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Multiplexed Loop-mediated Isothermal Amplification of the 16S rRNA Gene for the Diagnosis of Neonatal Sepsis in Resource-limited Environments

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Abstract

Sepsis, or dysregulated inflammation caused by bacterial infection, places a disproportionately high burden on newborns in developing countries. This is due in part to a lack of diagnostic tools suitable for sustainable use in resource-limited nurseries. One potential vehicle for a new diagnostic assay is loop-mediated isothermal amplification (LAMP), a high-yield DNA amplification method. LAMP has previously been used to detect genes from single species of bacteria in blood serum samples to aid in sepsis diagnosis. LAMP could be adapted to detect a broad set of bacteria, while retaining a degree of specificity that allows clinicians to begin directed antimicrobial therapy. Described herein is the successful design of a novel group of oligonucleotide LAMP primer sets that specifically bind to regions of the 16S rRNA gene of four bacterial orders. These could provide clinicians with a two-step sepsis-diagnosis technique that would provide a result in only one hour.

Introduction

Sepsis, or systemic inflammatory response syndrome in the presence of bacterial infection, is a leading cause of newborn morbidity and mortality, with the burden especially evident in developing countries; sepsis kills more than 1.6 million newborns annually in these resource-limited environments (Turner et al., 2013). The standard therapy for treating neonatal sepsis is administration of an antibiotic regimen, following clinical diagnosis. However, diagnosis of neonatal sepsis is complicated by its non-specific clinical presentation (Piantino, Schreiber, Alexander, & Hageman, 2013). While diagnostic tools must be used to aid in confirming sepsis diagnosis, current tools are not suitable for sustainable use in resource-limited environments since many of them are too expensive, require too much infrastructure, or are labor-intensive and too complicated for practical clinical application (Seale, Mwaniki, Newton, & Berkley, 2009; Vergnano, Sharland, Kazembe, Mwansambo, & Heath, 2005). Without diagnostic tools, clinicians must resort to prolonged broad-spectrum antibiotic therapies, operating under the assumption that clinical abnormalities connote sepsis. The unwarranted use of these antimicrobials continues to contribute to the increase of antibiotic-resistant strains (Kuppala, Meinzen-Derr, Morrow, & Schibler, 2011; Piantino et al., 2013). In addition, prolonged broad-spectrum antibiotic therapy can lead to adverse outcomes for the newborn, including increased risk of necrotizing enterocolitis (Cotton, 2009; Piantino et al., 2013). Diagnostic tools must be developed that are accessible to clinicians practicing in resource-limited environments, so that targeted antimicrobial therapy can be implemented. The ideal tool would be cost effective, simple to use, highly specific, and highly sensitive.

Background

Culture of bacteria from blood is the diagnostic gold standard for neonatal sepsis. Bacterial pathogens may be isolated and identified from a blood sample once incubated. However, blood culture requires a significant amount of infrastructure and trained lab personnel; thus, it is not practical for use in resource-limited settings (Simonsen, Anderson-Berry, Delair, & Davies, 2014). Additionally, blood cultures require taking at least 1mL of blood from the neonate. Removal of this significant amount of blood can lead to adverse effects (Piantino et al., 2013). Moreover, blood cultures take too long to give a definitive result; even the most advanced blood culture systems take at least 48 hours to yield a positive result (Raju, Bhat, Lewis, & Vandana, 2013). This waiting period necessitates administration of broad-spectrum antibiotics until the pathogen is identified, potentially leading to the previously mentioned adverse
outcomes. Therefore, a more rapid diagnostic test must be developed to quickly identify pathogens causing neonatal sepsis.

