA extraction procedure and therefore any small percentage loss in yield would have significantly less impact on the overall amount of bacterial DNA that is lost. As a result, I modified the DNA extraction from PBS by adding 200 ng of yeast genomic DNA to act as carrier DNA. This improved the detection of our
control organisms significantly. However, it did not significantly improve the efficiency of the *S. aureus* DNA extraction from PBS.

Another surprising problem in our control experiments was associated with the EMA treatment. I discovered that the MGS analysis of the *S. aureus* from the blood was always one order of magnitude less than the input cells, whereas all of the other three organisms were much more closely correlated to the amount of input cells. The lower detection of *S. aureus* prompted a review of the literature, which revealed that EMA had been shown previously to have adverse effects on certain bacterial species (61, 62), including *S. aureus*. I performed experiments evaluating the viability of our *S. aureus* strain after EMA exposure. I determined that the EMA-treated PBS used in the washing and dilution steps was killing the *S. aureus* cells. After switching to PMA for decontamination of the solutions to which the live *S. aureus* cells were exposed, more consistent results were observed for *S. aureus*. It is important to note that although this was important in these control experiments in which live cells were added to blood and PBS, this is not relevant to clinical samples as the DNA is already extracted from the cells as a first step.

The MGS assay was developed based on the Gram status identification of the 33 bacterial species commonly recovered in presumed septic patients in the STICU of Memorial Herman Hospital in Houston, Texas. I am certain that a greater range of pathogens would be common in different types of infections, such as osteomyelitis. One strongpoint of the MGS assay is that it is easily amendable to species not identified in its current configuration due to the low
variability of the probe target sites. I expect that by adding a mixture of degenerate probes containing single or double nucleotide substitutions for the Gram-positive and Gram-negative probes, with all degenerate variations using the same fluorophore, the assay's species coverage could be greatly increased. However, this addition to the assay requires experimental validation.

The MGS assay is currently configured as two duplex reactions that are compared as 'upstream' and 'downstream' reactions in separate wells of a 96-well assay plate. I plan that the final MGS assay will be configured as a quadruplex reaction in which all four primers and all four probes are present in one well. This configuration will create a situation in which the extension from the forward primer from the upstream amplicon and the reverse primer from the downstream amplicon will generate PCR products that I have termed the 'flanking amplification' products. These flanking products are of particular concern because they are anticipated to use an abundance of reaction components causing the amplification of the target amplicons to be less efficient.

I have addressed this concern by developing a ‘blocking’ oligonucleotide containing phosphorothioate modifications that will anneal between the upstream reverse primer and the downstream forward primer. This blocking oligonucleotide will function to stop the extension of polymerase by forming a stable duplex with the target site and preventing hydrolysis of the blocking oligo by the phosphorothioate modifications. This technique has been tested in standard PCR and shows great promise for further use (Fig. 19); however, a quadruplex qPCR reaction is required for final testing.
In summary, I have succeeded in the primary goal of this project, which was to develop the Molecular Gram-stain assay to detect and determine the Gram status of sepsis-associated bacterial pathogens. Based on previous experiences in our laboratory I concluded that an ideal assay should contain internal checks and balances, detect all bacteria by hybridization to a universally conserved DNA region, and differentiate Gram status by hybridization to specific DNA regions. I have developed a novel molecular diagnostic technology for the detection of sepsis-associated infections. I have shown that this technology has the ability to decrease the time to pathogen detection. It is anticipated that the use of this MGS assay will provide an increase in the standard of septic patient care, resulting in better patient outcomes with more rapid tailored antimicrobial use for those with bone fide infections and removal of antimicrobial therapy from those without infection.
PERSPECTIVES

The development of new molecular techniques to diagnose infections is of interest to academic and industrial communities alike. The current new techniques include various methods of PCR analysis, mass spectroscopy, automated microscopy, and NMR probe detection. The goal of this project was to develop and validate a rapid, quantitative, and sensitive molecular assay for the direct detection of pathogens in blood samples from suspected sepsis patients.

The bacterial 16S rRNA gene has been used commonly as a target for the identification of bacterial genera and species. The MGS assay follows suit as it is a method to detect bacterial pathogens and to differentiate their Gram-status. However, it is not necessary to limit the use of this assay to detection alone. The two amplicons (229 bp and 270 bp) can also be subjected to DNA sequence analysis, which should be useful in identification of the genus and possibility species of the pathogen if it is a single or predominant organism present in the blood sample.

There are many options for downstream species identification with species-specific probes. Additionally, I intend to develop a probe that will identify the DNA of fungal pathogens to use along side the MGS assay.

Finally, although this molecular technology is an improvement upon the current culture-based methods in that it is more rapid, sensitive and quantitative, it does not currently provide information concerning antimicrobial susceptibility. However, we anticipate that molecular technologies for identifying antibiotic susceptibility genes and their activities will be further developed in the future.
REFERENCES


VITA

Doug Litwin was born in 1984 on his mother's birthday, February 29, in Houston, Texas to his parents Bruce and Mary Litwin. He graduated from Humble High School in 2002, and entered the University of Houston-Downtown in 2008. After graduation, Doug worked as a research assistant for Millicent Goldschmidt Ph.D. at the University of Texas School of Dentistry at Houston. A year later he entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences in August 2012.