RAPID DETECTION OF BACTERIAL PATHOGENS USING BACTERIOPHAGE AMPLIFICATION COUPLED WITH SURFACE-ENHANCED RAMAN SPECTROSCOPY LATERAL FLOW IMMUNOCHROMATOGRAPHY

by

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ABSTRACT

The current gold standards for bacterial identification involve culture-based methods that can take weeks to months to provide definitive results. Because of this lengthy time period, a need for rapid diagnostics aimed at pathogenic bacteria exists. To this end, a lateral flow immunochromatographic (LFI) assay was developed that exploited bacteriophages (phages) as secondary markers. Phages are useful in diagnostic assay because they specifically target a bacterial host and produce many progeny phages in a matter of hours. In a process called phage amplification (PA), phages were input below the detection limit of the LFI and if the targeted bacteria was present, the phages rapidly multiplied. This increase in phage concentration above LFI limit of detection indicated the presence of the target bacteria.

The LFI devices presented here detected progeny phages using surface-enhanced Raman spectroscopy (SERS) nanoparticle reporters covalently linked with anti-phage antibodies. When interrogated with laser in the near IR range, a characteristic spectrum was produced by an organic reporter. This spectrum provided quantitative information of the LFI test line and eliminated dependence on visual determination.

The PA SERS LFI system was first developed for the foodborne pathogen *Listeria monocytogenes* using phage A511. Several assay parameters were optimized, such as the conjugation of anti-A511 antibodies onto the SERS reporters, LFI construction and proper sample flow the SERS LFI device. *L. monocytogenes* was detected in as little as 2 hours and at concentrations as low as $1 \times 10^5$ colony forming units (cfu)/mL in 5 hours.

PA SERS LFI for the detection of *L. monocytogenes* was further advanced to detect the pathogen in inoculated food matrices. Cantaloupe, ice cream, and two types of soft cheeses were inoculated with varying concentrations of *L. monocytogenes* and selectively enriched according
to FDA protocol. After a 24 hour enrichment, 1 cfu/g was detected by PA SERS LFI in all foods tested.

Finally, the PA SERS LFI strategy was adapted to detect the potential bio-warfare agent *Yersinia pestis*, using phage φA1122. Adjustments for the development a φA1122-specific SERS LFI included utilizing polyclonal rabbit anti-φA1122 antibodies for conjugation onto SERS reporters and for use as capture agents on the test line. PA SERS LFI detected *Y. pestis* in as short a time as 1 hour and at a concentration as low as $5.0 \times 10^4$ cfu/mL in 3 hours.

The work presented in this dissertation represents a combination of several scientific principles to develop a rapid, specific, easy-to-use diagnostic tool for the detection of bacterial pathogens *L. monocytogenes* and *Y. pestis*. Detection time was reduced from 4-6 days by conventional culture methods to hours by exploiting phages for their specificity and rapid reproduction. LFI provides an inexpensive, simple analytical test to detect progeny phages produced from PA. Furthermore, quantitative analysis of the test line of LFI devices was achieved using spectroscopic SERS reporters, which eliminated the need for visual determination and lowered detection levels below visual levels.
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<td>AK</td>
<td>Adenylate kinase</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
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<tr>
<td>BTM</td>
<td>Bulk tank milk</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
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<tr>
<td>CBD</td>
<td>Cell wall binding domain</td>
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<tr>
<td>CDC</td>
<td>U.S. Centers for Disease Control and Prevention</td>
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<tr>
<td>cfu</td>
<td>Colony forming units</td>
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<tr>
<td>CV</td>
<td>Coefficient of variance</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FAS</td>
<td>Ferrous ammonium sulfate</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>h</td>
<td>Hours</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>IMS</td>
<td>Immunomagnetic bead separation</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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LC ..............................................................Liquid chromatography
LFI ..............................................................Lateral flow immunochromatographic assay
LOD ............................................................Limit of detection
MALDI-TOF MS ........................................Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAP .........................................................Mycobacterium avium subsp. paratuberculosis
MBP ............................................................Maltose binding protein
mg .....................................................................Milligrams
min ........................................................................minutes
mL ..........................................................................Milliliters
MMBT ..........................................................MRSA and MSSA blood culture test
MOI .............................................................Multiplicity of Infection
MOPS .........................................................3-(N-morpholino)propanesulfonic acid
MRM ............................................................Multiple reaction monitoring
MRSA .........................................................Methicillin-resistant Staphylococcus aureus
MSPQC .......................................................Multichannel series piezoelectric quartz crystal
MSSA ........................................................Methicillin-sensitive Staphylococcus aureus
MTT ..........................................................3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH ..........................................................Sodium hydroxide
OADC .......................................................Oleate-albumin-dextrose-catalase
OD .............................................................Optical density
PA ............................................................Phage amplification
PCR ............................................................Polymerase chain reaction
pfu .............................................................Plaque forming units
PMS .......................................................... Peptide-mediated separation
PRE .......................................................... Pomegranate rind extract
qPCR .......................................................... Quantitative polymerase chain reaction
RT-PCR ..................................................... Reverse transcriptase polymerase chain reaction
SERS .......................................................... Surface-enhanced Raman spectroscopy
SDS .......................................................... Sodium dodecyl sulfate
TRT .......................................................... Turnaround time
µL ............................................................ Microliters
UV ............................................................ Ultraviolet
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CHAPTER 1

INTRODUCTION

A pressing need exists for more rapid and accurate detection of bacterial pathogens, particularly in the areas of health care and biodefense.\(^1\) The gold standards for bacterial detection include DNA-based tests (such as polymerase chain reaction, PCR, and DNA hybridization), enzyme-linked immunosorbent assay (ELISA), proteomic analysis, fatty acid analysis, and biochemical tests. These applications require culture-based methods in which the bacteria must be physically grown and isolated on selective and/or differential media. The major drawback to these methods is the turnaround time (TRT), the length of time required to grow viable colonies that are required for further biochemical and molecular tests in order to produce a confirmatory response. The total time for these processes can take many days to several weeks, leading to increased risk of widespread outbreaks. Bacteriophage (phage)-based strategies have been developed in efforts to reduce detection time.

Bacteriophages offer a tool for rapid, specific, and sensitive detection of bacterial pathogens.\(^2\)\(^-\)\(^4\) Phages are bacterial viruses that infect specific, target bacteria. Upon infection, phage nucleic acid hijacks host cell machinery and produces progeny phages.\(^5\) These progeny phages eventually lyse the host cell and are released into the surrounding milieu. This infection process has been exploited in many ways to indicate the presence of target bacteria. Examples include taking advantage of the hijacking of host cell machinery by reporter phages to produce reporter proteins encoded in the phage genome; identification of bacteria by the attachment of fluorescently labeled phages; and detection of intracellular products released after phage lysis.

This dissertation begins with a review that addresses the advantages of using phages for detection and the different methodologies in which phages have been employed for bacterial
diagnostics. This review specifically focuses on phage amplification (PA) assays and the numerous downstream analytical methods employed after infection of target bacteria to detect newly produced phages, called progeny phages. One of the discussed analytical methods is lateral flow immunochromatography (LFI), which provides a rapid, inexpensive, point-of-care test for progeny phages.

Next, this work transitions into a PA LFI method developed for the detection of *L. monocytogenes*. A511 was selected as the phage of choice due to its broad host range and lytic nature. The described LFI assay substituted conventional colorimetric-based reporters for surface-enhanced Raman spectroscopy (SERS) nanoparticles conjugated with anti-phage antibodies as reporter particles. SERS reporters provided quantifiable information of the test line and eliminate the dependence on visual determination which can be unreliable at low phage concentrations. Furthermore, the use of SERS reporters lowered detection limits below visual levels, improving the sensitivity of the assay.

After successful development of PA SERS LFI for the detection of *L. monocytogenes*, this technology was then applied in two different directions. The previously discussed A511 amplification SERS LFI assay was further evaluated for the detection of *L. monocytogenes* in inoculated food samples. Additionally, using the developed SERS conjugation protocol and LFI construction procedure, PA SERS LFI devices were constructed for the detection of the bioterrorism agent, *Yersinia pestis*, using *Y. pestis* specific phage ϕA1122.

### 1.1 Bacteriophage amplification as a bacterial detection method

Chapter 2 presents a review of PA methods used for bacterial detection. Phage amplification assays begin with the input of phages targeted towards specific bacteria. If the target is present, the phages will multiply rapidly and produce progeny phages. These progeny
phages are then used as indicators of the presence of the target bacteria and have been detected by various methods.

1.2 Rapid detection of *Listeria* by bacteriophage amplification and SERS-lateral flow immunochromatography

Chapter 3 describes the development of a PA SERS LFI assay for the detection of *L. monocytogenes*, the etiological agent of listeriosis.\(^{11}\) Mild symptoms of listeriosis include gastroenteritis and diarrhea, while more severe cases can lead to meningitis, spontaneous abortions, and death. Conventional culture-based strategies can take 4-6 days to identify *L. monocytogenes*.\(^{12}\) This TRT, combined with the pathogen’s deadly potential, highlights the need for rapid *L. monocytogenes* diagnostics. Much of the work described in this chapter focuses on the development of the conjugation of anti-A511 antibodies onto SERS reporters and optimization of the SERS LFI device, followed by the evaluation of A511 amplification detected by SERS LFI.

SERS LFI devices worked by the application of a sample containing progeny phages onto a sample pad. The sample then moved via capillary action to a release pad containing SERS reporters conjugated with anti-A511 antibodies which bound to the phage. A511-SERS complexes continued down a nitrocellulose membrane until reaching a test line, which contained immobilized anti-A511 antibodies. These complexes were captured at the test line and concentrated. The test line was interrogated with a laser in the near-IR (785 nm) and produced a characteristic spectrum of the SERS particles, which allowed for quantifiable detection and eliminated the need for visual determination.\(^{13}\) After the establishment of a visual limit of detection (LOD) and a SERS LOD, SERS LFI was used to detect *L. monocytogenes* after A511 amplification at various input phage and bacterial concentrations.
1.3 Detection of *Listeria monocytogenes* in inoculated food by bacteriophage amplification and surface-enhanced Raman spectroscopy coupled to lateral flow immunochromatography

*L. monocytogenes* is a foodborne pathogen, therefore Chapter 4 examines the application of the previously developed PA SERS LFI detection system for detection of *L. monocytogenes* in inoculated cantaloupe, ice cream, queso fresco, and Camembert cheese. These foods were chosen based on recent listeriosis outbreaks. Because current U.S. government regulations state a zero-tolerance policy for the presence of *Listeria* in foods intended for human consumption, a prior enrichment step was required to elevate concentrations of *L. monocytogenes* to detectable levels prior to PA SERS LFI analysis. For this purpose, the *L. monocytogenes* enrichment protocol developed by the FDA was used for pre-analysis bacterial growth. Samples were removed from the enrichment mixture at 24 and 48 h and evaluated by PA SERS LFI. Parallel analysis was performed by plating samples on chromogenic *Listeria* agar to confirm results of PA SERS LFI. PA SERS LFI was able to reduce detection time by 2-4 days compared to enrichment followed by plating on selective media and provided a user-friendly diagnostic for *L. monocytogenes* contamination.

1.4 Rapid detection of *Yersinia pestis* by bacteriophage amplification and SERS lateral flow immunochromatography

Chapter 5 discusses a novel approach to adapting PA SERS LFI to detect *Y. pestis* by applying the same antibody purification, SERS conjugation protocol, and LFI construction procedure discussed previously. *Y. pestis* is the etiological agent of the plague and current bacterial culture detection methods can take up to 5 days. Plague occurs in three forms: bubonic, septicemic, and pneumonic. Bubonic plague is a bacterial infection of the lymph nodes,
while septicemic and pneumonic forms are bacterial infections of the blood and lungs, respectively. Pneumonic plague is of current concern due to the bacteria’s ability to be aerosolized and used as a bioterrorism agent.

Pneumonic plague has a high mortality (100% if left untreated) and morbidity rates as symptoms begin to appear 1-6 days after exposure, which allows for rapid spread from person to person through repertory droplets, such as a cough.22-23 Therefore, due to the potential for deadly, widespread outbreaks and lengthy TRT, there is a pressing need for a rapid diagnostic for *Y. pestis*. To fulfil this need, φA1122 was selected as the phage in a PA SERS LFI assay to detect *Y. pestis* because of its large burst size and short latent period.24-25 Additionally, φA1122 is the diagnostic phage used by the U.S. Center for Disease Control and Prevention and has been shown to lyse thousands of *Y. pestis* strains.26 After successful development of φA1122-specific SERS LFI devices, PA experiments detected *Y. pestis* in as short as one hour.

### 1.5 SERS theory

A critical aspect of the research presented in this dissertation is the use of SERS nanoparticles as reporters. As mentioned earlier, when a laser in the near-IR (785 nm) was applied to these particles, a characteristic spectrum was produced and exploited for quantitative analysis of the test line of LFI devices. At low analyte concentrations, colorimetric-based LFI test lines can be weak and misread as false negatives; therefore, the application of SERS reporters eliminated reliance on a visual determination by providing quantifiable information. What follows is a brief description of Raman scattering and the SERS principle.

Raman scattering is the inelastic scattering of light from a molecule, to either higher (anti-Stokes) or lower (Stokes) energy than the incident light, producing a spectrum unique to the molecule of interest. Raman scattered photons represent only a small fraction (1 out of $10^7$) of
the total scattered photons, limiting the sensitivity of this technique.\textsuperscript{27} In a classical representation, the molecule can be represented as a harmonic oscillator in which the molecule is treated as small spheres connected by a spring. Raman scattering is produced by the change in the oscillating polarization of the molecule due to the incident light. The polarization of the molecule can be represented by the following equation:

\[
P_x(\text{inelastic}) = \frac{1}{2} E_x^0 Q_1 \frac{\partial \alpha_{xx}}{\partial q_1} \left[ \cos 2\pi (v_0 - v_l) t + \cos 2\pi (v_0 + v_l) t \right] \tag{1.1}
\]

\(P_x\) represents the polarization of the molecule along a particular vibrational mode and is affected by the incident field strength (\(E_x^0\)), vibrational amplitude (\(Q_1^0\), based on the stiffness of the bonds), and the polarizability of the molecule (\(\frac{\partial \alpha_{xx}}{\partial q_1}\)). Polarizability is the relative tendency of the electron cloud of the molecule to be displaced by an electric field. \(v_0 - v_l\) represents the Stokes shift, while \(v_0 + v_l\) represents the anti-Stokes shift.

SERS is the increase in Raman signal by molecules adsorbed onto rough metal surfaces. The increase in Raman signal is explained by the electromagnetic mechanism theory, in which an enhancement of the electric field (\(E_x^0\), Equation 1.1) is generated by the excitement of a localized surface plasmon resonance (LSPR) on the metal surface. LSPR occurs when valence electrons in the metal oscillate in resonance with incident light frequency.\textsuperscript{28} LSPR is illustrated in Figure 1.1. Gold, silver, and copper are typical metals of choice for SERS experiments because they provide higher enhancement than other metals (such as platinum, ruthenium, palladium, iron, cobalt, or nickel).\textsuperscript{29} The nanoparticles selected for this dissertation consisted of a gold nanoparticle core, coated with Raman reporter, and encased in silica (Figure 1.2). Gold nanoparticles 60 nm in diameter were selected because of their chemical inertness and display
ideal Raman scattering when excited at 785 nm.\textsuperscript{30} This wavelength is preferred because it minimizes autofluorescence in biological materials.\textsuperscript{10, 13, 30} These properties are essential because the original intent of these particles was for \textit{in vivo} studies. Silica encapsulation provided a biocompatible layer to prevent desorption of the Raman reporter. Furthermore, this silica layer was functionalized to allow for antibody attachment and for use as phage specific reporters in LFI devices.

\textbf{Figure 1.1:} Localized surface plasmon resonance effect.

\textbf{Figure 1.2:} Schematic of an antibody-linked SERS NP. (Figure credit: Dr. Christopher Cox)
1.6 Reference Cited


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CHAPTER 2

BACTERIOPHAGE AMPLIFICATION AS A BACTERIA DETECTION METHOD

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2.1 Introduction

DNA-based assays (PCR and DNA hybridization), ELISA, proteomic and fatty acid analysis, and biochemical testing represent the gold standards for bacterial detection. These methods rely on colony formation for use as a pure culture and subsequent testing for identification, a process which can take weeks to months. Due to the long TRT of these methods, disease outbreaks become widespread, impacting healthcare, agriculture, and biodefense.\textsuperscript{1} In order to reduce TRT, various culture-independent methods have been investigated, focusing on specific detection of target organisms without the need for laborious, time-consuming culturing.

PCR can be applied in a culture-independent approach and offer a rapid diagnostic method. PCR amplifies nucleic acids of target bacteria allowing for specific detection. The main shortcoming to PCR strategies is their inability to differentiate between living and dead cells, thus the requirement of viable colonies prior to analysis. This issue is particularly relevant as only living cells can cause an infection. Reverse transcription PCR (RT-PCR) potentially solves this problem by detecting mRNA instead of DNA, providing evidence of metabolically active cells.\textsuperscript{2,3} However, RT-PCR is costly for routine use and contaminants from matrices (such as food) can inhibit reagents.\textsuperscript{4}

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As an alternative, phage-based approaches have been utilized in bacterial detection strategies to reduce TRT from days to hours and provide live/dead differentiation. Phages are predatory bacterial viruses that have evolved to recognize specific cell surface structures of their target host. Infection begins with attachment and injection of nucleic acid in the bacterial host. Host cell machinery is hijacked to produce viral nucleic acids and synthesize phage proteins. Phage proteins are assembled and nucleic acids are packaged into the head to form mature virions. Many progeny phages can be produced in a matter of hours. The host cell lyses and releases progeny phages into the surrounding milieu. The ability for easy and inexpensive propagation makes phages an ideal reagent for bacterial diagnostics. Furthermore, phages can only replicate in a living host. Due to these advantages, phages have been employed in a wide variety of detection schemes to serve as biomarkers of targeted bacteria.