Recent novel approaches to aid in diagnosis of sepsis have decreased the amount of time required for a result. Simple laboratory tests such as C-reactive protein or white blood cell count can provide a rapid, sensitive result of biomarker levels (Piantino et al., 2013). These types of tests could potentially be used sustainably in resource-limited environments. However, these biomarkers are actually indicators of Systemic Inflammatory Response Syndrome (SIRS), which has a variety of causes and therefore a variety of treatments. These biomarkers often lead to false-positive sepsis diagnoses, since clinicians assume that SIRS connotes infection (Rajani & Philip, 2011). In fact, sepsis is defined as SIRS caused by an infection (Goldstein, Giroir, & Randolph, 2005). There are many other causes of SIRS that are not pathogenically based (Piantino et al., 2013). Thus, false-positive diagnosis of sepsis based on SIRS biomarkers can result in unnecessary antibiotic administration. A diagnostic test must be developed that is specific to bacterial infection so that appropriate treatment options can be considered.

One approach to specifically detect bacteria from blood serum samples is to target regions of the bacterial genome. Since the development of polymerase chain reaction (PCR), many diagnostic tests for bacterial infection have been developed that rapidly detect nucleic acid sequences specific to prokaryotes (Lu, Perng, Lee, & Wan, 2000). PCR involves using a heat-stable polymerase to rapidly replicate DNA, using two oligonucleotide primers to specify the replication region. One advantage of PCR is that the Taq polymerase employed can amplify large fragments (>1000bp) of DNA, allowing for the amplification of some whole genes (Wu et al., 2007). However, PCR must be used in combination with sequence-specific probes or further laboratory analysis to provide identity information when targeting a wide array of species (Lu et al., 2000). Additionally, in order to denature the DNA for replication to occur, the sample must be repeatedly heated and cooled, a process called thermal cycling (Notomi et al., 2000). This process requires a complex thermocycler, which is inaccessible to many resource-limited clinics.

To overcome the burden of sophisticated laboratory equipment, Notomi and colleagues (2000) developed a new method for gene amplification called loop-mediated isothermal amplification (LAMP). Since LAMP employs a Bst DNA polymerase that exhibits strand-displacement activity, there is no need to thermally cycle the sample. This negates the need for a complex thermocycler; the only equipment required to carry out LAMP reactions is a hot-water bath. Also, unlike PCR, LAMP utilizes four primers to recognize six distinct regions of target DNA sequence, resulting in greater amplification specificity. Several studies have shown the specificity of LAMP primers to detect individual bacterial species and closely related strains in less than one hour (Hill, Beriwal, Chandra, Paul, & Kapi, 2008; Huy et al., 2012; Kimura, Yanagisawa, Wachino, Shibayama, & Arakawa, 2013; Lim, Teh, & Thong, 2013; Seki et al., 2005). All of these studies have shown the specificity of LAMP primers to detect individual bacterial species and closely related strains in less than one hour (Hill, Beriwal, Chandra, Paul, & Kapi, 2008; Huy et al., 2012; Kimura, Yanagisawa, Wachino, Shibayama, & Arakawa, 2013; Lim, Teh, & Thong, 2013; Seki et al., 2005). While a diagnostic test that detects a single species could be helpful in some circumstances, it would not be a comprehensive detection method for all sepsis-causing bacteria. The ideal diagnostic test in a resource-limited environment would provide a broad result of definitely sepsis-negative or definitely sepsis-positive, with some indication of the type of bacteria present.

One gene target that is highly characterized and conserved across prokaryotes is the 16S ribosomal RNA gene. The conserved regions along this gene are common targets in PCR, since primers can be designed that are common to almost all prokaryotes (Lu et al., 2000; Wu et al., 2007). Flanking the highly conserved regions along the 16S rRNA gene are nine regions that are hypervariable across species. These hypervariable regions have been the targets of several species-specific detection and identification methods (Chakravorty, Helb, Burday, Connell, & Alland, 2007). Since LAMP can amplify small regions of DNA, it could be possible to amplify regions of the 16S rRNA gene along the boundary of the hypervariable and conserved regions. This could allow for an amplification of DNA from a broader group of species, while retaining a certain level of specificity. Since no single hypervariable boundary region could distinguish all possible prokaryotes causing sepsis, multiple hypervariable/conserved boundaries could be targeted with separate primer sets to distinguish bacterial groups present.

