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Griffin Thomas Sonaty
University of Arkansas, Fayetteville

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

An Undergraduate Honors College Thesis

in the

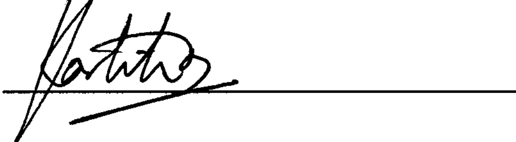
Department of Biomedical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

Griffin T Sonaty

This thesis is approved.

Thesis Advisor:



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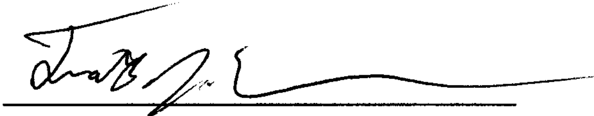
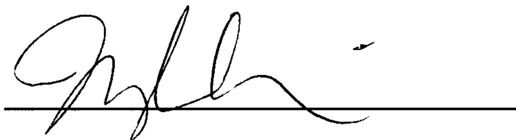




Summary

Bacterial infection, or sepsis, 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 been used to detect single genes from bacteria in blood serum samples to aid in sepsis diagnosis. While specific, this approach can only provide detection for one species at a time. LAMP could be adapted to detect a broader 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 regions lie on the transition area between sequences that are highly conserved and those that are hypervariable. This allows each primer set to be specific for one Order of bacteria. When primers bind, amplification occurs, and the large quantities of DNA produced could be detected using a fluorescent indicator. The four separate LAMP reactions could be multiplexed on a microfluidic chip to provide clinicians with a two-step sepsis-diagnosis technique that could give a result in only one hour. Future studies should examine fluorescent indicators, reaction multiplexing, and specificity of primers to recognize more species.

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

Introduction

Sepsis, or 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.[3] 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.[6] Diagnostic tools must be used to aid in confirming sepsis diagnosis. Current tools are not suitable for sustainable use in resource-limited environments—many of them are too expensive, require too much infrastructure, or are labor-intensive and too complicated for practical clinical application.[1,2] 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.[6,7] In addition, prolonged broad-spectrum antibiotic therapy can lead to adverse outcomes for the newborn, including increased risk of necrotizing enterocolitis.[6,8] 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, and highly sensitive.

Culture of 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.[4] 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.[6] 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.[5] This waiting period necessitates the 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.[6] These types of tests could potentially be used sustainably in resource-limited environments. However, these biomarkers are actually indicators of Systematic Inflammatory Response Syndrome (SIRS), which has a variety of causes and therefore a variety of treatments.[6] These biomarkers often lead to false-positive sepsis diagnoses, since clinicians assume that SIRS connotes infection.[10] In fact, sepsis is defined as SIRS caused by an infection.[9] There are many other causes of SIRS that are not pathogenically-based.[6] 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 detecting 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.[11] 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 of DNA.[12] However, PCR must be used in combination with restriction-digestion of products to provide any information about the identity of the pathogen present.[11] This requires further laboratory infrastructure and personnel. Additionally, in order to denature the DNA for replication to occur, the sample must be repeatedly heated and cooled—a process called thermal cycling.[13] This process requires a complex thermocycler, which is inaccessible to many resource-limited clinics.

Recently, however, Notomi, *et al.* developed a new method for gene amplification called loop-mediated isothermal amplification (LAMP).[13] Since LAMP employs a *Bst* DNA polymerase that exhibits strand-displacement activity, there is no need to thermally cycle the sample.[13] 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.[13] However, the *Bst* polymerase in LAMP can only amplify target

sequences between 100-300 base pairs.[13] Several studies have shown the specificity of LAMP primers to detect individual bacterial species and closely related strains in less than one hour.[15-19] All of these studies targeted small regions of DNA that are unique to given species. While a diagnostic test that detects a single species could be helpful in some circumstances, it is not 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. It is a common target of PCR universal primers, which can amplify the gene for almost all bacteria.[11,12] The gene contains within it nine regions that are hypervariable across species, which are flanked by highly conserved regions, as seen in Figure (1). These hypervariable regions have been the target of several species-specific detection and identification methods.[23] Since LAMP can amplify small regions of