Phage amplification assays provide a more rapid approach for bacterial diagnostics by directly targeting bacteria in culture. These assays involve the initial addition of phages (input) for specific infection of a target bacteria and the subsequent detection of an increase in phage concentration. The aim of this review is to discuss the many analytical approaches taken to detect these progeny phages as indicators of specific, viable bacteria. Critical aspects of these assays are to A) detect an increase in phage concentration above the detection limit of the analytical method or B) differentiate between progeny phage from the input phage.

While the focus of this review is on the phage amplification assay, it would be remiss not to briefly acknowledge the many other phage-mediated diagnostic approaches. These methods include reporter phages, which involve the manipulation of the phage genome to express reporter proteins upon phage infection. These proteins were induced to produce detectable signals indicating the presence of target bacteria. Additionally, several aspects of the phage lytic cycle
have been exploited for bacterial recognition, for example, detection of intracellular products that were released upon cell lysis by phages or the use of fluorescently labeled phages which were then used to visualize bacteria upon their attachment to a specific host. Lastly, phages have been exploited as bacterial capture agents in biosensor technology for detection of *Staphylococcus aureus*, *Salmonella*, *Bacillus anthracis*, and *Escherichia coli*.6-15

### 2.2 Other phage-based assays

Reporter phages are genetically modified phages that have a reporter gene inserted into their genome that upon infection is also transcribed and translated along with the phage genome. The expressed reporter protein can be detected, for example, by bioluminescence, fluorescence or the conversion of an exogenously added chromogenic substrate.16 The most common reporter phages to date involve the production of a bioluminescence luciferase protein, which produces a flash of light in the presence of an aldehyde substrate. Several applications of bioluminescent reporter phages utilized the insertion of the *lux* operon of marine bacteria *Vibrio fisheri* or *V. harveyi*, which contains 5 genes, *luxCDABE* (listed in order of occurrence). Bioluminescence is produced by a protein coded for by the *luxAB* genes, while *luxCDE* contains genes for proteins that generate the aldehyde substrate required for this reaction. A λ phage-based cloning vector was the first reporter phage to encode for the complete *lux* operon and used in the detection of *Escherichia coli*.17 This approach is illustrated in Figure 2.1. Due to the large size of the *lux* operon (~6000 base pairs), problems can occur in the packaging of phage genome in the progeny phage.16 Reporter phages inserting only *luxAB* have since been developed for the detection *Salmonella* spp., *Listeria monocytogenes*, *Yersinia pestis* and *Bacillus anthracis*.18-24 Because of the elimination of *luxCDE*, these assays required the addition of a long-chain fatty aldehyde reagent. This basic approach has been used for the detection of pathogens in complex food
matrices and surfaces. A further improvement of the bioluminescence reporter phage-based assay included the insertion of the luxI gene, which encodes for the production of quorum sensing acyl-homoserine lactone (AHL), into λ phage for the detection of E. coli. Upon infection of target E. coli cells, AHL was released into the surrounding milieu. Included in this assay was a λ-resistant E.coli strain, which contained the bioluminescence genes luxCDABE along with luxR. The exogenous AHL diffused into the λ-resistant E.coli and interacted with LuxR proteins to stimulate transcription of lux genes. This method was adapted for the specific detection of E. coli O157:H7 using reporter phage PP01. Bioluminescent reporter phages have also been developed employing firefly (Photinus pyralis) luciferase gene, luc, to detect Mycobacterium tuberculosis. A limitation to luciferase-based assays is the short-lived production of light that must be measured immediately. Other iterations have been developed to improve light output, specificity, and ability to detect dormant bacteria.

Several other reporter phages have exploited additional genes. One example was the use of green fluorescent protein (GFP) from jellyfish Aequorea victoria. GFP offers the advantage of not requiring an enzyme substrate in order to produce a signal and produces a green fluorescent signal in the presence of oxygen and an excitation light source. GFP reporter phages were first utilized for the detection of E. coli. A modification of this method involved a T4 GFP reporter coliphage in which the gene responsible for bacterial lysis (lysozyme) was inactivated by the insertion of an amber mutation. This mutation prevented the lytic activity of the phage and allowed for an accumulation of GFP inside the cell. This reporter phage was further extended for detection of E. coli in sewage effluent. However, due to the limited host range of T4, additional GFP reporter phages (IP008 and IP052) against E. coli with broader host ranges were prepared. In a different approach involving specificity, GFP reporter phage PP01
was developed for specific identification of *E. coli* O157:H7.\textsuperscript{40-41} Piuri *et al.* exploited phages producing GFP and yellow fluorescent protein (ZsYellow) for detection of *M. tuberculosis*.\textsuperscript{42} The GFP producing phage was further extended for detection of drug-resistant *M. tuberculosis*.\textsuperscript{43}

**Figure 2.1:** *lux*-based reporter phage bioluminescence assay. A) *lux* genes were inserted into a targeted host by species-specific reporter phage infection. B) Bioluminescence genes are expressed and read by a luminometer. (Figure credit: Dr. Christopher Cox.)
An alternative approach of reporter phages involved the production of the enzymes β-galactosidase and glycosidase. β-galactosidase catalyzes the hydrolysis of β-galactoside bonds within substrates, while glycosidase hydrolyzes glycosidic bonds. During reporter phage infection of target bacteria, these enzymes were produced and cleaved substrates that were exogenously added after phage infection to produce colorimetric, luminescent, chemiluminescent or fluorescent signals. Goodridge and Griffiths inserted the *lacZ* gene, which encodes β-galactosidase, into T4 for detection of *E. coli* upon the addition of the fluorescent substrate Mu-Gal.\(^{44}\) This technology led to the development of PhastSwab®, which incorporated a *lacZ* reporter phage for the detection of *E. coli* O157:H7 in a modified SnapValve tube and used colorimetric and luminescent substrates.\(^{45}\) Hagens *et al.* took a unique approach that incorporated the *celB* gene for the production of the hyperthermostable glycosidase, CelB, from *Pyrococcus furiosus* into *Listeria* phage A511.\(^{46}\) In this assay, all other enzymes were deactivated by raising the temperature to 85°C to eliminate background activity.

*Salmonella* detection has been investigated using the ice nucleation gene, *inaW*, of *Pseudomonas* integrated into *Salmonella* phage P22.\(^{47}\) The concept applied here is that water can be super cooled below 0°C and remain a liquid. The application of ice nuclei (a particle that acts as nucleus ice formation) to super cooled water causes a chain reaction of ice formation. After infection by the reporter phage, the InaW proteins were expressed on the surface of infected bacteria and acted as ice nuclei that caused the solution to freeze at approximately -10°C. To allow for visual determination, an indicator dye was added that turned orange if freezing occurred at -10°C compared to fluorescent green for no reaction.

Lastly, reporter phages in which the inserted genes encode for peptides that are displayed on the phage capsid are discussed. These expressed peptides were tagged by nanoparticles or
fluorescent dyes. Genetically engineered T7 produced progeny phages that expressed a small peptide on its capsid upon infection of *E. coli* cells. This peptide was then post-translationally biotinylated by host cell biotin-ligases. These progeny phages were then tagged by streptavidin-conjugated quantum dots, which bound to biotin, and were detected by flow cytometry and fluorescence microscopy. Phages that displayed a tetracysteine peptide were stained by adding fluorescent biarsenal dye and detected by flow cytometry and fluorescence microscopy for the presence of *E. coli*.

Upon cell lysis due to phage infection, progeny phages are released along with intracellular bacterial components. These bacterial components have served as biomarkers to signify a lysis event. One such example was the release of adenosine triphosphate (ATP). The release of ATP, detected by luminescence assays, for the detection of *E. coli* lysis by T4. Another approach focused on the release of the enzyme adenylate kinase (AK), which catalyzes the equilibrium of adenosine nucleotides (2ADP ⇔ ATP + AMP). Upon cell lysis by phages, ADP was added exogenously and converted into ATP by AK, which was then detected by luminescence assay. This approach was demonstrated for the detection of *E. coli* and *Salmonella*. To improve this assay, an immunomagnetic magnetic separation (IMS) step was added to capture and concentrate target bacteria prior to addition of phage.

The release of galactosidase and glucosidase enzymes after cell lysis has been identified by the addition of substrates and subsequent measurement of amperometric changes in solution. These approaches detected *E. coli*, *B. cereus* and *M. smegmatis*. Furthermore, it should be noted that small, charged molecules (such as acids) produced by microbial metabolism of larger uncharged molecules (such as carbohydrates) caused a change in the electrical properties of the
media and this change in conductivity can be measured. Phage lysis retarded the growth of a
target organism, thus slowing the change in electrical conductivity.\textsuperscript{60}

As previously stated, the first step of phage infection is recognition and attachment to
specific surface structures of their target host.\textsuperscript{5} This attachment process has been exploited to
detect target cells by labeling phage DNA with a fluorescent dye. Upon attachment to target
bacteria, the labeled phages produced dots of fluorescence around the cell that were visualized
by epifluorescence microscopy. Fluorescently-labeled phages have detected \textit{E. coli} and
\textit{Microlunatus phosphovorans}.\textsuperscript{61-63} Mosier-Boss \textit{et al.} applied phages with labeled DNA to detect
\textit{Salmonella}, but instead of recognizing phage-host attachment, the phage genome was observed
inside the host cell by epifluorescence microscopy.\textsuperscript{64}

Additional phage components employed in detection assays are endolysins. Endolysins
are peptidoglycan hydrolases produced by phages late in the infection process to destroy the cell
wall and allow for lysis and the release of progeny phages.\textsuperscript{65} These enzymes are composed of
two domains, an enzymatically active N-terminal domain, which cleaves bonds in the
peptidoglycan, and C-terminal cell wall binding domain (CBD). Endolysins can be classified
into categories based on which bonds are cleaved: 1) glucosaminidases, 2) muramidases, 3)
transglycosylases, 4) amidases, and 5) endopeptidases.\textsuperscript{66} CBDs of endolysins recognize species-
or strain-level cell wall carbohydrates with an affinity similar to antibodies ($K_A = 6 \times 10^8$).\textsuperscript{67}
CBDs have been fused with paramagnetic beads for specific capture of \textit{Listeria}, \textit{Bacillus} and
\textit{Clostridium}.\textsuperscript{68} For \textit{Listeria} detection, serovar-specific CBDs were linked with differently
colored fluorescent proteins and visualized by fluorescence microscopy.\textsuperscript{69}

Phages and phage components have also been exploited as bacterial capture agents in
biosensor technology. Biosensors are devices that are composed of a biorecognition element, a
transducer that converts the binding reaction into a measurable signal, and lastly, a signal display. Phage particles have been immobilized on the sensor surface by both physical adsorption and covalent bonds. Physical adsorption of phages to biosensors has been applied to the detection of *S. aureus*, *Salmonella* and *B. anthracis* spores.\(^6^{10}\) Physical adsorption of the phage to the biosensor has the drawback of being unable to direct the orientation of the phage onto the sensor to allow for cell capture. In order for a tailed phage to capture its host, the tail must be oriented in an outward direction, while the head is immobilized inward on the sensor. Several chemical approaches have been taken to direct orientation. Such strategies include genetically modified phages to 1) produce a biotinylated phage capsid and a sensor containing streptavidin, 2) express cellulose binding protein on the phage capsid and cellulose on the sensor, 3) linking the phage to the sensor by use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4) the use of glutaraldehyde activated amino acids on the sensor surface to covalently link phage particles.\(^11^{15}\)

As an example of applying phage components as capture agents, CBDs against *Listeria* were immobilized on a gold screen-printed electrode for detection by electrochemical impedance spectroscopy.\(^70\) It should be noted that CBDs are not effective for the capture of Gram-negative bacteria, due to the outer membrane denying access to the cell wall. To circumvent this problem, tail spike proteins and receptor binding proteins of phages to Gram-negative bacteria have been attached to biosensors for detection of *S. typhimurium* and *Campylobacter jejuni*, respectfully.\(^71^{72}\)

The previously discussed phage-based bacterial diagnostic applications using reporter phages, detection of intracellular products due to cell lysis, labeled phages and phages as bacterial capture agents in biosensors have been recently reviewed.\(^16,73^{75}\) The primary objective
of this review is to emphasize the phage amplification assay as a rapid method for bacterial detection. As mentioned earlier, phage amplification assays involve the addition of phage to a specific bacterial target and the subsequent detection of progeny phage to serve as secondary biomarkers. Detection of progeny phage is achieved in three ways: 1) by the addition of phage below the detection limit of the chosen analytical detector and subsequent detection in the rise of phage concentration above instrumental LOD to indicate the presence of the target bacteria, 2) differentiation of input phage versus progeny phage that can be distinguished by a detector, or 3) inactivation of input phage after initial phage infection and subsequent increase in phage concentration compared to a negative control. Such methods that will be described are plaque assay, high-performance liquid chromatography (HPLC), optical density (OD) measurements, PCR-based assays, mass spectrometry, lateral flow immunochromatography, staining and colorimetric assays and multichannel series piezoelectric quartz crystal (MSPQC) sensors.

### 2.3 Plaque assay

Phages were discovered in 1915 by William Twort and independently by Felix d’Herelle in 1917 by their ability to lyse bacterial cells. Since that time, a strategy called phage typing has been employed to allow for differentiation of bacterial isolates. Phage typing methods involved spotting a variety of phages with differing, known host ranges onto a bacterial lawn. If susceptible to the phage, a clearing, called a plaque, is visualized on the bacterial lawn due to cell lysis. Plaques are formed due to initial infection by the phage and subsequent production of progeny phages that go on to infect neighboring cells. Phage host range can vary in specificity from genus to species to strain level and based on which phages produced plaques, bacterial identification can be made. In 1938 the first described phage typing assay exploited phages for their specificity for the Vi antigen to differentiate strains of *S. typhi*. This technique has since
been applied to identify many bacteria, some examples include: *B. anthracis*, *C. jejuni*, *Enterococcus faecalis*, *L. monocytogenes*, *P. aeruginosa*, *S. typhimurium* and *S. aureus*.\(^{78-86}\) The major drawback to phage typing methods is the time involved, requiring at least overnight incubation in order for the formation of bacterial lawns to allow for plaques to be visualized.

To achieve faster results using the plaque assay, a phage amplification method employed the use of virucide to inactivate uninfected phages and incorporated the use of helper cells to decrease plaque formation time (Figure 2.2). The first step was the addition of phage to a sample containing a suspected target pathogen and allowing enough time for host infection. Upon infection, phage genetic information was inserted into the target cell. Next, the addition of a chemical agent, called a virucide, prior to lysis of the host cell inactivated exogenous phages while not disrupting host bacteria growth. Following neutralization of the virucide, progeny phages lysed the infected host and released into the surrounding milieu. These progeny phages were then mixed with a propagating strain (called helper, sensor, or signal-amplifying cells).\(^{87}\) Having previously inactivated residual-free phage, only progeny phages produced by the infection of the target will be present to infect the propagating strain. The propagating strain grows much faster than the targeted, pathogenic host, thus can produce progeny phages much more rapidly. The helper cell/phage mixture was then plated with soft agar and incubated for plaque formation. Negative control experiments (containing no target bacteria) were performed in parallel. Resulting plaques on suspect samples compared to negative controls served as indicators of phage infection of the initially targeted bacteria.

Wilson *et al.* developed a phage amplification assay combined with the plaque assay, termed phage amplified biologically (PhaB) assay, for the detection of *M. tuberculosis*.\(^{88}\) The PhaB assay detected *M. tuberculosis* in 3-4 days compared to 6-8 weeks with conventional
culture methods. The authors employed broad host mycobacterium phage D29 as the indicator phage with ferrous ammonium sulfate (FAS) as the virucidal agent. Subsequent dilution of the culture with oleate-albumin-dextrose-catalase (OADC) neutralized FAS. This assay utilized \textit{M. smegmatis} as helper bacteria because of its faster growth rate than \textit{M. tuberculosis}. The authors also evaluated

\begin{figure}[h]
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\caption{Phage plaque assay. A) Genus-specific phage is added to target bacterial culture. B) Phage adsorption and infection. A virucidal agent is added to destroy exogenous phage. C) Phage amplification by the host cells. D) Phage amplification results in bacterial lysis. E) Helper cells added and plated on agar. F) Phage plaques develop on the bacterial lawn. (Image credit: Dr. Christopher Cox)}
\end{figure}

this method for detection of drug-resistant \textit{M. tuberculosis} by incubating the sample with a selected drug for three days, followed by the phage amplification assay. If the drug killed the
bacteria, phage amplification did not occur, while drug-resistant bacteria allowed for phage infection and replication. The PhaB assay evaluated *M. tuberculosis* resistance to rifampicin and isoniazid, two frontline drugs used in the treatment of tuberculosis. The PhaB assay detected 100% (1/1) of the rifampicin-resistant/isoniazid-sensitive isolate, 94.6% (35/37) of rifampicin-sensitive isolates, 88.2% (15/17) of isoniazid-resistant/rifampicin-sensitive isolates, 81% (17/21) of isoniazid-sensitive isolates and 100% (8/8) of multidrug-resistant isolates (determined by resistance to both rifampicin and isoniazid). Eltringham *et al.* further evaluated PhaB as a rapid screening method for *M. tuberculosis* resistance to several other drugs; ethambutol, streptomycin, pyrazinamide and ciprofloxacin, in addition to an expanded isoniazid study. The authors compared PhaB assay results to data from the resistance ratio method for drug resistance with a correlation of 94% for isoniazid, 96% for streptomycin, 100% for ciprofloxacin, 88% for ethambutol and 87% for pyrazinamide. Advantages of the PhaB assay over conventional drug resistance determination were lower cost and a TRT of 2-3 days (compared to 10 days). Additionally, Eltringham *et al.* evaluated the PhaB assay against RT-PCR for detection of rifampin-resistant *M. tuberculosis*. This study selected *dnaK* mRNA, which encodes for heat shock protein, for detection by RT-PCR. Samples were exposed to rifampin overnight and heated at 45°C for 45 min to increase production of *dnaK* mRNA. The PhaB assay detected 100% (46/46) of sensitive and 100% (31/31) of resistant strains, while, RT-PCR detected 96% (46/48) and 97% (35/36) of sensitive and resistant strains, respectively. In addition to producing more accurate results, the PhaB assay had a lower technical demand and cost compared to RT-PCR.