To perform multiple LAMP reactions simultaneously, Fang and colleagues (2010; 2011) recently developed a
multiplexed microfluidic chip. Although these reactions targeted viral genes, the concept could be applied to isolate LAMP products from reactions targeted toward various hypervariable/conserved boundaries of the 16S rRNA gene. In order to detect amplification without complex equipment, visual detection with fluorescent reagents could be used. Tomita and colleagues (2008) have recently demonstrated that calcein can act as a fluorescent indicator of amplification, based on the generation of pyrophosphate ions. Herein is proposed a point-of-care multiplexed loop-mediated isothermal amplification system that targets the boundaries of hypervariable and conserved regions of the 16S rRNA gene in bacteria causing neonatal sepsis, utilizing a simple visual detection method based on fluorescence.

Materials and Methods

Selection of Target Sequences and Primer Design

To determine which bacterial species to target, a literature review was conducted to find the most common species identified in neonatal sepsis cases in resource-limited environments. The 16S rRNA genes of the most common strains of species contributing to neonatal sepsis cases were accessed from the NCBI GenBank (Benson, 2013). These genes were aligned using ClustalΩ multiple sequence alignment software (Goujon et al., 2010). Areas conserved across species were selected as target sequences. PrimerExplorer V4 software from Eiken Chemical Co. (Nogi, Japan) was used to generate novel oligonucleotide primer designs using the selected target sequences. Primer designs were run through a BLAST search against the prokaryote database to check for in silico specificity.

Culture of Bacteria

Eight species were used as representatives, two from each of the previously mentioned orders that include sepsis-causing bacteria. Plate cultures of Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa were obtained from the microbiology laboratory on the University of Arkansas campus. Lyophilized ampules of Acinetobacter baumannii (ATCC® 19606™), Streptococcus agalactiae (ATCC® 13813™), and Enterococcus faecalis (ATCC® 19433™) were obtained from the American Type Culture Collection (Manassas, VA). Lyophilized S. agalactiae and E. faecalis specimens were resuspended in 6 mL of brain-heart infusion (BHI) broth (Difco™) and incubated at 37°C for 24 hours. Lyophilized A. baumannii specimen was resuspended in 6mL of LB broth (Difco™) and also incubated under the same conditions. Glycerol stocks of all species were prepared with 50% of the appropriate broth (S. agalactiae and E. faecalis in BHI, all others in LB) and 50% glycerol, and then stored at -80°C. Following preparation of glycerol stocks, all specimens were individually quadrant streaked onto either BHI or LB agar plates and incubated at 37°C for 24 hours to isolate individual colonies. Plates were then stored at 4°C until DNA isolation.

Genomic DNA Extraction

Bacterial cell walls were lysed using boiling lysis technique, as described by Iwamoto and colleagues (2003), in order to extract genomic DNA. Briefly, a single colony of each strain was collected using an inoculating loop and individually suspended in 100µL of boiling lysis buffer (2mM EDTA, 1.2% Triton X-100, and 20mM Tris•HCl [pH=8], stored at 4°C). Cell suspension lysis tubes were then placed in a boiling water bath at 100°C for 20 minutes. Lysates were stored at -20°C until use in LAMP optimization reactions. Crude lysates were used directly in LAMP reactions.

Optimization of LAMP Reaction Conditions

Initial LAMP reactions were carried out to determine optimal conditions using Loopamp DNA Amplification Kit and calcein-based Fluorescent Detection Reagent, both from Eiken Chemical Co. (Nogi, Japan). Products from LAMP reactions were viewed under UV light at 365nm then run on agarose gels to confirm primer binding and amplification. To find optimal visual fluorescence results, LAMP was repeated for three sets of positive and negative controls using varying Mn²⁺ concentrations.

Determining Limit of Detection

To find the minimum amount of DNA that the primers could specifically amplify, the P. aeruginosa DNA sample was used as a representative. P. aeruginosa crude DNA lysate was serially diluted in lysis buffer.
LAMP was performed using Pseudomonadales primers with the lower dilution samples of the series. Results were viewed visually under UV excitation and with agarose gel electrophoresis.