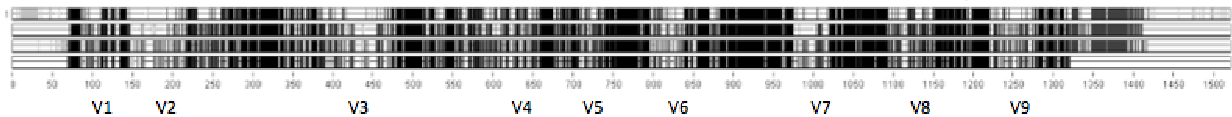


Figure 1. Conservation across consensus sequences of the 16s rRNA gene from the four bacterial Orders of interest in neonatal sepsis. Darker = higher conservation. V1-V9 are the nine hypervariable regions.

DNA, it could be possible to amplify regions of the 16s rRNA gene along the boundary of the hypervariable regions. This could allow for an amplification of a larger group of species, while retaining a certain level of specificity. Since no single hypervariable 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, *et al.* recently developed a multiplexed microfluidic chip.[20-21] Although these reactions targeted viral genes, the concept could be applied to separate LAMP reactions targeting 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, *et al.* have recently shown that calcein can act as a fluorescent indicator of amplification, based on the generation of pyrophosphate ions.[14] Herein is proposed a point-of-care multiplexed microfluidic 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

Review of Sepsis-causing Bacteria

To determine which bacterial species to target, the literature was reviewed to find the most common species identified in neonatal sepsis cases in resource-limited environments. Based on the study by Zaidi, *et al.*, the eight most-commonly identified isolates of sepsis-causing bacteria are listed in Table (1).[22]

Etiology of Sepsis-causing Bacteria in Resource-limited Environments[22]	
Organism	**% of Isolates from All Regions**
Escherichia coli	17.08
Klebsiella species	13.49
Staphylococcus aureus	13.30
Group B streptococci	8.06
Pseudomonas species	7.17
Streptococcus pneumoniae	5.67
Salmonella species	4.78
Acinetobacter species	4.20

Table 1. The vast majority of sepsis-causing bacteria come from four Orders: Enterobacteriales (green); Bacillales (blue); Lactobacillales (red); and Pseudomonadales (orange)

Further investigation was conducted of review articles determining the strains of those species responsible for neonatal sepsis.

Selection of Target Sequences

The 16s rRNA genes of the most common strains of species contributing to neonatal sepsis were accessed from the NCBI GenBank.[24] Genes accessed are listed in Table (2). Based on the relation of species responsible for sepsis, it was determined that target sequences should be specific 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 sequences from the same Order were aligned with ClustalΩ multiple sequence alignment software.[25] The consensus sequence from this first alignment was then aligned again with six total 16s rRNA gene sequences from the three other Orders. This alignment file was compared using Base-by-Base software[26] 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

16s rRNA Genes Accessed from NCBI GenBank		
Species	**Accession Number**	**Reference**
Bacillus cereus (ATCC 14893)	AJ310098	29
Listeria monocytogenes str. 4b F2365	AE017262	30
Staphylococcus aureus subs. aureus	CP000730	31
Staphylococcus epidermidis RP62A	CP000029	32
Enterobacter sakazakii ATCC (51329)	AY752937	33
Escherichia coli O7:K1 CE10	CP003034	34
Klebsiella pneumoniae K2	CP006648	35
Salmonella enterica sero. enteritidis	DQ344532	36
Salmonella enterica sero. typhimurium	DQ344533	37
Enterococcus faecalis	AF039902	38
Streptococcus agalactiae sero. V	AE009948	39
Streptococcus pneumoniae R6	AE007317	40
Streptococcus pyogenes M1	AE004092	41
Acinetobacter baumannii	X81667	42
Pseudomonas aeruginosa #796	AB037553	43
Pseudomonas oryzihabitans str. WB2003S	AY850170	44

Table 2. Species (with strain or serovar specified) of 16s rRNA genes accessed from NCBI GenBank. Color coding of bacterial Order follows that of Table (1).

noted, and the process was repeated for the genes of the remaining three Orders.