The PhaB assay has also been applied for detection of *M. tuberculosis* in spiked sputum samples. Sputum contains inhibitory factors that prevent phage infection; therefore, sample
preprocessing was required. Inhibitory factors were removed with SDS or NaOH without impacting the growth of *M. tuberculosis*. Additionally, sodium citrate along with calcium chloride presented a cost-effective alternative for the neutralization of FAS activity compared to the relatively expensive OADC, potentially lowering the cost of the assay.

PhaB research led to the commercial development of the *FastPlaqueTB®* assay (FPTB). The FPTB assay utilized phage D29, which has a broad host range against *Mycobacterium*. By exploiting the broad host of D29, Foddai *et al.* adapted the FPTB assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne’s disease in ruminants.\(^\text{93}\) D29 replication occurs at different rates depending on the host and an incubation time of two hours with D29 and MAP was determined ideal prior to the addition of helper cells. However, due to the broad host range, D29 plaques from naturally contaminated samples may not be solely due to the presence of MAP. Therefore, Foddai *et al.* performed immunomagnetic and peptide-mediated magnetic separation steps to isolate target MAP cells before evaluation by FPTB.\(^\text{94}\) Paramagnetic beads coated with two biotinylated peptides achieved 85-100% capture of MAP with <1% nonspecific recovery of other *Mycobacterium* spp. This peptide-mediated separation (PMS) strategy successfully detected MAP in bovine bulk tank milk (BTM) and fecal samples.\(^\text{95}\) In an expanded study, Foddai and Grant evaluated the PMS-phage assay for inclusivity (detected 100% of 43 MAP strains) and specificity (>99% specific for MAP).\(^\text{96}\) Furthermore, this study showed that the PMS-phage assay provided better sensitivity than PMS-qPCR and PMS-culture methods in raw milk and BTM samples from Johne’s affected herds. Stewart *et al.* combined PMS with enzyme-linked immunosorbent assay (ELISA) to detect progeny phages.\(^\text{97}\)
While the concept of phage amplification had been conducted for decades, Stewart et al. first introduced the term while describing the detection of *S. typhimurium* and *P. aeruginosa*. Phage Felix O-1 was used for *S. typhimurium* detection, while phages NCINB 10116 and NCINB 10884 were employed for *P. aeruginosa*. This approach involved infection for 15 min at 37°C for *S. typhimurium* and 25 min at 30°C for *P. aeruginosa* before the addition of a virucide, a combination of pomegranate rind extract (PRE) and ferrous sulfate. Following a 3 min incubation time with virucide, neutralization of the virucide was accomplished by the addition of an equal volume of 2% Tween-80. Lastly, helper cells mixed with soft agar were added to the culture and the mixture poured onto agar plates and incubated for plaque formation. The authors report detection limits of 600 cfu/mL and 40 cfu/mL for *S. typhimurium* and *P. aeruginosa*, respectively, in 4 h. Siqueira et al. later adapted this assay for detection of *S. typhimurium* in chicken meat using phage P22.

FAS and PRE are not effective virucidal agents against all phages. Oliveira et al. investigated FAS and PRE along several other chemical agents and laser treatments to inactivate *Listeria* phage A511. It was found that employing toluidine blue O with non-ablative laser light treatment were effective in reducing A511 concentrations without impacting the viability of *L. monocytogenes*. This method was further applied to detect 13 cfu/mL in skimmed milk. This work illustrated the virucidal agents may not be applicable for all phage amplification based strategies; therefore, other techniques are required.

A final iteration of plaque-based phage amplification assays discussed here incorporates the use of phage mutants to distinguish between input and progeny phages. The aim of these assays was to eliminate the need for inactivation of non-infecting, exogenous phages by a virucidal agent. Below are 3 examples of methods that utilized target bacteria to repair phage
mutants by recombination or complementation that would not be able to form plaques otherwise. The first version used a complementary pair of amber mutants of Felix-O1 that relied on infection by both phages in order to produce plaques for the detection of *S. typhimurium*. Complementation was achieved when target bacteria were co-infected by two different phage mutants with complementary genes that result in each phage mutant providing the missing gene in the other. This resulted in phenotypically rescued wild-type phages that can form plaques. The second procedure used a complimentary pair of temperature sensitive mutants that were unable to attach to the bacterium at a restrictive temperature for the detection of *E. coli* O157:H7. Two complimenting temperature sensitive mutants were added at the permissive temperature (37°C) and incubated for 35 min to allow for infection. Samples were then applied to a soft agar overlay and the temperature increased to the restrictive temperature (42°C) for three hours. Observation of plaque formation occurred if the mutation was repaired by complementation. The third strategy employed UV-irradiated mutants of phage OE for the detection of *E. coli*. These mutant phages were incubated with target cells under bright sunlight conditions to promote photo-reactivation. Then the sample was mixed with helper *E. coli* mutants that could not repair the phages, plaques that formed were from repaired phage mutants and not uninfected phage mutants.

2.4 First example of phage amplification detected by analytical instrumentation

In 1983, Hirsh and Martin first described a phage amplification method applying analytical instrumentation for the detection of *Salmonella* using phage Felix-O1. An increase in the number of phages was identified by high-performance liquid chromatography (HPLC) with a limit applications, this method required an input phage concentration lower than the instrumental LOD, which, for this method, was $3 \times 10^8$ pfu/mL. The goal of adding input phages
at levels below the instrumental LOD at the beginning of an amplification reaction was to prevent the detection of known reactive phages as a false positive in the case of a negative (no target bacteria) situation. In a later publication, 100 *Salmonella* spp. cells per mL of milk were detected after an overnight enrichment. This HPLC method was not very sensitive and has resulted in subsequent development of analytical techniques with lower detection limits.

### 2.5 Optical density differences as an indicator of phage amplification

Optical Density (OD) is a measurement of culture turbidity and increases during cellular growth while decreasing upon phage infection due to cell lysis. Favrin *et al.* exploited this difference in ODs as an endpoint in a phage amplification assay for detection of *S. enteritidis* in broth culture. First, immunomagnetic beads labeled with anti-*Salmonella* antibodies captured and concentrated bacterial cells. The concentrated cell culture was then inoculated with *Salmonella* phage SJ2 and incubated for 10 min to allow for infection of target cells. Exogenous phages were removed by washing the magnetic beads. Following phage amplification, progeny phages were transferred to a fresh culture of helper *S. enteritidis* cells and monitored by OD (at 600 nm) readings for 1.5-2 h. Negative controls of this assay consisted of *Salmonella*-free samples, in which no phage amplification occurred and displayed high OD readings due to the uninhibited growth of the helper cells. The presence of *Salmonella* led to the production of progeny phages, which then went on to infect helper cells and caused a decrease in OD. Samples were considered positive for *Salmonella* in the initial culture if the optical density was 70% or less than negative controls.

These authors further expanded the method for the detection of *Salmonella* in skimmed milk powder, chicken rinses, and ground beef. The assay detected 3 cfu of *S. enteritidis* in 25 g of each food sample with a total assay time, including enrichment, of 20 h. The authors also
adapted this method for the detection of *E. coli* O157:H7 after 20 h total assay time in ground beef at a concentration 2 cfu/g using phage LD1.

**2.6 Bacterial detection by phage amplification combined with polymerase chain reaction**

While PCR alone cannot distinguish between living cells and dead cells, PCR methods have been combined with phage amplification to increase species specificity. Two strategies have combined phage amplification and PCR. The first approach involved the use of a broad-host phage and the plaque assay described earlier in which phages were added against a specific target host, exogenous phage inactivated, helper cells added and mixture plated for plaque formation. For further specificity, bacterial DNA isolated from resulting clearings (plaques) was subjected to species-specific PCR analysis for the original target bacteria. The method combined the use of phage for live/dead determination, while PCR of the plaques provided increased specificity. Stanley *et al.* first described this phage-PCR combined method for detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

*FastPlaque*TB® (FPTB) assay reagents previously used for detection of *M. tuberculosis* were adapted to detect viable MAP because the phage used in this assay, D29, has a broad *Mycobacterium* host range. To achieve species-level specificity, samples taken from plaques formed in helper cell lawns were analyzed by PCR, amplifying the IS900 sequence of MAP and PCR products visualized by gel electrophoresis. Extensions of this method evaluated bulk tank milk and powdered infant milk samples for the presence of MAP by quantitative PCR (qPCR) of the IS900 sequence. Swift *et al.* employed an additional peptide-mediated magnetic separation step to detect MAP in blood samples.

Alternatively, PCR was used to directly amplify and detect the nucleic acids of progeny phages after phage amplification has occurred. The direct detection of progeny phage eliminated
the need for helper cells and the time required for plaque formation. This approach has been used in the detection of the plant pathogen *Ralstonia solanacearum*, a cause of bacterial wilt in plants such as tomatoes, potatoes, eggplant and peppers.\textsuperscript{109-110} Phage M_DS1 was selected for a qPCR/phage amplification assay for the detection of *R. solanacearum*.\textsuperscript{109} Approximately 3.3 cfu/mL of *R. solanacearum* were detected in pure culture after 1 h of incubation and $10^2$ cfu/mL could be detected in 0.1 g of leaf tissue from ginger plants inoculated with *R. solanacearum*.

A similar approach was applied for the detection of *B. anthracis* and *Y. pestis*.\textsuperscript{111-112} For *B. anthracis*, anthrax diagnostic gamma phage primers were developed in-house and utilized in a qPCR assay.\textsuperscript{112} This assay detected 207 cfu/mL of *B. anthracis* in 5 h. Primers for two yersiniophages, ϕA1122 and L-413C, were developed and used in phage amplification and progeny phages detected by qPCR.\textsuperscript{111} ϕA1122 and L-413 assays had detection limits of $10^3$ cfu/mL $10^5$ cfu/mL, respectively.

### 2.7 Detection of progeny phage proteins using mass spectrometry

Mass spectrometry-based phage amplification assays relied on detection of progeny phage proteins or peptides. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been the method of choice for several phage amplification-based schemes. For this technique, progeny phage proteins were applied onto a sample plate with a UV-absorbing matrix and then ablated by a laser. Madonna et al. separated and concentrated *E. coli* cells using immunomagnetic separation (IMS) and subsequently infected cells with coliphage MS2 below the detection limit of the instrument (\~$1.0 \times 10^5$ pfu/mL).\textsuperscript{113} After 40 min of amplification, samples were removed for analysis by MALDI-TOF MS. The capsid protein of MS2, with a mass of 13,726 Da, was detected. The capsid protein offered an
ideal target for detection because of its high copy number. For example, each MS2 phage has 180 copies. The LOD for this assay was $\sim 5.0 \times 10^4 \text{ cfu/mL}$.

Rees and Voorhees applied phage amplification with MALDI-TOF MS for the simultaneous detection of two bacterial pathogens, *E. coli* and *S. enterica*, in the same sample. Capsid proteins of *E. coli* phage MS2 and *Salmonella* phage MPSS-1 had masses of 13.7 kDa and 13.5 kDa, respectively. The authors reported a detection limit for both phages of $1 \times 10^7 \text{ pfu/mL}$ and presented three scenarios that demonstrated how each phage could be detected in the presence of the target bacteria. In the first scenario, a mixture of *E. coli*, MS2 and MPSS-1 was monitored by MALDI. Observation of the MS2 capsid protein at 13.7 kDa appeared after a 3 h amplification. The second scenario involved *S. enterica* mixed with MS2 and MPSS-1 and in this example; the MPSS-1 capsid protein peak became present in the spectrum after 3 h. The final scenario mixed *E. coli* and *S. enterica*, along with both phages, MS2 and MPSS-1. Both phage capsid protein peaks occurred in the spectrum, showing the utility of this method to simultaneously detect two different bacterial targets by the amplification of two different phages and to distinguish between two phages that had similar molecular weights.

While some phage capsids can be dissociated by the acidic nature of the matrix, other phages require additional steps to free the capsid monomer. One such example was the use of β-mercaptoethanol (BME) or tris(2-carboxyethyl)phosphine to break disulfide bonds between capsid proteins containing cysteines (Figure 2.3). McAlpin *et al.* employed this approach in the study of 6 *Y. pestis* specific phage capsid proteins. BME combined with phage amplification detected *Burkholderia pseudomallei* by targeting the capsid protein of *B. pseudomallei* phage ϕX216 (37.6 kDa), with a phage LOD of $2.6 \times 10^7 \text{ pfu/mL}$. With an initial bacterial concentration of $3.2 \times 10^5 \text{ cfu/mL}$ and an initial
phage concentration below the detection limit of the instrument at \(1.6 \times 10^5\) pfu/mL, detection of the \(\phi X216\) capsid protein occurred in two hours. This strategy was applied in the detection of ceftazidime-resistant \(B.\ pseudomallei\).\(^{116}\) Ceftazidime was added at the onset of phage infection and progeny phage capsid detection was observed in resistant strains due to phage amplification. The drug susceptible strain was killed and did not promote phage amplification, thus, no phage proteins were detected in the MALDI spectrum.

**Figure 2.3:** Phage amplification detected by MALDI-TOF MS. A) Species-specific phage infection at starting concentration below the limit of detection. B) Phage amplification. C) \(\beta\)-mercaptoethanol (BME) added to infection. D) MALDI matrix spotted with an aliquot of phage lysis reaction. E) MALDI analysis. F) Phage-specific protein spectral profile indicates phage amplification due to the presence of target bacterial host in the original sample. (Figure credit: Dr. Christopher Cox)
The requirement of input phage concentrations below the detection limit of the instrument increases the incubation time for phage amplification to reach the instrumental LOD at low bacterial concentrations. This is because, at low concentrations of bacteria, fewer infections occur and this increases the time for the rise in phage concentration above instrumental LOD. To reduce detection time and increase phages infections, Pierce et al. developed a method that employed a way to input phages at a concentration above instrumental LOD and distinguish them from progeny phages. Differentiation of input phages from output phages was achieved by isotopically labeling input phage 53 with $^{15}$N for the detection of S. aureus. $^{117}$ $^{15}$N input phage was produced by infection of $^{15}$N S. aureus in $^{15}$N rich media. Detection of S. aureus was achieved by the addition of $^{15}$N phage, which upon infection of S. aureus, generated wild-type $^{14}$N progeny phages. $^{14}$N progeny phage capsid proteins were distinguished from the input $^{15}$N phage capsids by MALDI mass spectrometry. Isotopically-labeled phages contained capsid proteins with a mass of 35.46 kDa, while the wild type phage capsid proteins were 35.04 kDa. By differentiating the masses of input phage from progeny phage, a high concentration ($2 \times 10^8$ pfu/mL) of input phage above instrumental LOD was employed to increase interactions between input phage and a low concentration of target bacteria. This mass difference was successfully observed by MALDI MS and overcame the need for the input phage concentrations below detection limits of the instrument, thus reducing incubation time by 90 min (from 180 min to 90 min). Isotopically labeled phage amplification was also applied for antibiotic resistance determination. In the presence of cefoxitin (an analog for methicillin), methicillin-resistant S. aureus (MRSA) exhibited phage amplification of $^{15}$N phage K, as opposed to methicillin-sensitive S. aureus (MSSA), which did not show phage
amplification.\textsuperscript{118} \textsuperscript{14}N K progeny phages were digested with trypsin prior MALDI MS analysis and the appearance of 3 phage specific peptide peaks were used to indicate phage amplification.