**Finding Specificity, Sensitivity, PV(+), and PV(-) of Primers and Calcein**

LAMP reactions were performed using the optimized LAMP conditions. Primer sets were tested with DNA samples from species within orders for which they were designed. In addition, primers were cross-reacted with DNA from a species within an opposing order. Sensitivity, specificity, positive predictive value [PV(+)], and negative predictive value [PV(-)] were determined according to Equations (1)-(4), based on Rajani and Philip (2011), for both simple visual fluorescence and agarose gel electrophoresis detection:

\[
\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}} \quad (1)
\]

\[
\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}} \quad (2)
\]

\[
\text{PV(+)} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Positives}} \quad (3)
\]

\[
\text{PV(-)} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}} \quad (4)
\]

‘True positives’ for simple visual detection method constitute an emission of fluorescence when the primer set is tested with DNA for which it is designed (i.e. a positive result is obtained when expected); ‘true negatives’ are a lack of fluorescence when the primer set is tested with DNA for which it is not designed; ‘false positives’ are an emission of fluorescence when the primer set is tested with DNA for which it is designed (Rajani & Philip, 2011). For primer sensitivity, specificity, PV(+), and PV(-), the same definitions apply, with ladder-like streaks on agarose gel in lieu of fluorescence.

**Results**

**Target Sequences Selected**

Based on the study by Zaidi and colleagues (2009), the eight most-commonly identified isolates of sepsis-causing bacteria are listed in Table 1. Further investigation was conducted of review articles determining the strains of those species responsible for neonatal sepsis.

**Table 1. Etiology of sepsis-causing bacteria in resource-limited environments. Data based on Zaidi (2009).**

<table>
<thead>
<tr>
<th>Order</th>
<th>Organism</th>
<th>% of Isolates from All Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriales</td>
<td><em>Escherichia coli</em></td>
<td>17.08</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella species</em></td>
<td>13.49</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella species</em></td>
<td>4.78</td>
</tr>
<tr>
<td>Lactobacillales</td>
<td><em>Group B streptococci</em></td>
<td>8.06</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus pneumoniae</em></td>
<td>5.67</td>
</tr>
<tr>
<td>Pseudomonadales</td>
<td><em>Pseudomonas species</em></td>
<td>7.17</td>
</tr>
<tr>
<td></td>
<td><em>Acinetobacter species</em></td>
<td>4.20</td>
</tr>
<tr>
<td>Bacillales</td>
<td><em>Staphylococcus aureus</em></td>
<td>13.30</td>
</tr>
</tbody>
</table>

The 16S rRNA genes accessed from NCBI GenBank are listed in Table 2. Based on the phylogenies of species responsible for sepsis, it was determined that target sequences should be common to the order-level of classification. This resulted in four orders of bacteria to be separately targeted: Bacillales, Lactobacillales, Enterobacteriales, and Pseudomonadales.

To find regions conserved within an order, three to five dissimilar 16S rRNA gene sequences from the same order were aligned with ClustalΩ multiple sequence alignment software (Goujon et al., 2010). The consensus sequence from this first alignment was then aligned with two 16S rRNA gene sequences from each of the three opposing orders, since the phylogenies of the orders differed from one another.