Primer Design

Four novel oligonucleotide primer sets were designed using PrimerExplorer V4 software (Eiken Chemical Co.). 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.[27] An example of this is shown in Figure (2). These mutation sites thus have

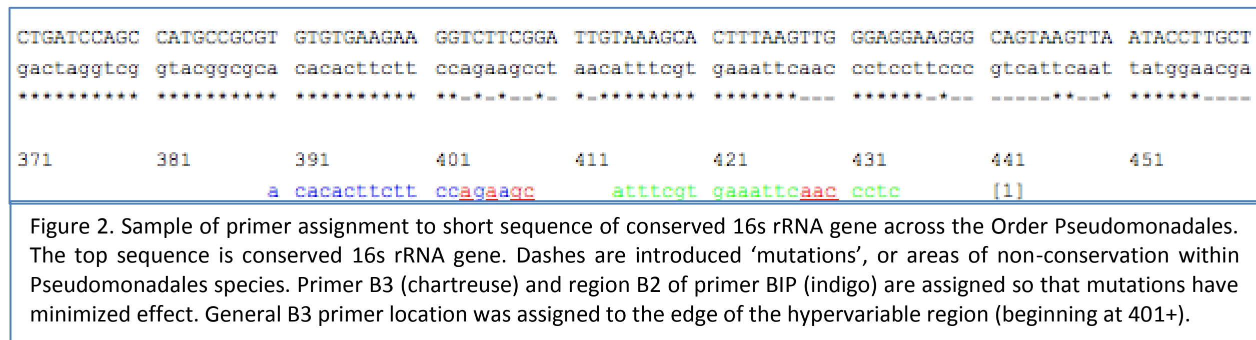


Figure 2. Sample of primer assignment to short sequence of conserved 16s rRNA gene across the Order Pseudomonadales. The top sequence is conserved 16s rRNA gene. Dashes are introduced 'mutations', or areas of non-conservation within Pseudomonadales species. Primer B3 (chartreuse) and region B2 of primer BIP (indigo) are assigned so that mutations have minimized effect. General B3 primer location was assigned to the edge of the hypervariable region (beginning at 401+).

minimal effect on primer specificity. Therefore, primers bind to regions on the conserved consensus sequence within an Order. 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. This process was repeated three times for each of the remaining Orders. Each primer set design was then run in a BLAST search to confirm theoretical primer specificity for all species within the prescribed Order. The four primer sets were obtained from Invitrogen.

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. 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 step.

Genomic DNA Extraction

Bacterial cell walls were lysed using boiling lysis technique, as described by Iwamoto, *et al.*, in order to extract genomic DNA.[28] 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 min. Lysates were stored at

-20˚C until LAMP optimization reactions. Crude lysates were used directly in LAMP reactions.

Primer Resuspension and Dilution

Lyophilized primers obtained from Invitrogen were each first resuspended to 100µM stock solutions using microbiology-grade H_2O. Primer solutions were then aliquoted and diluted accordingly, so that all FIP and BIP primers had a final concentration of 40 pmol/µL, and all F3 and B3 primers had a final concentration of 5 pmol/µL. Primers of the same set were then combined into single solutions at a 1:1:1:1 volume ratio for ease of primer addition to LAMP reaction, for a total of four primer sets. Primer stocks solutions and primer set working concentrations were stored at -20˚C.