Not every phage capsid can be readily disassembled by BME treatment. Therefore, trypsin digestion was applied after phage amplification and subsequent phage peptides used as biomarkers. Isotopically labeled phage amplification was combined with liquid chromatography/mass spectrometry (LC/MS) and multiple reaction monitoring (MRM) for the detection and quantification of \textit{S. aureus}.\textsuperscript{119} In this study, the authors digested phage 53 with trypsin and three resulting capsid peptides were detected by LC electrospray ionization MS using MRM analysis. The amino acid sequence of these peptides was determined by MS/MS and searched in a protein database (National Center for Biotechnology); thus verifying that the three peptides were derived from the major capsid protein. \textsuperscript{15}N-labeled phage amplification produced progeny phages that contained \textsuperscript{14}N, allowing differentiation between input and progeny phages. Quantification of \textit{S. aureus} was accomplished by constructing a calibration curve in which known concentrations of \textit{S. aureus} infected with \textsuperscript{15}N phage. The calibration curve consisted of the ratio of \textsuperscript{15}N phage peptide peak area to \textsuperscript{14}N phage peak area versus \textit{S. aureus} concentration. \textit{S. aureus} LOD was determined to be $3 \times 10^4$ cfu/mL using an amplification time of 3 h. Another study combined \textsuperscript{15}N T7 amplification with LC/MS-MRM for the quantified detection of \textit{E. coli} in LB media, coconut water and apple juice. LODs of $3.0 \times 10^3$ cfu/mL in LB media, $4.1 \times 10^4$ cfu/mL in coconut water, and $1.9 \times 10^3$ cfu/mL in apple juice were found using a 4 h amplification time.\textsuperscript{120}

Rees \textit{et al.} combined phage amplification with LC/MS-MRM for antibiotic resistance determination of \textit{S. aureus} by dividing a sample into two fractions, with the first fraction containing phage and the bacteria strain of interest, while the second fraction contained phage,
antibiotic and bacteria. The first fraction showed that the phage was specific to the strain, while phage amplification in the second fraction indicated the presence of an antibiotic resistance strain. Broad host range phage K was used for detection and antibiotic resistance to clindamycin and cefoxitin of 4 strains of *S. aureus*.

Martelet *et al.* joined MRM with unlabeled phage amplification and LC-ESI MS for detection of *E. coli* and *B. subtilis*. This study utilized phages T4 and SPP1 for detection of *E. coli* and *B. subtilis*, respectively. This study investigated the use of two T4 structural proteins (internal protein I and small outer protein) and one SPP1 nonstructural protein (scaffolding protein) as biomarkers. After phage amplification, lysis supernatant was subjected to trypsin digestion and resulting peptides were analyzed by LC/MS-MRM. T4 amplification detected *E. coli* in LB medium, orange juice and cassoulet (a French stew) with LODs of $1 \times 10^5$ cfu/mL, $5 \times 10^5$ cfu/mL and $1 \times 10^6$ cfu/mL, respectively. This paper also demonstrated how non-structural proteins can be utilized as biomarkers, using phage SPP1 for the detection *B. subtilis*. Non-structural proteins, such as scaffolding proteins, are absent from the infecting phage but are expressed during the infection process to aid in phage assembly. Detection of non-structural proteins allows for input phage concentrations above instrumental LOD. To improve the sensitivity of this method, Martelet *et al.* employed immunomagnetic separation (IMS) along with LC/MS-MRM for detection of T4 amplification in food matrices. This study determined a phage LOD in both milk and orange juice to be $5.0 \times 10^6$ pfu/mL. Subsequent phage amplification performed on these foods for 3 h with an input phage below the LOD ($1.0 \times 10^4$ pfu/mL) followed by the addition of superparamagnetic beads linked to anti-phage antibodies to isolate T4. Isolated phage was digested with trypsin and peptides detected by LC/MS-MRM. For a 3 h amplification, *E. coli* LOD was $1.0 \times 10^4$ cfu/mL and $5.0 \times 10^4$ cfu/mL in milk and
orange juice, respectively. This method successfully detected 1 cfu/mL of *E. coli* in 10 mL of milk after 8 h amplification. Because of the low starting concentration of bacteria and phage, the probability of phage and bacterial host recognition was very low. Therefore, longer incubation times were required for detection.

2.8 Lateral flow immunochromatographic detection of progeny phage

Lateral flow immunochromatography has been demonstrated as an endpoint detection system for numerous analytes. Figure 2.4 illustrates how LFI has been applied to detect progeny phages from phage amplification. The mechanism of LFI begins with the application of a sample solution containing the analyte of interest to a test strip. This solution moves via capillary action through a release pad, which contains reporter particles that are covalently linked to analyte-specific antibodies. The reporter particles attach to the analyte and continue down a porous membrane until they become arrested at a test line containing immobilized analyte antibodies and at a high enough concentration, a detectable colored line is produced.

The use of LFI for phage detection led to a Colorado School of Mines spinoff company, Microphage, whose KeyPath® MRSA and MSSA blood culture test (MMBT) received FDA approval in 2011. The MMBT was performed on clinical blood culture samples positive by BactT/Alert and consisted of two tubes, one for identification of *S. aureus* and one containing cefoxitin for antibiotic resistance determination. Both tubes contained culture medium and a bacteriophage cocktail specific to *S. aureus*. Samples added to the tubes were incubated for 5 hours at 35°C to allow for phage amplification and then samples were removed and applied to LFI strips. MMBT evaluated 119 clinical blood samples, of which 46 were positive for *S. aureus*. Of the *S. aureus* positive samples, 26 were classified as MSSA and 20 MRSA. MMBT had a sensitivity of 87% for *S. aureus* (40/46), 81% for MSSA (21/26), and 95% for
MRSA (19/20). Of the 6 samples that failed to identify *S. aureus* in 5 h, three tested positive when MMBT was repeated within the 24 h recommended incubation window. MMBT also showed 100% specificity, not falsely identifying *S. aureus* in any negative samples (0/69). In a much broader study containing 1116 clinical blood samples, MMBT correctly identified *S. aureus* in 336 of 366 (92%) samples, while incorrectly identified 13 out of 750 (98%) non *S. aureus* as *S. aureus*.\(^{129}\) Twelve of the 13 misidentified strains were determined to be coagulase-negative staphylococci, with remaining the strain identified as enterococcus. MMBT correctly identified 99.4% MRSA (178/180) and 98.7% MSSA (153/154).

\[\text{Figure 2.4: Phage amplification detected by lateral flow immunochromatography (LFI).} \]

A) Species-specific phage infection at starting concentration below the limit of detection, phage amplification and cell lysis. B) Progeny phages attach to colored anti-phage reporter at reporter release pad. Phage/reporter complexes are captured at the test line. C) Diagram of LFI device for phage-based bacterial detection. (Figure credit: Dr. Christopher Cox)
LFI combined with phage amplification was developed for detection of *B. anthracis*, the etiological agent of anthrax, using gamma phage.\textsuperscript{130} Current *B. anthracis* detection by culture-based methods requires 12 to 120 h followed by an additional 24 h for confirmatory plaque assay by gamma phage. Because of *B. anthracis* ability to be weaponized and its associated high mortality and morbidity rates, rapid detection strategies are needed to reduce detection TRT. Gamma phage amplification detected by LFI detected *B. anthracis* in as little as 2 h and a bacterial LOD of $2.5 \times 10^4$ cfu/mL. This gamma phage assay and MMBT were colorimetric-based assays that required the presence of visible test line in order for determination of a positive test. At low analyte concentration, this line can become difficult to observe and lead to false negative interpretations. This dissertation discusses an approach using surface-enhanced Raman spectroscopy nanoparticles as LFI reporters to provide quantitative analysis of the test line, which eliminated reliance on visual determination and reduced detection limits below visual levels.\textsuperscript{131} Briefly, SERS nanoparticles, which consisted of a gold-core coated with a Raman active organic reporter, produced a characteristic Raman spectrum when interrogated with a laser in the near IR. This SERS LFI assay was first applied to detect *L. monocytogenes* (Chapter 3 and 4) and further adapted for detection *Y. pestis* (Chapter 5).

Another approach to phage amplification-based LFI employed the use of 2 different bioengineered T7 phages for *E. coli* detection, one that expressed maltose-binding protein (MBP) and one that expressed alkaline phosphatase (ALP).\textsuperscript{132} After phage amplification, MBP LFI was performed by mixing sample with biotinylated polyclonal anti-MBP antibodies in a tube and an LFI strip with a test line consisting of anti-MBP antibody was inserted into the tube. Next, the LFI strip was removed and added to a tube which contained a solution of streptavidin-conjugated quantum dots followed by fluorescent analysis of the test line. ALP LFI did not require the use
of a reporter as alkaline phosphatase produced by phage amplification was directly captured at the test line by anti-alkaline phosphatase antibodies. After application of the sample, LFI strips were placed into a tube with a solution that contained the substrates 5-bromo-4-chloro-3’-indolylphosphate and nitro-blue tetrazolium, which was converted to a purple substrate by captured alkaline phosphatase to produce a visible line at the test line. MBP LFI and ALP LFI produced 10-fold and 100-fold, respectively, increase in sensitivity over LFI detection of native T7.

### 2.9 Phage amplification and live/dead determination by staining and colorimetric assays

Because the endpoint of phage lysis is cell death, live/dead fluorochromic staining methods have been applied to detected phage amplification. Fluorochromic staining identified damage to cell walls created by phage infection. To differentiate between intact cells and infected cells, a combination of SYTO9 and propidium iodide stains were used. SYTO9 is a nucleic acid dye that can penetrate the cell membranes of both live and dead or damaged cells and fluoresces green, while propidium iodide, which fluoresces red, can only penetrate damaged membranes and causes a reduction in SYTO 9 fluorescence when both dyes are present. The presence of damaged membranes due to phage amplification was indicated by red fluorescence. Such method was applied for detection of *P. aeruginosa*. In this assay, the authors exposed samples with *Pseudomonas* phage NCIMB 10116 and incubated for two hours, followed by the addition of helper cells to amplify phage from initial infection and further incubation for 1-2 h. Bacterial capture was achieved by filtration through a 0.45 μm membrane and a wash with lambda buffer, SYTO9. Bacteria were recovered by the addition of the filter to test tubes containing lambda buffer and vortexed for one minute. Propidium iodide and SYTO 9 dyes were
added to the resulting bacterial suspension and the fluorescence was measured. This assay detected approximately $1 \times 10^1 \text{cfu/mL}$ within 4 h.

A colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) combined with phage amplification for detection of rifampin-resistant *M. tuberculosis*. In the presence of bacterial growth, water-soluble, yellow MTT was reduced to insoluble, purple formazan by NAD(P)H-dependent cellular oxidoreductase in metabolically active cells. In this assay, bacterial cells were incubated overnight with rifampin followed by 90 min incubation with phage D29. The addition of 0.3% sulfuric acid for 5 min removed exogenous phage and neutralized by the addition of NaOH. For phage detection, samples were transferred to cultures of helper *M. smegmatis* for overnight incubation followed by the addition of MTT. The presence of a purple color indicated that helper cells were not infected by newly amplified phage and that these *M. tuberculosis* samples were susceptible to rifampin. If growth of helper cells was inhibited due to phage lysis, the color remained yellow.

2.10 Multichannel series piezoelectric quartz crystal sensor technology used to indicate phage amplification

Multichannel series piezoelectric quartz crystal (MSPQC) sensor technology measures the change in electrical parameters in culture due to bacterial growth. Bacterial growth caused an increase in frequency shift. MSPQC demonstrated an endpoint for phage D29 amplification for detection of *M. tuberculosis* as indicated by an absence of a frequency shift, resulting from cell death. Similar to the previously discussed phage amplification assay in which progeny phages were detected by plaque assay, phage D29 was added to a sample suspected of containing *M. tuberculosis* and exogenous phage inactivated with ferrous ammonium sulfate (FAS), followed by the addition of helper *M. smegmatis*. However, instead of plating the phage/MM.}
*smegmatis* mixture and observing for plaques, a MSPQC sensor measured the changes in electrical parameters in the growth medium. The killing of helper bacteria by phage amplification did not cause a frequency shift and signaled the presence of target *M. tuberculosis* cells. In the absence of phage, bacterial growth by the helper cells was detected as a frequency shift. In less than 30 h, $10^2$ cfu/mL of *M. tuberculosis* were detected, significantly less than the 30 days required by culture.

### 2.11 Conclusion

Numerous methods have been developed employing phages for bacterial detection. These detection strategies utilize phage specificity for a targeted host, rapid reproduction and their ability to only carry out their infection process in living cells. The capability to discriminate between live and dead cells also makes phages ideal candidates for drug resistance studies. Several bacterial diagnostic techniques employed phages. One example is the use of reporter phages in which reporter genes are inserted into the phage genome and are transcribed and translated upon phage infection. Other phage-based methods include the detection of intracellular products that were released upon cells lysis by the phage. Additionally, bacteria have been detected due to the attachment of fluorescently labeled phages. Furthermore, phages have been used as bacterial recognition elements on biosensors.

Phage amplification methods rely on the initial infection and subsequent detection of progeny phage to indicate the presence of a target bacterium. Several downstream techniques have been described here, each offering a unique approach to progeny phage detection. Such approaches included the use of input phage below instrumental LOD and the subsequent detection of a rise in phage concentration due to the presence of the target host, differentiating input phage proteins from progeny phage proteins and detection by mass spectrometry, and the
use of a virucide to inactivate exogenous input phage. Phage amplification techniques reduce
detection time compared to the current, culture-based gold standards. This time reduction can
help prevent widespread bacterial disease outbreaks, reducing the number of illnesses. Phage
amplification assays offer exciting avenues for rapid bacterial diagnostics.

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CHAPTER 3

RAPID DETECTION OF LISTERIA BY BACTERIOPHAGE AMPLIFICATION AND SERS-LATERAL FLOW IMMUNOCHEMOTGRAPHTY

Modified slightly from a paper published in Viruses\textsuperscript{1}

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3.1 Abstract

A rapid \textit{Listeria} detection method was developed utilizing A511 bacteriophage amplification combined with surface-enhanced Raman spectroscopy (SERS) and lateral flow immunochromatography (LFI). Anti-A511 antibodies were covalently linked to SERS nanoparticles and printed onto nitrocellulose membranes. Antibody-conjugated SERS nanoparticles were used as quantifiable reporters. In the presence of A511, phage-SERS nanoparticle complexes were arrested and concentrated as a visible test line, which was interrogated quantitatively by Raman spectroscopy. An increase in SERS intensity correlated to an increase in captured phage-reporter complexes. SERS limit of detection was $6 \times 10^6$ pfu/mL, offering detection below that obtainable by the naked eye (LOD $6 \times 10^7$ pfu/mL). Phage amplification experiments were carried out at a multiplicity of infection (MOI) of 0.1 with 4 different starting phage concentrations monitored over time using SERS-LFI and validated by spot titer assay.

Detection of \textit{L. monocytogenes} concentrations of $1 \times 10^7$ colony forming units (cfu)/mL, $5 \times 10^6$

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cfu/mL, $5 \times 10^5$ cfu/mL and $5 \times 10^4$ cfu/mL was achieved in 2, 2, 6, and 8 h, respectively. Similar experiments were conducted at a constant starting phage concentration ($5 \times 10^5$ pfu/mL) with MOIs of 1, 2.5, and 5 and were detected in 2, 4, and 5 h, respectively.