---

**Table 2. 16S rRNA Genes Accessed from NCBI GenBank, with strain or serovar of species listed with accession numbers.**

<table>
<thead>
<tr>
<th>Order</th>
<th>Species</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillales</td>
<td><em>Bacillus cereus</em> (ATCC 14893)</td>
<td>AJ310098</td>
</tr>
<tr>
<td></td>
<td><em>Listeria monocytogenes</em> str. 4b F2365</td>
<td>AE017262</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus subsp. aureus</em></td>
<td>CP000730</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus epidermidis</em> RP62A</td>
<td>CP000029</td>
</tr>
<tr>
<td>Enterobacteriales</td>
<td><em>Enterobacter sakazakii</em> ATCC (51329)</td>
<td>AY752937</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em> O7:K1 CE10</td>
<td>CP003034</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella pneumoniae</em> K2</td>
<td>CP006648</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enterica</em> sero. enteritidis</td>
<td>DQ344532</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enterica</em> sero. typhimurium</td>
<td>DQ344453</td>
</tr>
<tr>
<td>Lactobacillales</td>
<td><em>Enterococcus faecalis</em></td>
<td>AJ039002</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus agalactiae</em> sero. V</td>
<td>AJ009948</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus pneumoniae</em> R6</td>
<td>AJ007317</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus pyogenes</em> M1</td>
<td>AJ004092</td>
</tr>
<tr>
<td>Pseudomonadales</td>
<td><em>Acinetobacter baumannii</em></td>
<td>X81667</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em> N796</td>
<td>AB037553</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas oryzihabitans</em> str. WB2003S</td>
<td>AY850170</td>
</tr>
</tbody>
</table>
enough to be represented by only two species each. This alignment file was compared using Base-by-Base software (Hillary, Lin, & Upton, 2011) to view regions of the consensus sequence of the first alignment that are unique from the 16S rRNA gene of other orders. Unique regions of a conserved sequence were noted, and the process was repeated for the 16S rRNA genes of the remaining three orders. All four consensus sequences were aligned again and compared using Base-by-Base software to show the nine hypervariable regions along the 16S rRNA gene, seen in Figure 1.

**Novel Oligonucleotide Primers Designed**

Four novel oligonucleotide primer sets were designed using PrimerExplorer V4 software. Each set consisted of four primers: F3, B3, FIP (containing regions F1c and F2), and BIP (containing regions B1c and B2). Briefly, the consensus sequence of an order-specific alignment was used as a target DNA sequence. Mutations were introduced in the PrimerExplorer design window at positions on the target sequence where the consensus sequence was nonconserved across species within the order. This caused the primers to be assigned accordingly, with the 3’ end of internal primers F1c or B1c; 5’ end of F2 or B2; and 5’ end of F3 or B3 being the only primer regions overlaying the mutations (A Guide to LAMP Primer Designing). An example of this is shown in Figure 2. These mutation sites thus have minimal effect on primer specificity.

Using the Base-by-Base results as a reference, primers were then assigned locations unique to consensus sequences of a given order on the boundary of the hypervariable and conserved regions of the 16S rRNA gene. Therefore, primers bind to regions conserved within an order, but variable across orders. This process was repeated three times for each of 16S rRNA genes of the remaining orders. Each primer set design was then run in a BLAST search against prokaryotic genomes to confirm theoretical primer specificity for all species within the prescribed order. The final primer designs are listed in Table 3.

**LAMP Confirmation and Cross Reactions: Primers Specifically Bind**

For each LAMP optimization reaction: 40 pmol of FIP and BIP, 5 pmol of F3 and B3 from the prescribed primer set, 12.5 µL LAMP reaction mix (2x), 1 µL

![Figure 2. Sample of primer assignment to short sequence of conserved 16S rRNA gene across the order Pseudomonadales. The top sequence is conserved 16S rRNA gene. Dashes are introduced ‘mutations’, or areas of non-conservation within the Pseudomonadales order. Primer B3 (chartreuse) and region B2 of primer BIP (indigo) are assigned so mutations fall at the 5’ ends, reducing the mutations’ effect on binding. General B3 primer location was assigned to the edge of the hypervariable region (beginning at 401+).](image_url)
Bst DNA polymerase, 2 µL of the prescribed crude DNA lysate, 1 µL Fluorescent Detection Reagent, and 4.5 µL microbiology-grade H₂O were added to a sterile µcentrifuge tube, for a total reaction mixture of 25 µL. For positive control, Loopamp positive control DNA and positive control primers were added to LAMP reagents in lieu of sample DNA and novel primers. Positive control primers from Eiken are specific to recombinant pDNA with HindIII insertion (provided in Loopamp Kit). For negative control, sample DNA template was replaced by water. Each reaction mixture was incubated in a 63°C water bath for 60 minutes. LAMP reactions were then terminated by incubation at 80°C for 2 minutes to inactivate the polymerase. This procedure was repeated, but with 6 µL of Enterobacteriales primer set added to E. coli and K. pneumoniae reactions. In this second optimization reaction, the incubation temperature was increased to 65°C. Results of primer confirmation reactions, using optimized LAMP conditions, are shown in Figures 3-5. Amplification products were viewed under UV light then measured with a Qubit 2.0 fluorometer to find relative fluorescence units (RFU) in the green wavelength (500-525nm). Amount of fluorescence did not correspond to amount of DNA amplified, when compared with the products run on a 2.5% agarose gel. Positive fluorescence was not significantly higher than negative fluorescence. LAMP cross-reactions were also viewed under UV light and run on an agarose gel, see Figures 5 and 6.