Optimization of LAMP Reaction Conditions

Initial LAMP reactions were carried out to determine optimal conditions using Loopamp DNA Amplification Kit (Eiken Chemical Co.) and calcein-based Fluorescent Detection Reagent (Eiken). For each reaction: 4 µL of the prescribed primer set, 12.5 µL LAMP reaction mix (2x), 1 µL *Bst* DNA polymerase, 2 µL of the prescribed crude DNA lysate, 1 µL Fluorescent Detection Reagent, and 4.5 µL microbiology-grade H_2O 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

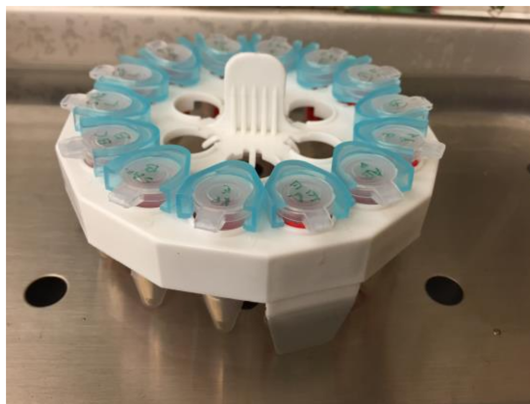


Figure 3. The only equipment needed to carry out LAMP is a simple hot-water bath.

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. The reaction tubes were incubated in a hot water bath at 63˚C for 60 min, as seen in Figure (3). They were briefly heated at 80˚C for 2 min following incubation to inactivate the polymerase. Qualitative results were visualized on a UV lamp at 365nm. 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. To find optimal visual fluorescence results, LAMP was repeated for three sets of positive and negative controls.

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 to a concentration equivalent to a DNA amount from 10^0 CFU/mL—this is assuming that starting cell concentration from one colony was 10^9 CFU/100µL. LAMP was performed using Pseudomonadales primers with the lower dilution samples of the series. Results were viewed visually and with agarose gel electrophoresis.

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

LAMP reactions were performed using the optimized LAMP conditions previously mentioned. 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. Amplification products were viewed with a UV lamp then electrophoresed on a 2.5% agarose/TAE gel to view amplified DNA. Sensitivity, specificity, positive predictive value [PV(+)], and negative predictive value [PV(-)] were determined according to Equations (1)-(4):[10]

$$Sensitivity = \frac{True\ Positives}{(True\ Positives + False\ Negatives)} \tag{1}$$

$$Specificity = \frac{True\ Negatives}{(True\ Negatives + False\ Positives)} \tag{2}$$

$$PV(+) = \frac{True\ Positives}{(True\ Positives + False\ Positves)} \tag{3}$$

$$PV(-) = \frac{True\ Negatives}{(True\ Negatives + False\ Positives)} \tag{4}$$

Where true positives for calcein constitute an emission of fluorescence when the primer set is designed for the DNA present; true negatives are a lack of fluorescence when primer set is not designed for DNA present; false positives are fluorescence when primer set is not designed for DNA present; and false negatives are lack of fluorescence when primer is designed for DNA present.[10] For primer sensitivity, specificity, PV(+), and PV(-), the same definitions apply, with ladder-like streaks on agarose gel in lieu of fluorescence.

Design of mμLAMP Chip

A student colleague in the Department of Biomedical Engineering designed the multiplexed microfluidic (mμLAMP) chip that could potentially facilitate simultaneous, separate LAMP reactions. Briefly, the chip design consists of a poly-(dimethylsiloxane) [PDMS] replica adhered to glass substrate. The PDMS replica contains six separate channels extending from a central loading chamber. The PDMS replicas were cast from a 3D-printed mold.

Technical issues prevented testing LAMP reactions on the chip within the timespan of research.

Results

Novel Oligonucleotide Primer Sets

Four novel primer sets were generated using PrimerExplorer V4, which were designed for four Orders of interest in neonatal sepsis. The final primer designs are listed in Table (3).