3.2 Introduction

*Listeria monocytogenes* is a Gram-positive, motile, facultative anaerobic rod and the etiological agent of food-borne listeriosis. Symptoms of listeriosis include gastroenteritis, diarrhea, meningitis and bacteremia. It also contributes significantly to spontaneous abortions.\(^1\) Listeriosis is responsible for approximately 1600 food-related illnesses and 260 deaths in the U.S. annually\(^2\) and is the third leading cause of death among foodborne pathogens (behind *Salmonella* spp. and *Toxoplasma gondii*), with 94% of cases leading to hospitalization and a mortality rate of 20-30%.\(^2\)\(^-\)\(^3\) The U.S. has adopted a zero tolerance policy for *Listeria* on food. However, because of the protracted turnaround times (TATs) of conventional detection methods, the majority of food products potentially contaminated with *L. monocytogenes* are not tested before entering the marketplace, increasing the risk for widespread outbreaks. Given *Listeria*’s natural occurrence in soil and among animal reservoirs, many processed foods are at risk for *Listeria* contamination. These include ready-to-eat meats and cheeses, unpasteurized dairy products, hot dogs, smoked seafood and raw produce.\(^4\) Further adding to difficulties in outbreak prevention is *Listeria*’s ability to grow over a wide range of temperatures, including those as low as 1°C, which allows it to propagate even when refrigerated (~4°C). One example of this is the 2011 Jensen Farms outbreak associated with contaminated cantaloupes.\(^5\) One hundred forty seven people were infected across 28 states resulting in 33 deaths and one miscarriage, making it the second deadliest food-borne related outbreak in U.S. history.
The most commonly employed *Listeria* detection/differentiation methods include culture-based biochemical tests (such as the Remel MICRO-ID® *Listeria*, BioMérieux *Listeria* API® test system and VITEK® 2 Compact), and the Christie, Atkins, Munch-Petersen (CAMP) test. These commercially available tests have average TATs of 4-6 days.\textsuperscript{6-7} Polymerase chain reaction (PCR)-based methods, such as the DuPont BAX® system, offer an alternative for rapid detection, however, DNA amplification can be negatively impacted by polymerase inhibitors found in food components, leading to false negatives.\textsuperscript{8} Another limitation with PCR is the inability to differentiate between live and dead cells, a critical issue in the food industry due to the fact that many food products undergo treatment to kill bacteria.\textsuperscript{9}

The Jensen Farms outbreak and others that have occurred since 2011 strongly suggest the need for more rapid detection methods for food-borne *Listeria*. This study exploited *Listeria* phage A511 and its natural, rapid and host-specific amplification. A511, a large (134,494 bp), well-characterized myovirus was chosen for this study because of its lytic nature, broad-host range, and ability to produce many progeny phage (burst size 40-50) in a short amount of time, with a latent period of 55-60 min at 30°C.\textsuperscript{10-12} While only genus specific, A511 still proves useful in detection systems, as government regulatory agencies such as, the Food and Drug Administration, U.S. Department of Agriculture and Food Standards Australia and New Zealand, do not differentiate between *Listeria* spp. because the presence of non-pathogenic *Listeria* spp. indicate poor hygiene and indicate conditions that promote the growth of pathogenic *L. monocytogenes*.\textsuperscript{13-14} As such, A511 has been used in a variety of detection schemes.\textsuperscript{11, 15-17} Phages are predatory bacterial viruses that have long been utilized for bacterial detection and identification and are simple and inexpensive to produce.\textsuperscript{9, 18-20} Fundamentally, phage amplification involves phage attachment to the surface of a host bacterium, followed by insertion
of its nucleic acid. This results in hijacking of the host replication machinery and subsequent transcription and translation of phage genes. Replication and assembly of progeny phages follows, culminating in host cell lysis and release of new infectious virions (progeny phage) into the surrounding milieu to repeat the cycle. This leads to a rapid increase (termed burst) in phage concentration in less than three hours. In phage amplification assays, such as the one described here, input phage are added below detection limits of the detection of choice and allowed to specifically infect target bacterial pathogen and multiply. An increase in the number of detectable progeny phage signals a successful amplification event, which therefore indicates the presence of the target bacteria. By employing contemporary, analytical techniques, such as matrix-assisted laser desorption/ionization time of flight mass spectrometry, progeny phage have been used as secondary biomarkers for species-specific bacterial detection.\textsuperscript{21-23}

Alternatively, LFI offers a less expensive, rapid, point-of-need biosensor for phage-based bacterial detection. LFI combined with phage amplification (diagrammed in Figure 3.1) has previously been used for the rapid detection of \textit{Staphylococcus aureus} and \textit{Bacillus anthracis}.\textsuperscript{24-26} The LFI system used in these assays employed colorimetric particles for visual determination. The drawback to visual-based LFI systems lies in the difficulty in the reading of the test line at low analyte concentrations, where faint lines can be misread and lead to false negative results. Various reporter particles have been developed to address this issue and can include fluorescent, spectroscopic or contain enzymatically labeled particles.\textsuperscript{27-29}

\textbf{Figure 3.1:} Schematic of phage-based LFI device. (Figure credit: Dr. Christopher Cox)
LFI biosensors have been combined with spectroscopic techniques, such as SERS, in the past for detection of cancer antigens and influenza. Figure 1.2 shows a schematic of a SERS nanoparticle used in this study which consisted of a gold core covered with a layer of an organic, Raman-active reporter dye, encased in a thin layer of silica and surface functionalized with thiol groups to facilitate antibody attachment. The dye in the SERS nanoparticles produces a unique Raman spectrum to allow for identification and signal quantification. The use of several particle types, each producing unique Raman spectra makes possible simultaneous detection of multiple target analytes. While SERS particles at high concentrations allow visual observation of LFI test lines, at low concentrations test line visibility can be faint and unreliable. It is hypothesized that the use of SERS extends sensitivity below visual levels and provide a quantifiable signal, thus eliminating the need for visual conformation. Figure 3.2 displays a characteristic spectrum of the organic reporter molecule trans-1,2-bis(4-pyridyl)-ethylene, used in this study.

**Figure 3.2:** Raman spectrum of trans-1,2-bis(4-pyridyl)-ethylene, SERS NP S440.
In this work, we describe the development of a novel SERS-LFI device utilizing anti-A511 conjugated SERS nanoparticles. This was combined with phage amplification for rapid and specific detection of *L. monocytogenes*. A511 amplification experiments were monitored via SERS-LFI and confirmed by spot titer assay. Raman signal quantitation and overall test sensitivity is discussed. While the aim of this study was specifically for *Listeria* detection, this platform can be adapted to other bacterial pathogens for which a suitable lytic phage is available.

### 3.3 Materials and Methods

#### 3.3.1 A511 propagation

*Listeria ivanovii* ATCC 19119 was obtained from American Type Culture Collection (Manassas, VA, USA) and used for A511 propagation (provided by Martin Lossener, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland) using soft agar overlays.\(^{34}\) Briefly, 80 μL aliquots of A511 (10^8 pfu/mL) were spotted onto lawns of *L. ivanovii* mixed with soft agar (0.5% agar in Brain Heart Infusion, BHI) and incubated at 23°C for 24 hours. Resulting plaques were harvested by addition of three mL of phosphate buffered saline (PBS), pH 7.4, followed by centrifugation of the resulting slurries at 9000 × g for 15 min at 4°C. Supernatants were filter sterilized with 0.22 μm PES 1000 mL Rapid Flow Filter Units (Nalgene, Rochester, NY, USA). Polyethylene glycol phage precipitation (PEG 8000) (OmniPur, Gibbstown, NJ, USA) was conducted as previously described.\(^{35}\) Further phage purification was conducted by cesium chloride gradient ultrafiltration.\(^{35}\) Briefly, CsCl (Fisher Scientific, Fairlawn, NJ, USA) was mixed in varying ratios with TE buffer (CsCl:TE; 1:0, 2:1, 1:1, 1:2). TE buffer consisted of 10 mM Tris-HCl (Trizma® hydrochloride buffer solution) and 1 mM EDTA (Sigma Aldrich, St. Louis, MO, USA). PEG purified phages were placed on CsCl:TE gradients in ultracentrifuge tubes and centrifuged at 35,000 rpm for 24 h at 4°C, followed by
collection of phage bands. Residual CsCl was removed by dialysis in PBS. All anti-phage antibodies were prepared using this purified phage.

3.3.2. Production and purification of anti-A511 antibodies

Polyclonal rabbit anti-A511 phage IgG was prepared by Antibodies Incorporated (Davis, CA, USA). Antibodies were Protein G purified (Nab™, Thermo Scientific, Rockford, IL, USA) and specificity was confirmed by enzyme-linked immunosorbent assay (ELISA). Purified antibodies were dialyzed in PBS, concentrated by ultrafiltration (Amicon® Ultra, 30 kDa cutoff) (Millipore, Billerica, MA, USA) and filter sterilized with 0.22 μm PES filters (Thermo Scientific, Rockford, IL, USA).

3.3.3 Nanoparticle reporter and control particle preparation

Anti-phage SERS reporter particles were prepared by conjugation of 50-60 nm diameter SERS-S440 Nanotags (SERS NPs, OD 24) (Becton Dickinson, Research Triangle Park, NC, USA) with purified polyclonal anti-A511 antibodies. The conjugation method described here is a modification found in Wang, et al. A 1 mg/mL solution of the crosslinker, sulfo-succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Scientific, Rockford, IL) was prepared in degassed conjugation buffer, 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS) (Sigma Aldrich, St. Louis, MO, USA)(pH 7.2). Next, 1.17 μL of the sulfo-SMCC was reacted with 8.55 μL of purified anti-A511 antibodies (1 mg/mL) (50 molar excess sulfo-SMCC to antibody) for 30 min at 23°C. Antibody-crosslinker complexes were reacted with SERS NPs (350 molar excess antibodies to SERS NPs) by mixing 200 μL of conjugation buffer and 200 μL SERS NPs (600 pM), followed by addition of the antibody-crosslinker complex, and reacted with continuous inversion at 23°C for three hours. A 1 mg/mL solution of N-ethylmaleimide (NEM) (Thermo Scientific, Rockford, IL, USA) was prepared in degassed conjugation buffer.
and used as a blocker for unreacted, free thiols; 12.43 μL of this solution was added (650,000 molar excess NEM to SERS NPs). Concomitantly, 40 μL of Blocker™ Casein in PBS (Thermo Scientific, Rockford, IL, USA) was added to block the surface of the SERS NPs. Blocking was performed at 23°C for 2 h with continuous inversion. Unreacted sulfo-SMCC malimide groups were quenched with 40 μL of 10 mg/mL 2-mercaptoethanesulfonic acid (MESA) (MP Biomedicals, Santa Ana, CA, USA) at 23°C with continuous inversion for 45 min. Excess reagents were removed by centrifugation (1000 × g for 10 min) and the supernatant removed and replaced with 200 μL of storage buffer, 50 mM sodium borate (Fisher Scientific, Fairlawn, NJ, USA), 1% v/v gelatin (telostean gelatin from cold water fish skin, Sigma Aldrich, St. Louis, MO, USA), 0.05% w/v sodium azide (Fisher Scientific, Fairlawn, NJ, USA) (pH 7.5). This was repeated 4 times and conjugated particles were stored at 4°C. Figure 3.3 shows a schematic overview of the conjugation.

**Figure 3.3:** Schematic illustration of anti-phage antibody conjugation onto SERS NP.
Control particles were prepared by passive conjugation of blue carboxy-modified polystyrene Seradyn particles (Thermo Scientific, Waltham, MA, USA, 223 nm diameter, 2.5% solids) to ImmunoPure® biotinylated bovine serum albumin (Biotin-LC-BSA) (biotin BSA) (Thermo Scientific, Rockford, IL). Briefly, particles were centrifuged at 9000 × g for 5 min and the supernatant discarded. Particles were resuspended in 200 μL of 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (Research Organics, Cleveland, OH, USA) (pH 7.4) with 50 μL of 2 mg/mL biotin BSA added and mixed with continuous inversion for 90 min at 23°C. The resulting suspension was centrifuged at 10000 × g for 5 min, the supernatant discarded, and the pellet resuspended in HEPES. This was repeated twice and particles were resuspended in 200 μL of TNGA (0.025 M Tris base, 0.1 M NaCl, 1% v/v fish gelatin, and 0.05% w/v sodium azide) (pH 8.4) and blocked for one hour at 23°C with continuous inversion. Particles were pelleted by centrifugation (10000 × g, 5 min) with the supernatant removed and replaced with fresh TNGA. This centrifugation step was repeated twice more, then resuspended to 0.625% solids and stored at 4°C.

3.3.4. LFI device fabrication

Nitrocellulose membranes (Millipore Hi-Flow 180, Billerica, MA, USA, SHF1800425) were prepared by applying purified anti-A511 antibodies as a test line and NeutrAvidin™ Biotin-Binding Protein (Thermo Scientific, Rockford, IL) as a control line using an IVEK Digispense 2000 stripper (IVEK Corporation, North Springfield, VT). Anti-A511 (2 mg/mL) and NeutrAvidin (1.25 mg/mL) were applied at a rate of 4 μL/s and dried for 15 min at 35°C. Antibody/control line-stripped membranes were stored desiccated at 23°C. Release pads (Schleicher & Schuell, Keene, NH) were prepared by impregnation of glass fiber release medium with a solution of SERS reporter nanoparticles (0.02% solids) and control particles (0.01%...
solids) in 2 mM sodium borate, 0.1 M NaCl, 1% v/v fish gelatin, 0.05% w/v sodium azide, and 3% w/v sucrose (Baker, Phillipsburg, NJ) (pH 8.4) dried for 30 min at 35°C and stored desiccated at 23°C until assembly.

LFI devices were fabricated by mating sample pad, release pad, nitrocellulose membrane, and absorbing pad (Schleicher & Schuell, Keene, NH) to an adhesive backboard (G&L Precision Die Casting, San Jose, CA) with an overlap of approximately 2 mm between layers (Figure 3.1). Assembled LFI strips were cut to a width of 3.7 mm using a programmable sheer (Kinematic Automation Inc., Twain Harte, CA) and desiccated at 23°C until use.

3.3.5. Determination of LFI limit of detection.

LFI LOD was determined by serial dilution of filter sterilized A511 in tryptose and 1mM CaCl$_2$. Triplicate analysis of phage dilutions ranging from $1 \times 10^9$ plaque forming units (pfu)/mL to $2 \times 10^0$ pfu/mL were tested, in addition to phage-free controls. Prior to application, phage samples were mixed with running buffer at a 1:1 ratio to a volume of 100 µL and applied dropwise to the sample pad. The running buffer consisted of 0.1 M sodium borate, 3% w/v bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO), 1% v/v Tween®20 (Sigma Aldrich, St. Louis, MO) (pH 8). LFI was conducted for 30 min, wicking pads were removed to prevent backflow and LFI strips were dried in a desiccator for 10 min. Test lines were interrogated by Raman spectroscopy (Advantage 785, DeltaNu Inc., Laramie, WY) at 785 nm with a laser power of 51 mW using the NuScope attachment. The laser power setting was set on high as this produced the highest signals with lowest background. Fifty one mW was the measured laser power produced at this setting with the NuScope attachment. Twelve measurements were collected along the test line with an interrogation time of 3 sec per measurement. Longer interrogation time saturated the detector at high concentration of A511.
while shorter times produced lower intensities. The polarization setting was set to not polarized and the baseline feature was turned on to a setting of 160 to subtracted fluorescence from the spectrum. One spectrum was collected for each measurement. Whole spectra analyses of SERS samples were performed by assuming sample spectra were a linear combinations of a SERS nanoparticle reference spectrum and a nitrocellulose reference spectrum and solved using least squares (see Appendix A).\textsuperscript{28,37}

3.3.6. Phage amplification and LFI analysis

Four phage amplification experiments were performed using A511 and \textit{L. monocytogenes} ATCC 19115 at a multiplicity of infection (MOI) of 0.1 with varying A511 starting concentrations (\textbf{1} × \textbf{10}^6, \textbf{5} × \textbf{10}^5, \textbf{5} × \textbf{10}^4, and \textbf{5} × \textbf{10}^3 pfu/mL). Two additional phage amplification experiments were conducted at MOIs of 2.5 and 5 with the same starting phage concentration (\textbf{5} × \textbf{10}^5 pfu/mL). A511 starting concentration was always below the determined phage LOD to avoid false positive results caused by initial infecting phage. Overnight cultures of \textit{L. monocytogenes} were back diluted and grown to an OD\textsubscript{620} of 0.3, corresponding to \textbf{1.0} × \textbf{10}^8 colony forming units (cfu)/mL, then diluted to the appropriate starting concentration at a 10 mL final volume. A511 (\textbf{5} × \textbf{10}^7 pfu/mL) was added at appropriate volumes for the desired starting phage concentrations. Aliquots were taken from amplification reactions every hour and filtered to remove bacteria. Fifty microliters of resulting filtrates were mixed with 50 μL running buffer and applied to LFI strips as described in the previous section. Time was required to run the LFI device (~30 min), dry the strip (~10 min) and interrogate the LFI strip with the Raman spectrometer (~5 min). Phage concentration was also followed in parallel for the duration of the experiment by spot titer assay as previously described to confirm results of the SERS-LFI.\textsuperscript{38}
3.4 Results and discussion

3.4.1 SERS-LFI device optimization

Phage biology, SERS, and LFI are independent concepts previously utilized for bacterial detection. The current study combined aspects of each of these principles to overcome obstacles associated with manufacturing reliable SERS-LFI devices. Consistent, optimized antibody conjugation to SERS NPs and LFI construction were particularly important to reproducibility, thus improving these methods was a major focus of the current study. All changes to the original NP manufacturer’s conjugation protocols were intended to minimize agglomeration of conjugated NPs on the membrane. Agglomerated NPs did not consistently travel down the nitrocellulose, which created an artificially high signal with phage-free controls. Problems addressed in this paper included optimization of conjugation and running buffer composition; antibody:SERS NP ratio; blocking of non-specific phage-particle interactions; capture antibody application; membrane flow rate and SERS reporter release pad concentration. Troubleshooting these parameters lead to the development of a new, robust protocol for fabrication of SERS-LFI devices. These adjustments are summarized in Table 3.1.

<table>
<thead>
<tr>
<th>Parameter Investigated</th>
<th>Optimized Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugation Buffer</td>
<td>10 mM MOPS, pH 7.15</td>
</tr>
<tr>
<td>Running Buffer</td>
<td>0.1 M sodium borate, 3%, 1% Tween 20, pH 8</td>
</tr>
<tr>
<td>Antibody:SERS NP ratio</td>
<td>350:1</td>
</tr>
<tr>
<td>Thiol blocker</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>Capture antibody concentration</td>
<td>2 mg/mL</td>
</tr>
<tr>
<td>Membrane Flow Rate</td>
<td>180 mm/4 s</td>
</tr>
<tr>
<td>SERS reporter release pad concentration</td>
<td>0.02% solids</td>
</tr>
</tbody>
</table>

Original manufacturer instructions called for a 50 mM phosphate (pH 7.15) solution; however, it was observed that this caused an aggregation of NPs during conjugation. A 10 mM
MOPS (pH 7.15) conjugation buffer was observed to minimize agglomeration. Precise antibody:SERS NP molar ratios were also critical for preventing agglomeration. High antibody:NP molar ratios (above 350:1) increased phage-free control signal, and even higher ratios (above 700:1) gave rise to faint visual false positives. Molar ratios below 350:1 gave less sensitivity but minimized false positives with phage-free controls. The blocking of free reactive thiols on NPs with NEM further prevented agglomeration and particle precipitation during conjugation.