**Sensitivity, Specificity, PV(+), and PV(-)**

The results of the LAMP confirmation and cross-reactions are summarized in the matrix in Figure 7. Calcein fluorescent probe statistically displayed theoretical 100% sensitivity, but very low specificity, between 0-67% depending on reaction. In addition, it displayed 100% PV(-), but varying PV(+) between 50-100%, depending on reaction. Each of the primer...
Figure 6. LAMP cross-reactions: primer sets paired with DNA from species of the closest-related order, or with a more phylogenetically distant order from the primer’s designated order. Calcein non-specifically emits fluorescence, even with no amplification. Viewed under UV lamp at 365nm.

sets, however, displayed 100% sensitivity, specificity, PV(+), and PV(-) in this small trial—each primer set successfully bound to its target and no other targets.

Limit of Detection: LAMP More Efficient when Diluted

Crude DNA lysate from a single colony of *P. aeruginosa* was serially diluted to achieve a 1/10⁹ dilution. LAMP reaction products from the six most dilute *P. aeruginosa* DNA lysates and Pseudomonadales primers are shown in Figures 8-9. The primer set tested here displayed higher efficiency at lower theoretical concentrations, with more intense bands appearing on the agarose gel. Amount of fluorescence by calcein was not proportional to intensity of bands on agarose gel.

Discussion and Future Direction

Successful Novel Primer Design

Agarose gel electrophoresis results of LAMP products suggest that each primer set specifically binds to the target sequence for which it was assigned. These target sequences consist of hypervariable/conserved boundary regions of the 16S rRNA genes of species within four orders containing sepsis-causing bacteria. The electrophoresis results of both confirmation and cross-reactions showed non-specific streaks on the lower halves of lanes. The appearance of these streaks could be due to the presence of calcein in the loaded sample, which has similar excitation and emission wavelengths to SYBR® Safe, the DNA stain used in the electrophoresis. However, intensities of these lower streaks on the agarose gel were not consistent with intensity of fluorescence observed under UV. Alternatively, these streaks could be attributed to unreacted primers. The absence of these streaks in the more efficient LAMP reactions of the serial dilution products supports this hypothesis, where all primers would be utilized and incorporated into DNA product. Additionally, the presence of these streaks in negative cross-reactions and no-template controls supports that these bands are due to the presence of unreacted

![Figure 6](image6.png)

![Figure 8](image8.png)

Figure 6. LAMP cross-reactions: primer sets paired with DNA from species of the closest-related order, or with a more phylogenetically distant order from the primer’s designated order. Calcein non-specifically emits fluorescence, even with no amplification. Viewed under UV lamp at 365nm.

Figure 8. LAMP products of serial dilution reaction, showing dilution factor of crude DNA lysate from one colony. Viewed on UV lamp at 365nm.

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primers. Positive results in agarose gels appear as dense ladder-like streaks from the top well down, due to the formation of varied-molecular weight stem-loop DNA produced from the Bst DNA polymerase (Notomi et al., 2000). These products are not visible in cross-reactions, since primers do not bind to target sequences, and Bst polymerase does not synthesize any new DNA. Very high levels of specificity, sensitivity, PV(+), and PV (-) for these primer sets are promising. However, these were calculated from a small data set, and should be re-evaluated using a larger breadth of species for both confirmation and cross-reactions.