Order for Primer Specificity	F3	B3	FIP	BIP
Bacillales	GGTGAGTAACACGTGGAT	TATGCATCGTTGCCTTGG	CATGCGGTTCAAAATATTATCCGGT-AACCTACCTATAAGACTGGGAT	GAAAGACGGTCTTGCTGTCAC-CGTTACCTTACCAACTAGCT
Lactobacillales	CTCGAAAGCGTGGGGAGC	GTAAGGTTCTTCGCGTTG	GGAAAGGGCCTAACACCTAGC-GGATTAGATACCCTGGTAGTC	GGAGTACGACCGCAAGGTTG-TCGAATTAAACCACATGCTC
Enterobacteriales	AAGACTGACGCTCAGG	CAGGTAAGGTTCTTCGCG	AGGGCACAACCTCCAAATCG-GCAAACAGGATTAGATACCCT	GAGTACGGCCGCAAGGTTAA-TAAACCACATGCTCCACC
Pseudomonadales	AGGCCTACCAAGGCGAC	CTCCCAACTTAAAGTGCTTTA	TAGGAGTCTGGACCGTGTCT-CGTAACTGGTCTGAGAGG	GAGGCAGCAGTGGGGAATAT-CGAAGACCTTCTTCACACA

Table 3. Final design of novel oligonucleotide primers, listed 5’→3’. Each primer set consists of four primers with six regions that specifically bind to the edge of hypervariable regions of 16s rRNA gene.

LAMP Confirmation and Cross Reactions: Primers Specifically Bind

Results of primer confirmation reactions are shown in Figures (4)-(6). 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: Figures (6) and (7).

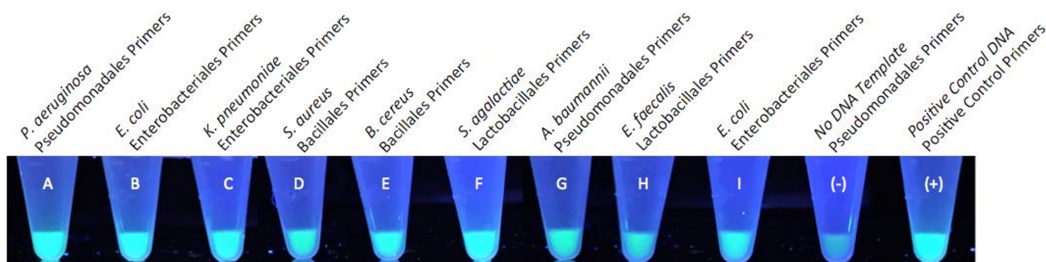
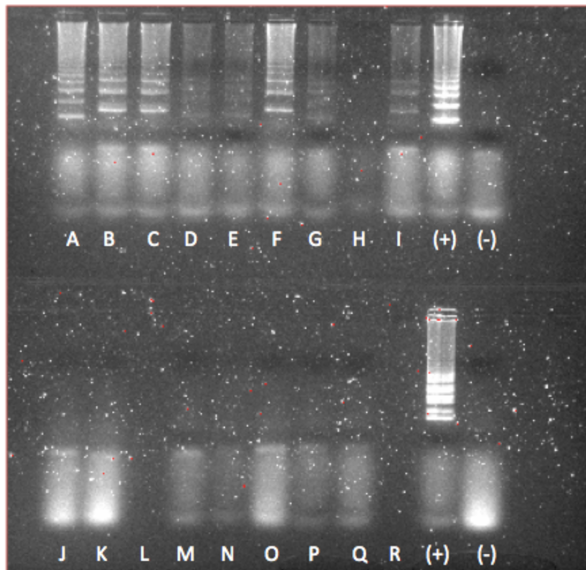


Figure 4. LAMP confirmation reactions. Primer sets paired with DNA samples from species within Orders for which they were designed. Visualized with UV lamp at λ=365nm.