Once particle conjugation was optimized, maximal LFI running conditions were investigated. First, the running buffer composition was examined. The running buffer solubilized both reporter and control particles in the release pad and served as a mobile phase to carry particles down the membrane. A 50 mM HEPES, 0.05% Tween (pH 8) solution was initially used; however, it did not adequately transport the NPs through the LFI device to the absorbing pad, resulting in heterogeneous distribution of particles throughout the membrane. The running buffer used in Noble et al., which consisted of 0.1 M sodium borate, 3% w/v BSA, 1% v/v Tween 20, pH 8, was examined with phage-free controls and observed to give the least non-specific binding. Particles were also observed to completely transit the membranes to the wicking pads.

Other factors that were investigated included LFI capture antibody concentration, membrane flow rate and NP release pad concentration. Capture antibody application concentration was optimal at 2 mg/mL. Concentrations higher than 2 mg/mL resulted in lower overall device sensitivity. A manufacturer-determined, membrane-limited capillary flow rate of 180 s/4 mm was observed as optimal, while rates of 120 or 90 s/4 mm decreased sensitivity. SERS NP release pad concentration also affected Raman sensitivity. A final concentration of
0.02% solids SERS NPs was determined to be optimal. Higher particle concentrations caused an increased in phage-free control false positive signal, while lower concentrations decreased sensitivity.

3.4.2 Determination of LFI limit of detection

Both visual and spectroscopic limits of detection were determined for prototype SERS-LFI. Visual positives were determined by formation of a pink test line. As shown in Figure 3.5A and B, phage concentrations of $1 \times 10^9$ pfu/mL and $2 \times 10^8$ pfu/mL produced clearly visible pink lines. The minimum concentration for which a visually observable colored line formed was $6 \times 10^7$ pfu/mL (Figure 3.4C). It should be noted that this concentration produced a very weak response, which could be mistaken as a negative result. The possibility of such outcomes emphasizes the difficulty associated with qualitative visual analysis and interpretation by the untrained user.

![Figure 3.4](image)

**Figure 3.4:** Visual LOD was determined by the appearance of a pink line at the test line. The column on the left represents the phage concentration sampled, while phage number is indicated in the parenthesis.
Raman signals measured from the same serial dilutions of A511 are shown in Figure 3.5. These intensities represent the interquartile range (IQR) of 12 random shots along a single test line, shown by the vertical error bars. The IQR was necessary, because heterogeneous membrane pore distribution was observed to lead to areas of higher and lower Raman intensity along the length of the test line. Horizontal error bars in Figure 3.5 represent standard deviation of phage titers done in triplicate. The lowest phage dilution to produce a Raman signal above phage-fee controls was $6 \times 10^6$ pfu/mL. Thus, this phage dilution, represented by a dotted line in Figures 3.6B and 3.7B, and the resulting Raman signal (intensity of 1.37), represented by a dashed line in Figures 3.6A and 3.7A, were accepted as the phage and SERS LOD, respectively. A comparison between SERS and visual limits of detection ($6 \times 10^6$ pfu/mL and $6 \times 10^7$ pfu/mL, respectively) demonstrated an order of magnitude greater sensitivity for SERS. All IQR measurements below SERS LOD represent instrument noise.

Figure 3.5: Phage concentration vs. Raman intensity for dilution of phage. The SERS LOD is indicated by a dashed line.
3.4.3 Phage amplification and SERS-LFI analysis

Four phage amplification experiments at a MOI of 0.1 were monitored by SERS-LFI (Figure 3.6A, Table 3.2) and parallel spot titer assay (Figure 3.6B). The purpose of this experiment was to investigate how decreasing phage and bacterial concentrations affected the time it took to get a positive result. Phage concentrations were varied from $1 \times 10^6$ pfu/mL to $5 \times 10^3$ pfu/mL, and corresponding bacterial concentrations were varied from $1 \times 10^7$ cfu/mL to $5 \times 10^4$ cfu/mL. SERS detection of the highest phage concentrations ($1 \times 10^6$ pfu/mL and $5 \times 10^5$ pfu/mL) was achieved in two hours. Additional time was required before decreased phage concentrations reached detectable levels (6 h for $5 \times 10^4$ pfu/mL and 8 h for $5 \times 10^3$ pfu/mL). Parallel spot titer assays confirmed that all positive tests represented phage concentrations greater than the established LOD and that increased SERS intensities correlated with phage amplification.

Figure 3.6: Four phage amplification experiments carried out at MOI 0.1 and decreasing concentrations of phage and bacteria to investigate the time needed for a positive detection, monitored by (A) SERS-LFI and (B) parallel spot titer assay. SERS and phage LODs are represented by dashed and dotted lines, respectively.
Figure 3.7: Phage amplifications carried out at 3 different MOIs utilizing a constant starting phage concentration of $5 \times 10^5$ pfu/mL. SERS and phage LODs are represented by a dashed and dotted lines respectively.

Table 3.2: Summary of SERS-LFI coupled with phage amplification detection times.

<table>
<thead>
<tr>
<th>Phage Amplification Series 1</th>
<th>Phage Concentration (pfu/mL)</th>
<th>Bacterial Concentration (cfu/mL)</th>
<th>MOI</th>
<th>SERS-LFI Detection Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$1 \times 10^6$</td>
<td>$1 \times 10^7$</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^5$</td>
<td>$5 \times 10^6$</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$</td>
<td>$5 \times 10^5$</td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^3$</td>
<td>$5 \times 10^4$</td>
<td>0.1</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phage Amplification Series 2</th>
<th>Phage Concentration (pfu/mL)</th>
<th>Bacterial Concentration (cfu/mL)</th>
<th>MOI</th>
<th>SERS-LFI Detection Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$5 \times 10^5$</td>
<td>$1 \times 10^6$</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^5$</td>
<td>$2 \times 10^5$</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^5$</td>
<td>$1 \times 10^5$</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

A second series of amplifications was conducted to investigate the relationship between MOI and detection time (Figure 3.7, Table 3.2). As previously discussed, an MOI of 0.1 resulted in detection in two hours. Detection times increased with increasing MOIs. MOIs of 2.5 and 5 surpassed detection limits at 4 h and 5 h, respectively. Parallel spot titer assays are shown in Figure 3.7B. Error bars in Figures 3.6A and 3.7A correspond to the IQR of 12 random shots along the test line of a single LFI strip, while error bars in Figures 3.6B and 3.7B represent
standard deviation of phage titers measured in triplicate. Table 3.2 summarizes Figures 3.6 and 3.7.

The results displayed in Figures 3.6 and 3.7 highlight a drawback of phage amplification assays. Low concentrations of phage and bacteria decrease the chance of a phage and a bacterium meeting, irrespective of the binding efficiency of the phage to the bacterium. This increases the time necessary for the phage concentrations to reach the detection limit of the detector device. Hagens and Loessner have previously discussed this phenomenon.

In conclusion, this is the first report of phage amplification combined with the use of SERS and LFI for *Listeria* detection. This study focused on establishing a robust anti-phage conjugation protocol and on optimization of LFI construction, with the goal of minimizing nanoparticle agglomeration and improving reproducibility. The resulting devices are capable of detecting progeny A511 at concentrations as low as $6 \times 10^6$ pfu/mL. The shortest detection time for *L. monocytogenes* was two hours, while, in a separate experiment, detection of bacteria at a concentration of $1 \times 10^4$ cfu/mL was achieved in 8 h. While an enrichment step is still needed to obtain bacterial concentrations that allow for propagation of progeny phage to detectable levels, phage amplification eliminates the need for downstream plating on selective media, and for further biochemical or molecular tests (reducing detection by 24–48 h), while providing evidence of viable cells. SERS-LFI allows for positive identification in as little as 30 min, while traditional plaque assays can take up to 24 h.

### 3.5 Acknowledgments

We would like to thank Martin Loessner, Jochen Klumpp, Roy Mondesire, Patrick McVey and William Navidi for their input in the study. This work was funded by Colorado
Office of Economic Development and International Trade’s Bioscience Discovery Evaluation Grant Program.

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CHAPTER 4

DETECTION OF *LISTERIA MONOCYTOGENES* IN INOCULATED FOOD BY BACTERIOPHAGE AMPLIFICATION AND SURFACE-ENHANCED RAMAN SPECTROSCOPY COUPLED TO LATERAL FLOW IMMUNOCHROMATOGRAPHY

A paper submitted to *Letter in Applied Micobiology*

Nicholas R. Stambach\textsuperscript{1,2}, Christopher R. Cox\textsuperscript{3}, Kent J. Voorhees\textsuperscript{4}

4.1 Significance and impact of the study

The current gold-standard for detection of the deadly pathogen *Listeria monocytogenes* in food is a series of culture-based techniques requiring 4-6 days to produce results. Rapid diagnostic techniques that can reduce detection time can prevent widespread outbreak of *L. monocytogenes* and reduce the number of illnesses. To this end, a bacteriophage (phage) amplification (PA) method was previously developed in which progeny phages were detected by lateral flow immunochromatography (LFI) coupled with surface-enhanced Raman spectroscopy (SERS) reporters. In the present study, we report the application of this PA SERS LFI system for detection of *L. monocytogenes* directly in various food matrices. This effectively eliminated the need for downstream culture and confirmatory testing, with the result of reducing detection time by days and extending device sensitivity with quantifiable SERS signal detection at concentrations below those observable by visual LFI alone.

4.2 Abstract

Current culture-based strategies for detection of the food-borne pathogen *Listeria monocytogenes* in contaminated ready-to-eat products involve selective enrichment followed by

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4. Emeritus Professor, Department of Chemistry, Colorado School of Mines
a series confirmatory test on resulting colonies, a process that can take 4-6 days. Because of the long turnaround time (TRT) of these methods, it is possible for contaminated foods to be distributed before test results are available, which could increase the likelihood of a widespread outbreak. Therefore, there is a pressing need for more rapid diagnostics against this organism. To this end, a phage amplification (PA) method was applied at the initial enrichment step to dramatically reduce detection time by 2-4 days. Progeny phages produced during infection of \textit{L. monocytogenes} with phage A511 were directly detected using surface-enhanced Raman spectroscopy lateral flow immunochromatography (SERS LFI). The use of SERS reporters increased assay sensitivity by adding quantification signal detection at bacterial concentrations below those detectable by visual LFI. PA SERS LFI was evaluated for its application as a rapid diagnostic for determining the presence of \textit{L. monocytogenes} in inoculated cantaloupe, ice cream, queso fresco, and Camembert cheese. These food products were chosen specifically because of their recent involvement in listeriosis outbreaks in the United States. Detection of approximately 1 cfu/g by PA SERS LFI was attained after 24 h of enrichment, three days faster than conventional enrichment and selective chromogenic plating. Additionally, the use of PA provided live/dead differentiation and specificity, eliminating the need for colony formation and downstream biochemical or molecular confirmation tests.

4.3 Introduction

\textit{Listeria monocytogenes} is an important pathogen in the food industry and the etiological agent of listeriosis. Illness can result from ingestion of as few as 10 organisms, and as few as a single bacterium in individuals with increased risk of infection.\textsuperscript{1} Disease symptoms include gastroenteritis, diarrhea, meningitis, and bacteremia. In the most severe cases, \textit{L. monocytogenes} can cause spontaneous abortion and death.\textsuperscript{2} \textit{L. monocytogenes} is of particular concern due to its
ability to survive and grow on food at refrigerated conditions (4°C), high salinity, and low pH.³ Cases of listeriosis are relatively rare, however, the disease has high hospitalization (94%) and mortality rates (20-30%).⁴⁻⁵

The current gold standard for *L. monocytogenes* detection involves homogenizing food samples, selective enrichment followed by characterization by colony morphology, carbohydrate fermentation, and isolate hemolytic properties.⁶ These culture-based *L. monocytogenes* detection methods take 4-6 days, often resulting in distribution of *Listeria*-contaminated foods before testing is completed.⁷⁻⁸ Due to the length of this turnaround time (TRT), recent outbreaks have occurred in the U.S. on a wide range of produce and ready-to-eat food products. In an effort to reduce TRT, many commercially available tests have been reported by numerous manufacturers.⁹ Many facilities and third party labs have employed some of these tests with varying levels of success. Rapid test kits, some requiring 24-76 hours, include DNA amplification and hybridization methods and immunological assays. These tests have been validated by independent organizations, such as AOAC, AFNOR, MicroVal, NordVal, and are listed by the Food Safety and Inspection Service.⁹⁻¹⁰ These tests potentially allow for producers of foods at risk of *L. monocytogenes* contamination to recall products before they change ownership or are otherwise distributed for sale to the general public. Furthermore, retail testing by state and federal agencies has become increasingly reliant on genomic testing, with PCR-based detection providing faster analysis time compared to culture-based strategies. However, PCR-based assays are not without drawbacks. Indeed, DNA amplification-based tests are unable to differentiate between living and dead cells. This has the potential to lead to false positive results. Further, PCR can be cost prohibitive for routine high throughput use. More importantly,
contaminants from food matrices have been observed to inhibit PCR resulting in false
negatives.\textsuperscript{11-12}

The 2011 Jensen Farms outbreak was associated with contaminated cantaloupes and
resulted in 147 infections across 28 states leading to 33 deaths and one miscarriage, making it the
second deadliest food-borne outbreak in U.S. history.\textsuperscript{13} An outbreak associated with ice cream
in 2015 led to 10 hospitalizations in 4 states resulting in 3 deaths.\textsuperscript{14} In addition, \textit{L. monocytogenes}
contamination of soft cheeses was also responsible for a 2015 outbreak that
resulted in 28 hospitalizations, 3 deaths, and one miscarriage.\textsuperscript{15}

The recent increase of \textit{Listeria} outbreaks highlights the need for improved quality control
and more rapid diagnostic techniques for user-friendly, field deployable identification of
foodborne pathogen-contaminated foods before they reach consumers. Bacteriophage (phage)
amplification (PA)-based detection systems provide a potential solution to this problem due to
their ability to specifically infect target bacteria and produce many detectable progeny phages in
a short time. Detection of progeny phage at concentrations above those used to initiate infection
indicate the presence of the viable host bacteria, which can be observed by various contemporary
analytical techniques such as real-time PCR, matrix-assisted laser desorption/ionization time-of-flight
mass spectrometry, and lateral flow immunochromatography (LFI).\textsuperscript{16-18} Additionally, the
use of phage amplification offers an advantage over PCR-based detection methods due to its
capability for differentiating between living and dead cells. This is of particular interest in the
food processing industry, as many foods are treated to kill bacteria that would still be detected by
PCR-based approaches, resulting in false positive results.\textsuperscript{19}

We recently reported a novel LFI test for rapid detection of viable \textit{Listeria} utilizing PA
with \textit{Listeria} phage A511.\textsuperscript{20} This test employed surface-enhanced Raman spectroscopy (SERS)
nanoparticles conjugated with anti-phage A511 specific antibodies as quantifiable reporters. The following study extends the application of PA SERS LFI to demonstrate its utility for detection of *L. monocytogenes* in inoculated cantaloupe, ice cream, and soft cheeses. PA SERS LFI was applied at the enrichment step, eliminating the need for downstream selective plating and colony characterization.

### 4.4 Results and discussion

Lytic phage A511 was selected for this study because of its broad host range (infecting 95% of *L. monocytogenes* strains of serovars 1/2 and 4), sufficient burst size (40-50 viruses) for an LFI approach, and short latent time (55-60 min). Importantly, this phage has previously been successfully used in several other unrelated *Listeria* detection schemes. A511 phage amplification was utilized in the development of SERS LFI with a PA detection limit of $1 \times 10^5$ cfu/mL of *L. monocytogenes*. In this application, A511 provided a useful tool for rapid detection of *L. monocytogenes* in view of government zero tolerance regulatory policies, such as those of the U.S. FDA, U.S. Department of Agriculture and Food Standards Australia and New Zealand. From a food hygiene perspective, these regulatory agencies do not differentiate between *Listeria* species because the presence of any *Listeria* species constitutes conditions conducive to contamination by *L. monocytogenes*. An additional species-specific differentiation step can be achieved by performing PCR or mass spectrometric analysis on positive PA SERS LFI samples to confirm *L. monocytogenes*. For example, PCR targeting the virulence gene *hly*, which encodes for the hemolysin listeriolysin O, has been used to distinguish *L. monocytogenes* from other *Listeria* species. Additionally, *L. monocytogenes* has been identified to species level with the Bruker Biotyper MALDI MS system. Because of the zero tolerance policy for *L. monocytogenes*, an enrichment step was required using buffered *Listeria* media.
enrichment broth (BLEB) in order to increase low initial bacterial numbers to detectable concentrations.

Cantaloupe, ice cream, queso fresco, and Camembert cheese were selected because of their involvement in recent listeriosis outbreaks. Table 4.1 shows triplicate PA SERS LFI detection results for various inoculation concentrations of *L. monocytogenes* in these foods. Values above the previously established SERS LOD of 1.37 arbitrary units (AU) were considered positive for *L. monocytogenes*. Visual positives were determined by the formation of an observable colored test line. Additionally, Table 4.1 provides *L. monocytogenes* colony counts taken at 48 h of enrichment. Colonies were confirmed as *Listeria* by plating on chromogenic agar. Plates were incubated for 48 h because halos around some indicator colonies were not always obvious at 24 h. Results were consistent with previous reports.