Agarose gel electrophoresis results of LAMP products from serial dilutions of P. aeruginosa DNA lysate showed successful primer binding, even with template DNA at a 1/10⁹ dilution. It is interesting to note that the electrophoresis products of these serial-dilution amplifications suggest that the reaction was more efficient with less initial target DNA. The presence of the most intense ladder-like streak on the 10⁹ lane

**Potential of Primers for Clinical Application**

While many other LAMP primers have been designed to detect individual sepsis-causing species, no broad-spectrum LAMP primers have been developed. Large groups of bacteria are amplified by the primers described herein, which could provide a broad diagnostic method for sepsis-positive or sepsis-negative neonates. Though PCR primers can also amplify the 16S rRNA gene for bacteria, they provide no insight regarding the identity of the species present (Lu, 2000). Further laboratory analysis or additional probes are required to gain any indication of the type of bacteria present, since the PCR universal primers amplify the entire 16S rRNA gene for most bacteria (Chakravorty et al., 2007). In contrast, the novel LAMP primer sets described here are specific to four orders of bacteria. Future work should include clinical testing of samples compared with the gold standard of blood culture to confirm primer specificity. If these primer sets are separated and simultaneous reactions are carried out, clinicians could know the identity of any bacteria present in serum samples down to the order-level—enough information to begin a targeted antimicrobial regimen (Simonsen et al., 2014). This information could be attained in under an hour, with as little infrastructure as a hot-water bath and simple UV light, provided the specificity of the fluorescent reagent. However, simple visual detection with calcein fluorescent reagent did not correspond to the results of agarose gel electrophoresis.

**Non-specific Fluorescence of Calcein Probe**

Calcein should only exhibit fluorescent activity when an amplification reaction occurs; however, fluorescence was detected in many products not containing any amplified DNA, as shown by agarose gel electrophoresis. This could be due to the presence of metal chelators in the reaction solution, which would remove the Mn²⁺ quencher from calcein. These chelators, such as EDTA, could be present from the crude bacterial lysates used as DNA targets. Fluorescence was not consistent over similar reaction conditions, however, and non-specific results were detected each time. In addition, negative controls still emitted some fluorescence, where the intensity was not significantly different from positive reactions (p=.05, n=10). Therefore, calcein is an unreliable fluorescent indicator of amplification reactions in this circumstance.
Other avenues of visual fluorescent detection of DNA should be investigated. LAMP reactions should be repeated using SYTO-82 DNA stain. Initially it was hypothesized that SYTO-82 may result in non-specific fluorescence due to the presence of genomic DNA. However, given the magnitude of amplification product from LAMP reactions, it is estimated that there would be a significant difference in fluorescence emission intensities from amplified DNA (positive results) from non-amplified genomic DNA (negative results) stained with SYTO-82. If successful, SYTO-82 could be used as an indicator of simultaneous amplification reactions on a multiplexed microfluidic chip.

**Potential for Multiplexing Reaction**

To differentiate the orders present in a clinical serum sample, the four novel primer sets could be separated in distinct channels in a microfluidic chip. The LAMP reagents and lysed serum sample could be loaded into a central filling chamber that equally distributes the solution across the separate channels. A \( \mu \)-LAMP chip designed by a student colleague could facilitate these multiplexed reactions, pending the successful casting of PDMS replicas from 3-D printed molds. If successful, clinicians could use the \( \mu \)-LAMP chip as a two-step detection method—one step to expose the bacterial DNA and one step to load LAMP reagents—for diagnosing neonatal sepsis in about an hour. The successful design of novel oligonucleotide primers warrants further investigation into the visual detection mechanisms of the reaction.

**Acknowledgements**

The author would like to thank the Arkansas Department of Higher Education for research funding through the Statewide Undergraduate Research Fellowship. In addition, the author thanks Dr. Kartik Balachandran for further research advising. The author also thanks Grace Morrison for designing the \( \mu \)-LAMP chip. This research is dedicated to the memory of Dr. Jerry Umanos, the pediatrician in Kabul who first made the author aware of the burden of sepsis in Afghanistan and similar developing countries.

**References**