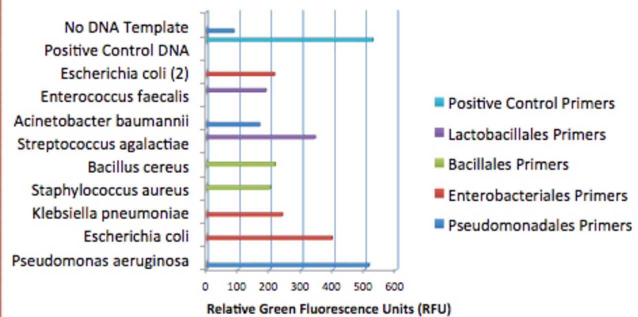


Figure 5. Relative fluorescence units of calcein probe following LAMP confirmation reactions. Positives are not significantly higher than negatives (p=.05,n=10). $\lambda_{excitation}$=470nm, $\lambda_{emission}$=510nm

Figure 6. Electrophoresis of LAMP confirmation and cross-reactions. *Lanes A-I*: LAMP confirmation reaction products (no product added to lane H). Typical ladder-like bands from amplified stem-loop DNA structures supports specificity of primers to bind to designated target sequences.[5] *Lanes J-Q*: LAMP cross-reaction products (no product added to lanes L, R). Lack of ladder-like LAMP pattern supports hypothesis that primers do not bind to 16s rRNA from species outside of designated Order. Faint streaks at the bottom of each lane are estimated to be unreacted primer. Run on TAE/agarose (2.5%) gel at 100V for 40min.

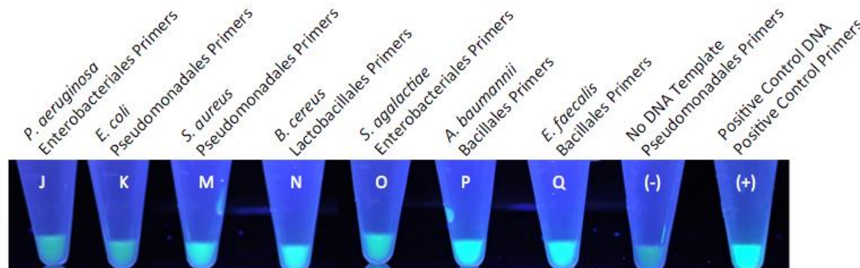


Figure 7. LAMP cross-reactions: Primer sets paired with DNA from species of the closest-related Order, or with a more phylogenetically distant Order from the primer's designated Order. Calcein non-specifically emits fluorescence, even with no amplification. This may be due to a lack of Mn^{2+} to quench unreacted calcein. LAMP reactions titrated with $MnSO_4$ (data not shown) resulted in full quenching, even for positive reactions.

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

The results of the LAMP confirmation and cross-reactions are displayed in a matrix in Figure (8). 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 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.

	Primer Set	Pseudomonadales		Enterobacteriales		Bacillales		Lactobacillales		Positive Control	
		Fluorescence	Electrophoresis	Fluorescence	Electrophoresis	Fluorescence	Electrophoresis	Fluorescence	Electrophoresis	Fluorescence	Electrophoresis
Order	Species	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result
Pseudomonadales	Pseudomonas aeruginosa	+	+	-	-						
Enterobacteriales	Escherichia coli	-	-	+	+						
Enterobacteriales	Klebsiella pneumoniae			+	+						
Bacillales	Staphylococcus aureus	+	-			+	+				
Bacillales	Bacillus cereus					+	+	+	-		
Lactobacillales	Streptococcus agalactiae			+	-			+	+		
Pseudomonadales	Acinetobacter baumannii	+	+			+	-				
Lactobacillales	Enterococcus faecalis					+	-	+	o		
Enterobacteriales	Escherichia coli (2)			+	+						
Positive Control	Positive Control DNA									+	+
Negative Control	No DNA Template	-	-								
	Sensitivity	1	1	1	1	1	1	1	1	1	1
	Specificity	0.666666667	1	0.5	1	0	1	0	1		
	PV(+)	0.666666667	1	0.75	1	0.5	1	0.666666667	1	1	1
	PV(-)	1	1	1	1	0	1	0	1		

Figure 8. LAMP results and statistical matrix for both calcein fluorescent probe and primer sets. Statistics referring to electrophoresis results are assumed to apply to primers themselves. Those referring to fluorescence results correspond to calcein characteristics. False positives are marked in red. The electrophoresis result from the *E. faecalis* reaction with Lactobacillales was excluded, since the LAMP product was not loaded properly into the gel.