### 4.4.1 Detection of *L. monocytogenes* in cantaloupe.

As shown in Table 4.1, PA SERS LFI signals for a given starting inoculum of *L. monocytogenes* were dependent on the food matrix tested. All food samples inoculated with *L. monocytogenes* produced positive SERS detection at 24 and 48 h enrichment, while negative controls were not detected by SERS analysis or confirmatory selective culture. At 24 h enrichment, cantaloupes inoculated with ~10 and ~100 cfu/g produced visible positives, while approximately one cfu/g spiked samples were observed as positive only by SERS analysis. Interestingly, cantaloupes spiked with approximately one cfu/g produced visual positives at 48 h of enrichment, while ~10 and ~100 cfu/g samples were negative by visual determination. This is likely because lower initial inoculation concentrations took longer to reach concentrations above the threshold of detection in the exponential growth phase, and thus resulted in stronger signal at 48 h as opposed to 24 h. In contrast, experiments performed at higher starting concentrations
Table 4.1: Detection of *L. monocytogenes* in various inoculated foods by PA SERS LFI

<table>
<thead>
<tr>
<th>Food sample</th>
<th>Approximate initial contamination (cfu/g)</th>
<th>SERS signal(a)</th>
<th><em>L. monocytogenes</em> enumeration at 48 h (log cfu/g)(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td>Cantaloupe</td>
<td>0</td>
<td>1.06±0.16 (---)</td>
<td>0.87±0.36 (---)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.54±0.10 (+++)</td>
<td>1.87±0.13 (+++)***</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.95±0.15 (+++)***</td>
<td>1.64±0.11 (++)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.31±0.25 (+++)***</td>
<td>1.59±0.14 (++)</td>
</tr>
<tr>
<td>Ice cream</td>
<td>0</td>
<td>1.04±0.21 (---)</td>
<td>1.07±0.09 (---)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.58±0.10 (+++)</td>
<td>2.01±0.10 (+++)***</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.72±0.27 (+++)***</td>
<td>2.00±0.13 (++)***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.25±0.20 (+++)***</td>
<td>2.07±0.11 (++)***</td>
</tr>
<tr>
<td>Queso fresco</td>
<td>0</td>
<td>1.04±0.17 (---)</td>
<td>1.02±0.14 (---)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.49±0.07 (+++)</td>
<td>1.49±0.10 (++)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.68±0.17 (++)**</td>
<td>1.67±0.09 (++)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.84±0.26 (++)**</td>
<td>1.68±0.25 (++)*</td>
</tr>
<tr>
<td>Camembert</td>
<td>0</td>
<td>1.07±0.22 (---)</td>
<td>1.03±0.18 (---)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.48±0.08 (+++)</td>
<td>1.68±0.17 (++)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.55±0.11 (++)</td>
<td>1.76±0.27 (++)*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.75±0.32 (++)*</td>
<td>2.04±0.19 (++)**</td>
</tr>
</tbody>
</table>

\(-\) Indicates number SERS-negative samples.  (\+) Indicates number of SERS-positive samples.  *Indicates number of visual positive results.  \(a\)SERS signals above 1.37 indicated positive results.  \(b\)*Listeria* detection suggest that a culture starting at \(~100\) cfu/g is well past the window for optimal phage infection.  This results in

began to enter the stationary or death phase at around 48 h, resulting in decreased signals. For

\(~100\) cfu/g samples, *Listeria* colony counts at 24 h were 7.45±0.09 log cfu/mL.

Colony counts of 9.45±0.10 log cfu/mL were observed for \(~100\) cfu/mL samples at 48 h.

Previous reports detailing the use of phage A511 for *Listeria* detection suggest that a culture starting at \(~100\) cfu/g is well past the window for optimal phage infection. This results in
reduced concentrations of detectable progeny phage with these conditions.\textsuperscript{23, 26, 32} Further, PA-SERS-LFI was shown to be highly reproducible for detection of \textit{L. monocytogenes} as none of three replicates produced visible LFI results at 48 h while positive SERS results were consistently observed with a coefficient of variance of 8.8%.

\textbf{4.4.2 Detection of \textit{L. monocytogenes} in ice cream.}

One \textasciitilde 10 cfu/g ice cream sample produced visual positive results at 24 h enrichment. All \textasciitilde 100 cfu/g ice cream samples were positive by visual analysis at 24 h enrichment. At 48 h enrichment, all ice cream samples produced positive visible LFI results. A representative set of strips for detection of \textit{L. monocytogenes} in inoculated ice cream are shown in Figure 4.1. Visual positives are represented by + signs. The faint lines in Figure 4.1 highlight the limitation of visual determination and the potential of this approach on its own to result in false negative interpretations. The use of SERS reporters eliminated this concern by providing a quantifiable Raman signal at the LFI test line.

\textbf{4.4.3 Detection of \textit{L. monocytogenes} in cheese.}

No single cfu/g queso fresco samples were visually positive at 24 or 48 h. Only one \textasciitilde 10 cfu/g queso fresco sample exhibited a visual positive after 24 h enrichment while \textasciitilde 100 cfu/g samples showed two visual positives at 24 h and one at 48 h. Camembert samples inoculated with \textasciitilde 1 cfu/g showed similar results to queso fresco with none resulting in visual positives. Only one \textasciitilde 10 cfu/g sample was visually positive at 48 h while \textasciitilde 100 cfu/g displayed one visual positive at 24 h and two at 48 h. Recovery of \textit{L. monocytogenes} in Camembert was poor relative to the other foods tested. This is consistent with previously reported results, where the authors found that naturally occurring bacteria in Camembert proved refractory to efficient recovery of \textit{L. monocytogenes}.\textsuperscript{33}
**Figure 4.1:** Representative PA SERS LFI results for ice cream after enrichment for A) 24 h and B) 48 h. Initial concentrations per gram of food were as follows: 0 cfu/g (A1, B1), ~1 cfu/g (A2, B2), ~10 cfu/g (A3, B3), and ~100 cfu/g (A4, B4). + indicates visual positive result.

The overall coefficient of variance observed for all SERS-positive samples in the present study was 9.0%. The coefficient of variance for negative controls was below the established LOD and matched that of inherent instrument noise. The coefficient of variance for cantaloupe was 7.9%, whereas ice cream was 8.0%, and queso fresco and Camembert were 9.3% and 10.9%, respectively.

**4.5 Conclusion.**

The results reported here illustrate the strong reproducibility of PA SERS LFI for *L. monocytogenes* detection at low concentrations in a range inoculated food samples. The specific foods evaluated have well-documented involvement in widespread listeriosis. The protracted TRT of most if not all commercially available *L. monocytogenes* detection technologies is a significant cause for concern as contaminated products continue to enter the marketplace causing
repeated outbreaks and preventable mortality. Therefore, a PA SERS LFI detection system was developed with the aim of reducing detection time in comparison to current culture-based approaches. Application of PA SERS LFI at the enrichment phase used by most, if not all, other approaches allowed for detection of *L. monocytogenes* at 24 h, compared to 4-6 days required by other strategies. *L. monocytogenes* was detected by PA SERS LFI in cantaloupe, ice cream, and two soft cheeses at 24 h enrichment at starting concentrations as low as ~1 cfu/g. The ability to detect low concentrations is critical as low levels (10^2-10^4 cfu/g) of contamination have caused listeriosis in immunocompromised individuals. This substantial reduction in time was accomplished by use of A511 phage amplification. A511 specifically infected and multiplied in *L. monocytogenes*. Progeny phages were detected by SERS LFI as an indicator of the presence of viable *L. monocytogenes*. The use of A511 amplification eliminated the need for further incubation of samples on selective media for colony formation and subsequent confirmatory testing. In this fashion, PA SERS LFI afforded a user-friendly, inexpensive and quantitative detection method in a field deployable form factor amenable to onsite point-of-need testing. While further evaluation of PA SERS LFI for *L. monocytogenes* detection in other sample types is needed (surface swabs taken from food processing equipment as one possible example) and investigation of lower contamination concentrations, the results discussed here are promising. We clearly demonstrate proof-of-principle for tractable *L. monocytogenes* detection in complex food matrices with known involvement in recent listeriosis outbreaks and eliminate the reliance on a less sensitive qualitative visual read out.
4.6 Materials and methods

4.6.1 Bacteriophage preparation

Phage A511 was provided by Dr. Martin Loessner (Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland). A511 cultures were prepared by soft agar overlay as previously described. Briefly, lawns of *L. ivanovii* ATCC 19119 were prepared by mixing 200 µL overnight culture with 3 mL of soft agar (0.5% agar in brain heart infusion, BHI). Plates were spotted with 80 µL of stock A511 (10^8 pfu/mL) and incubated for 24 h at 23°C. Phages were recovered by harvesting plaques with 3 mL of phosphate buffered saline, followed by centrifugation at 9000 × g for 15 min and filtration through a 0.22 µm filter.

4.6.2 *L. monocytogenes* culture

Frozen stocks of *L. monocytogenes* ATCC 19115 was streak onto BHI agar plates and incubated at 37°C for 24 h. After incubation, plates were sealed with parafilm and stored at 4°C. Overnight cultures were prepared by removal of a well-separated colony and transferred to tryptose broth supplemented with 1 mM CaCl₂ and incubated overnight at 30°C. For use in inoculated foods, overnight cultures were back diluted in a 1:4 ratio into fresh tryptose broth with 1 mM CaCl₂ and grown to an OD of 0.3 (1 × 10^8 CFU mL⁻¹) and serially diluted to appropriate starting concentrations.

4.6.3 Inoculation of food samples with *L. monocytogenes*

Cantaloupe, ice cream, queso fresco, and Camembert cheese were purchased from local retailers. Each food sample was homogenized in a surface-decontaminated blender, aliquoted into 25±1 g portions and stored at -20°C until needed. Each food type was thawed to 4°C and tested for the presence of *L. monocytogenes* (i.e. by natural contamination) using the FDA Bacteriological Analytical Manual (BAM) enrichment protocol, as previously described. After
48 h incubation, samples were removed and cultured on Brilliance™ Listeria agar base (Oxoid CM1080) containing Listeria selective supplement (Oxoid SR0227) and chromogenic Listeria differential supplement (Oxoid SR0228). Plates that were incubated for 48 h and samples that did not produce blue colonies with opaque halos were considered negative for L. monocytogenes and used in trials of inoculated food. Samples inoculation was conducted by addition of 1 mL dilutions of log-phase L. monocytogenes ATCC 19115 to thawed (4°C) food samples (25±1 g) to obtain initial concentrations as described.\textsuperscript{35} L. monocytogenes-free negative controls consisted of identical food samples inoculated with 1 mL of sterile tryptose broth containing 1 mM CaCl\textsubscript{2}.

4.6.4 Bacterial pre-enrichment

Previous work showed that a prior selective enrichment step was needed to achieve detectable concentrations of L. monocytogenes by PA SERS LFI.\textsuperscript{20} For this purpose, buffered enrichment Listeria broth (BLEB, Becton Dickinson 290720), detailed in the FDA BAM protocol, was utilized as described.\textsuperscript{7} Briefly, contaminated homogenates were mixed with 225 mL of BLEB and incubated at 30ºC for 4 h without addition of antimicrobials. At 4 h, selective agents were added as follows: 0.455 mL of 0.5% w/v acriflavine HCL (Sigma Aldrich A8251), 1.8 mL of 0.5% w/v nalidixic acid (Sigma Aldrich N4382), and 1.15 mL of 1% w/v cycloheximide (Sigma Aldrich O1810) in 40% v/v water/ethanol solution.

4.6.5 PA SERS LFI detection of L. monocytogenes in inoculated food matrices

SERS LFI strips were constructed as previously described.\textsuperscript{20} Figure 4.2 illustrates the workflow for enriched samples prior to PA SERS LFI analysis. After 24 or 48 h enrichment, 2 mL samples were used to inoculate 8 mL tryptose broth (Becton Dickinson 211713) containing 1 mM CaCl\textsubscript{2}. Following a 2 h incubation, phage A511 was added to a final concentration of 5 x 10\textsuperscript{5} pfu/mL and incubated a further 5 h. Two mL were removed from each sample and filtered.
through 0.22 µm filters to remove food debris and residual bacteria. Fifty microliter aliquots were mixed with an equal volume of running buffer (0.1 M sodium borate, 3% BSA, 1% Tween 20, pH 8) and pipetted onto A511-specific SERS LFI strips. Each PA aliquot was allowed to flow for 15 min and absorbent pads were removed to prevent backflow. Strips were dried in a vacuum desiccator for 10 min, followed by Raman spectroscopic analysis of test lines at 785 nm with a laser power of 51 mW. Twelve random SERS spectra per LFI device were acquired and processed in MATLAB (The MathWorks, Inc., Natick, Massachusetts, United States) using a linear combination least squares method. Each inoculation was performed in triplicate with the means and standard deviations presented in Table 4.1.

![Figure 4.2: Representative PA SERS LFI workflow for detection of *L. monocytogenes* in food samples.](image-url)
4.6.6 Bacterial enumeration

To confirm the presence of viable *L. monocytogenes* in samples analyzed by PA SERS LFI, triplicate aliquots were removed from the individual enrichment mixtures at 48 h, serially diluted, and quantified on chromogenic differential selective agar plates as described. Blue colonies with opaque haloes were counted after a 48 h incubation, with mean and standard deviations calculated (Table 4.1).

4.7 Acknowledgments

This work was funded by Colorado Office of Economic Development and International Trade’s Bioscience Discovery Evaluation Grant Program.

4.8 Conflicts of interest

The authors declare that there are no conflicts of interest.

4.9 References


http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm (accessed February 9).


5.1 Abstract

The work described discusses the development of a bacteriophage (phage) amplification (PA) based *Yersinia pestis* detection system. PA offers rapid, specific pathogen detection along with providing live/dead determination, providing an advantage over PCR-based detection methods. *Y. pestis* typing phage ϕA1122 was selected for this study because of its broad host range, large burst size and short latent period. Progeny phage were detected by surface-enhanced Raman spectroscopy (SERS) lateral flow immunochromatography (LFI) utilizing SERS nanoparticle reporters conjugated with anti-ϕA1122 antibodies. In the presence of progeny ϕA1122, reporter-phage complexes were arrested at the LFI test line, which was quantitatively analyzed by Raman spectroscopy. A visual limit of detection (LOD) of $6.7 \times 10^7$ pfu/mL was determined based on the concentration of colored reporter-phage complexes at the test line. The SERS LOD was found to be $7.7 \times 10^6$ pfu/mL. PA experiments were conducted with input bacterial concentrations of $5 \times 10^6$, $5 \times 10^5$ and $5 \times 10^4$ cfu/mL, all providing positive *Y. pestis* detection in a range of one to three hours, respectively.
5.2 Introduction

_Yersinia pestis_ is a nonmotile, Gram-negative, non-spore-forming coccobacillus and the etiological agent of plague.\(^1\) Plague is a zoonotic disease primarily affecting rodents that is transferred animal to animal through flea bites. Humans can become infected by the flea vector, exposure to open wounds when handling infected animals, or through inhalation. The disease presents in three primary forms: bubonic, septicemic, and pneumonic. Bubonic plague is the most common with transmission of _Y. pestis_ from flea bites followed by dissemination of bacteria to the nearest lymph node. This form is characterized by the appearance of swollen lymph nodes (buboes) resulting from infection. Primary septicemic plague arises from blood infections without formation of buboes. In contrast to bubonic and septicemic forms, pneumonic plague is spread through aerosolized respiratory droplets from individual to individual. Untreated bubonic plague has a mortality rate of 50-90%, while, if left untreated, the septicemic and pneumonic forms are fatal in most cases.\(^2\)

Because of its acute virulence, _Y. pestis_ has been utilized as a biological weapon, dating as far back as 1346, when the Tatar army catapulted plague-infected corpses at Genoese sailors.\(^3\) During World War II, the Japanese released fleas infected with plague from planes against the Chinese. These methods promoted the spread of bubonic plague, however, the use of aerosolized _Y. pestis_ as biological weapon is now a current concern due to the potential for pneumonic plague outbreaks in exposed populations accompanied by high fatality rates and risk of rapid person to person spread.\(^4\) _Y. pestis_ has been classified as a Category A pathogen and Tier 1 select agent by the Centers for Disease Control and Prevention (CDC) due to its potential for use in bioterrorism and because most untreated pneumonic plague cases are fatal. Currently, bacterial culturing is required for biochemical confirmation, a process which can take up to 5
days.\textsuperscript{5} Clearly, there exists a pressing need for rapid, field deployable, diagnostic detection of \textit{Y. pestis} in order to implement swift public health action in the event of an attack.