Theoretical Limit of Detection: 10^0-10^1 CFU/mL

LAMP reaction products from *P. aeruginosa* DNA lysate serial dilutions and Pseudomonadales primers are shown in Figures (9)-(10). The primer set tested showed higher efficiency at lower theoretical concentrations.

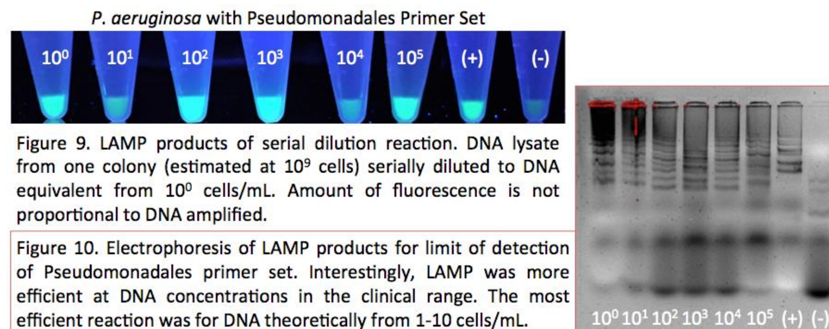


Figure 9. LAMP products of serial dilution reaction. DNA lysate from one colony (estimated at 10^9 cells) serially diluted to DNA equivalent from 10^0 cells/mL. Amount of fluorescence is not proportional to DNA amplified.

Figure 10. Electrophoresis of LAMP products for limit of detection of Pseudomonadales primer set. Interestingly, LAMP was more efficient at DNA concentrations in the clinical range. The most efficient reaction was for DNA theoretically from 1-10 cells/mL.

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. Positive results on the gel display 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.[13] 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.

The theoretical limit of detection of the Pseudomonadales primer set was estimated to be 10^0-10^1 CFU/mL of *P. aeruginosa*. It is interesting to note that the electrophoresis products of these serial-dilution amplifications suggested that the reaction was more efficient with less initial target DNA. The presence of the most intense ladder-like streak on the 10^0 lane of Figure (10) suggests that DNA amplification was most efficient with target DNA equivalent to that from 1 CFU/mL. This is surprising, given the method of serial dilution: one colony of *P. aeruginosa* was suspended in 100μL of lysis buffer and boiled for 20 min. The products of this lysis were serial diluted. This would prevent lysis bias where lower concentrations of cells would be more efficiently lysed. The most efficient LAMP reactions of the serial dilution products in the clinical range of neonatal sepsis inoculation (10^0-10^2 CFU/mL) are a promising prospect. Limits of detection for the other primer sets should be determined in the future.

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.[11] Further laboratory analysis is 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.[23] 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.[4] 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.

Non-specific Fluorescence of Calcein Probe

While calcein should only exhibit fluorescent activity when an amplification reaction occurs, fluorescence was detected in many products not containing any amplified DNA. This could be due to the presence of metal chelators in the reaction solution, which would remove the Mn^{2+} quencher from calcein. These chelators 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 SYBR Green DNA intercalating dye. Initially it was hypothesized that SYBR Green 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 of amplified DNA (positive results) from non-amplified genomic DNA (negative results). If

successful, SYBR Green 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 then be loaded into a central filling chamber that equally distributes the solution across the separate channels. The mμLAMP 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 mμLAMP chip as a two-step detection method—one step to expose the bacterial DNA; one step to load LAMP reagents—for diagnosing neonatal sepsis in under an hour.

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