Many rapid plague identification techniques focus on the detection of surface proteins, specifically the fraction 1 capsular antigen (F1) and the plasminogen activator protein (Pla). F1-based detection methods were evaluated by Tomaso \textit{et al.} for detection limits and procedure times and found the lowest LOD to be $3 \times 10^3$ colony forming units per milliliter (cfu/mL) detectable in 20 min using an in-house immunochromatographic assay.\textsuperscript{6} For this assay and the ones that follow, bacterial LODs were determined by growth of overnight culture of \textit{Y. pestis} to high concentrations ($10^9$ cfu/mL), followed by serial dilution. Another immunochromatographic assay with improved analysis time of less than 10 min had an LOD of $10^5$ cfu/mL.\textsuperscript{7} Immunochromatographic assays are advantageous due to their potential for use in the field. The major drawback to F1-based assays, however, is that some virulent \textit{Y. pestis} strains do not express the F1 antigen.\textsuperscript{8} Furthermore, expression of F1 is temperature regulated (>$33^\circ$C) and is not expressed in flea-vectors.\textsuperscript{8-9} As an alternative to F1-based strategies, the surface protein Pla has been investigated. Pla-based ELISA and immunochromatographic assay LODs have been reported as $2 \times 10^4$ and $10^7$ cfu/mL, respectively.\textsuperscript{10} However, the ELISA method required 4 h to perform while the immunochromatographic assay was completed in 25 min. Additionally, ELISA is not field deployable, requiring the use of ancillary equipment, such as a centrifuge and plate reader. In a different approach, a mass spectrometric (MS) assay was investigated that first involved isolating \textit{Y. pestis} cells using anti-PLA antibodies linked to magnetic particles followed by mass analysis.\textsuperscript{11} After separation of \textit{Y. pestis} from the sample, cells were lysed and the proteins enzymatically cleaved followed by analysis of the peptides by liquid chromatography-MS using selected reaction monitoring. \textit{Y. pestis} was detected as low as $2 \times 10^4$ cfu/mL.
DNA amplification strategies (real-time polymerase chain reaction and isothermal amplification) also offer potential for rapid detection of *Y. pestis*. Several real-time PCR methods have been developed targeting both plasmid and chromosomal sequences. Loïez *et al.* selected the *pla* gene, located on *Y. pestis*-specific pPCP1 plasmid, because of its high copy number (186 per bacterium) as a marker for *Y. pestis* detection. This assay had a reported LOD of $10^2$ cfu/mL in the absence of PCR inhibitors in less than 5 h. However, it was recently discovered that the *pla* gene is not specific to *Y. pestis*. This highlights the pitfalls of selecting solely plasmid genes for PCR assays. Additionally, targeting genes on virulence plasmids can be problematic for atypical strains that can be altogether devoid of these plasmids. A potential solution for this problem is selecting chromosomal genes for PCR-based assays, though, care must be taken in selecting a chromosomal target that differentiates *Y. pestis* from the closely-related species, *Y. pseudotuberculosis*. To this end, Chase *et al.* developed a real-time PCR assay targeting the *yp48* gene located on the *Y. pestis* chromosome. This gene was determined to be a homologue to the *Escherichia coli* gene for MalK, an ATP-binding component of the maltose transport system. Real-time PCR differentiated *Y. pestis* from *Y. pseudotuberculosis* due to a 25-bp sequence that is present in *Y. pseudotuberculosis* but absent in *Y. pestis*. Similarly, *Y. pestis* and *Y. pseudotuberculosis* differentiation was also achieved by a quadruplex real-time PCR assay. This assay targeted *Y. pestis* genes on the three virulence plasmids (*lcrV* on pCD1, *caf1* on pMT1 and *pla* on pPCP1) and a fourth target on the *Y. pestis* chromosome (*yihN*). The approach allowed for identification of *Y. pestis* based on a unique chromosomal sequence and targets from *Y. pestis*-associated virulence plasmids. This multiplexed assay was able to detect 1.5 ng of stock DNA. It should be noted that this method and the following methods do not translate units of DNA into bacterial cells detected. Real-time PCR assays
largely are restricted to laboratory use due to the requirement of a thermocycler with real-time
detection capability. Isothermal amplification offers potential as a DNA amplification method
that can be used in the field. Because the target DNA is amplified at a constant temperature, the
use of an expensive thermocycler is eliminated. Loop-mediated isothermal amplification
(LAMP) targeting the *Y. pestis* F1 antigen-encoding *caf1* gene on pMT1 detected 10 pg of DNA
in 45 min.\textsuperscript{19} Recombinase polymerase amplification (RPA) is another isothermal DNA
amplification method that has been applied for detection of *Y. pestis* DNA. RPA had detection
limits of $4.04 \times 10^{-13} \text{M}$ and $3.14 \times 10^{-16} \text{M}$ for *Y. pestis* specific ssDNA and dsDNA sequences,
respectively, in less than 1 h, however, culture time was not taken into account.\textsuperscript{20}

While DNA amplification-based techniques offer potential for rapid, specific detection of
*Y. pestis*, they do suffer from drawbacks. Sample matrix interference can decrease PCR
sensitivity or lead to false negative responses by inhibiting enzyme activity.\textsuperscript{21} In addition, a
major downfall to any amplified DNA diagnostic method is the inability to discriminate between
living and dead cells.

Bacteriophage (phage) amplification (PA) is a possible candidate for detection of viable
*Y. pestis* due to the ability of phages to rapidly infect a targeted bacterial host and multiply in a
highly species-specific fashion. Modern analytical techniques, such as matrix assisted
laser/desorption ionization mass spectrometry (MALDI MS) have been utilized to detect progeny
phage as secondary biomarkers.\textsuperscript{22-24} Lateral flow immunochromatography (LFI) offers an
inexpensive, rapid method with potential to be used for detection of progeny phage has been
utilized in the detection of *Staphylococcus aureus* and *Bacillus anthracis*.\textsuperscript{25-27} LFI normally
depends on a colorimetric determination which can lead to false negatives at low concentrations
where the indicator line is weak. Recent work has demonstrated the use of surface-enhanced
Raman spectroscopy (SERS) nanoparticle reporters coupled to PA LFI provided quantitative information with greater sensitivity and eliminated the problems with the detection of low concentration samples. The work described here demonstrates the application of PA SERS LFI for rapid, specific, and sensitive detection of *Y. pestis*.

**5.3 Methods**

**5.3.1 \( \phi A1122 \) propagation**

\( \phi A1122 \) and its propagation strain, *Y. pestis* A1122, were provided by the Division of Vector-Borne Infectious Disease of the Centers for Disease Control and Prevention (CDC), Fort Collins, CO. Phage propagation was carried out by plaque assay. Briefly, *Y. pestis* A1122 lawns were prepared by mixing 300 μL of overnight culture with three mL of a soft agar overlay (0.5 % agar in Brain Heart Infusion, BHI) and spotted with 80 μL aliquots of \( \phi A1122 \) at a concentration of \( 10^9 \) plaque forming units (pfu)/mL and incubated overnight at 37°C. Resulting plaques were harvested by application of three mL phosphate buffered saline (PBS, pH 7.4) and scraping of the agar surface with a sterile metal rod followed by collection into sterile conical tubes. The resulting slurries were separated by centrifugation at 9000 × g for 15 min at 4°C followed by filter sterilization of supernatants through 0.22 μm polyethersulfone filters (Millipore, Billerica, MA). Crude lysates were purified by polyethylene glycol precipitation (PEG 8000, OmniPur, Gibbstown, NJ) and cesium chloride gradient ultrafiltration as previously described. Removal of residual CsCl was carried out by dialysis (Pierce Slide-A-Lyzer, 20 kDa molecular weight cutoff, Thermo Scientific, Rockford, IL) in PBS with three buffer exchanges. The resulting purified phage preparation was used for production of anti-phage antibodies.
5.3.2 Production and purification of anti-ϕA1122 antibodies

Anti-ϕA1122 phage polyclonal rabbit IgG antibodies were prepared by Antibodies Incorporated (Davis, CA, USA) using purified phage. Purification of anti-phage antibodies was performed using Protein G spin columns (Nab™, Thermo Scientific) and specificity confirmed by ELISA. Further purification was carried out by dialysis in PBS and antibodies were concentrated by ultrafiltration, following manufacturer’s instructions (Amicon® Ultra, 30 kDa cutoff, Millipore).

5.3.3 Nanoparticle reporter and control particle preparation

Anti-ϕA1122 SERS reporter and control particles consisting of blue polystyrene particles were prepared as previously described. Modifications were made for SERS reporter antibody conjugation as follows. Fifty molar equivalents of purified anti-ϕA1122 were reacted with crosslinker sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Scientific) for 30 min at 23°C in degassed conjugation buffer, 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS) (Sigma Aldrich, St. Louis, MO, USA) (pH 7.2).

5.3.4 LFI device fabrication

LFI devices designed for detection and identification of *Y. pestis* by ϕA1122 amplification with SERS reporter particles were constructed as previously described. Modification consisted of a one-tenth mm wide phage capture/test lines applied to nitrocellulose membranes (Millipore Hi-Flow 180) with anti-ϕA1122 antibodies (2 mg/mL) using an IVEK Digispense 2000 stiper (IVEK Corporation, North Springfield, VT, USA) at a rate of 4 μL/s. Membranes were dried for 15 min in a forced air oven at 35°C and stored desiccated at 23°C. Release pads consisted of glass fiber release medium (Schleicher & Schuell, Keene, NH) impregnated with anti-ϕA1122 SERS reporters by application of a solution of SERS (0.02%
solids) and control particles (0.01% solids) in 2 mM sodium borate, 0.1 M NaCl, 1% v/v fish gelatin, 0.05% w/v sodium azide, and 3% w/v sucrose (Baker, Phillipsburg, NJ, USA) (pH 8.4). Impregnated release pads were dried for 30 min at 35 °C and stored desiccated at 23 °C until device assembly.

Nitrocellulose membrane, impregnated glass fiber, sample pad (Ahlstrom, Helsinki, Finland) and absorption pad (Schleicher & Schuell) were applied to an adhesive backboard (G&L Precision Die Casting, San Jose, CA, USA) with an approximate 2 mm overlap between LFI components. A programmable sheer (Kinematic Automation Inc., Twain Harte, CA, USA) was used to cut assembled LFI strips to a width of 3.7 mm. Combined LFI components were assembled into handheld plastic LFI cassettes.

5.3.5 Determination of LFI limit of detection

Visual and Raman spectroscopic LFI limits of detection (LOD) were determined by triplicate analysis of serial dilutions of filter-sterilized ϕA1122 in BHI media with concentrations ranging from $2.0 \times 10^0$ to $9.3 \times 10^9$ pfu/mL. Negative controls consisted of phage free sterile BHI. Fifty μL of each phage sample were mixed with 50 μL running buffer, which consisted of 0.1 M sodium borate, 3% w/v bovine serum albumin (BSA) (Sigma Aldrich), and 1% v/v Tween® 20 (Sigma Aldrich) (pH 8). Sample solutions were applied to LFI devices and allowed to flow for 15 min, after which time absorption pads were removed. Strips were then desiccated for 10 min prior to SERS analysis. Visual LOD was determined as the lowest concentration that produced a detectable test line. SERS analysis of the test line was carried out as previously described 28. Briefly, test lines were interrogated by Raman spectroscopy (Advantage 785, DeltaNu Inc., Laramie, WY, USA) at 785 nm with a laser power of 51 mW. Twelve random
spectra were collected across the width of test lines and were analyzed using a linear least squares curve fit.\textsuperscript{31}

\section*{5.3.6 Phage amplification and SERS LFI analysis}

Three independently performed PA experiments were conducted in triplicate using \(\phi\text{A1122}\) and \(Y.\text{pestis}\) A1122 and monitored by SERS LFI and plaque titer assay. Input phage for each experiment was below the experimentally determined SERS LOD. Overnight cultures were back-diluted in BHI and grown to an \(\text{OD}_{620}\) of 0.35, which corresponded to approximately \(2 \times 10^7\) cfu/mL (as determined by triplicate growth curves). This culture was subsequently diluted to appropriate starting concentrations in 50 mL final volumes. Finally, phage was added at multiplicities of infection (MOI) of 0.1, 1.0, and 10.0. Additionally, bacteria-free experiments were performed as negative controls. Cultures were grown at 28\(^\circ\)C with continual aeration (200 rpm). Aliquots were removed at 1 h increments for 3 h and filter sterilized to remove bacteria. Amplification samples were analyzed by SERS LFI similar to LOD samples with 50 \(\mu\text{L}\) sample mixed with 50 \(\mu\text{L}\) of running buffer and applied to LFI devices. To confirm SERS LFI results, phage concentrations were also determined by triplicate spot titer assay.\textsuperscript{30}

\section*{5.4 Results and Discussion}

\subsection*{5.4.1 Bacteriophage selection}

\(\phi\text{A1122}\) was selected for use in a PA SERS LFI diagnostic assay for \(Y.\text{pestis}\) because of its ability to lyse all but two of thousands of \(Y.\text{pestis}\) isolates, including both virulent and avirulent strains, large burst size (180), and short latent period (30 min).\textsuperscript{24, 32-33} \(\phi\text{A1122}\) is a plague diagnostic standard used in \(Y.\text{pestis}\) confirmatory assays by the CDC, the U.S. Army Medical Research Institute of Infectious Disease, and the World Health Organization.\textsuperscript{5, 34-35} Additionally, \(\phi\text{A1122}\) has been used in detection schemes that involved bioluminescent reporter
phage assays and an assay that detect amplified progeny phage DNA.\textsuperscript{32, 36-37} \textit{Y. pestis} A1122 strain was chosen to demonstrate the proof of concept of PA SERS LFI. \textit{Y. pestis} A1122 lacks the \textit{pgm} locus and the pCD1 plasmid that encode for essential virulence factors and is therefore avirulent and exempt from the select-agent list, making it useful for research conducted in BSL2 laboratories.\textsuperscript{38} Attenuated strains have been used to demonstrate feasibility for detection systems against potential biowarfare agents \textit{Y. pestis}, \textit{B. anthracis} and \textit{Burkholderia pseudomallei} and \textit{B. mallei}.\textsuperscript{24, 27, 36-37, 39-40}

5.4.2 PA SERS LFI

Previous work in our group utilized ϕA1122 amplification for the detection of \textit{Y. pestis}. In this work, progeny ϕA1122 were detected by conventional qualitative LFI, with a limit of detection of $2 \times 10^9$ pfu/mL.\textsuperscript{41} Subsequent work demonstrated the use of PA SERS LFI for detection of the foodborne pathogen \textit{L. monocytogenes}. The use of SERS allowed for quantitative analysis with a three-fold increase in sensitivity demonstrated by an LOD of $6 \times 10^6$ pfu/mL.\textsuperscript{28} SERS nanoparticle reporters consisted of a Raman-active organic dye coating a gold core enclosed in a silica layer with thiol surface modification that allowed for anti-phage antibody attachment.\textsuperscript{42-43} When a laser in the near IR was applied, SERS nanoparticles produced a characteristic spectrum that was exploited for quantitative analysis, eliminating the reliance on visual confirmation and potential for human error while increasing the ultimate device LOD significantly.\textsuperscript{44}

5.4.3 Determination of ϕA1122 LFI limit of detection.

Visual and SERS LODs were determined for ϕA1122-specific SERS LFI devices by triplicate analysis of ϕA1122 serial dilutions. Figures 5.1 and 5.2 are representatives of this triplicate analysis. Positive visual results were indicated by formation of a colored test line.
Figure 5.1A and B (9.3 × 10^9 and 7.7 × 10^8 pfu/mL, respectively) displayed strong positive visual results, while a phage input of 6.7 × 10^7 pfu/mL produced a weak visual positive result (Figure 5.1C). A phage concentration of 7.7 × 10^6 pfu/mL and phage free negative controls were visually negative (Figure 5.1D and E, respectively). Based on these observations, the visual LOD was determined to be 7.7 × 10^7 pfu/mL. The weak positive produced in Figure 5.1C demonstrates an instance where visual determination can be missed when a weak response is observed, leading to the potential for false negative visual interpretation.

Figure 5.1. Determination of visual limit of detection for PA SERS LFI. A) 9.3 × 10^9 pfu/mL, B) 7.8 × 10^8 pfu/mL, C) 6.7 × 10^7 pfu/mL D) 7.7 × 10^6 pfu/mL, E) negative control.
Figure 5.2 shows comparative SERS measurements from the same serial phage dilutions used for visual determination. Vertical error bars represent the interquartile range (IQR) of 12 random Raman acquisitions across each test line. Heterogeneous pore distribution of the nitrocellulose membrane produced areas of higher and lower Raman intensities, which necessitated the use of IQR. The standard deviations of triplicate phage titer measurements are represented by horizontal error bars. A phage concentration of $7.7 \times 10^6$ pfu/mL was the lowest observed to produce a positive SERS result. Thus, an input phage concentration below SERS LOD was used in all subsequent phage amplification experiments. A phage concentration of $7.7 \times 10^6$ pfu/mL corresponded to 1.34 arbitrary units (AU) as the SERS baseline. Samples measured above this value were considered positive. LODs determined here (both visual and SERS) were similar to previous SERS LFI LOD results observed with a different phage-antibody system used for detection of Gram-positive Listeria.

**Figure 5.2.** Comparison of visual and Raman limits of detection by PA SERS LFI. Visual LOD, dotted line. SERS LOD, dashed line. Vertical error bars represent the IQR of 12 Raman spectra collected at the test line. Horizontal error bars represent standard deviation of triplicate spot titer assays.
5.4.4 Phage amplification and SERS LFI analysis.

Anti-ϕA1122 SERS LFI devices were evaluated for detection of *Y. pestis* utilizing a series of PA experiments with an input ϕA1122 concentration of $5.0 \times 10^5$ pfu/mL and a *Y. pestis* cfu/mL of $5.0 \times 10^6$ (MOI of 0.1) to $5.0 \times 10^5$ (MOI of 1.0) to $5.0 \times 10^4$ (MOI of 10.0) (Table 5.1). All experiments were monitored in real time by SERS LFI and confirmed by overnight spot titer assays (Figure 5.3 and 5.4). Figure 5.3 shows representative visual results at varying MOIs over 3 h taken at 1-hour intervals. $5.0 \times 10^6$ cfu/mL (MOI = 0.1) (Figure 5.3A) produced a faint visual positive at 1 h, while a $5.0 \times 10^5$ cfu/mL (MOI = 1.0) produced a visual positive at 2 h (Figure 5.3B). $5.0 \times 10^4$ cfu/mL (MOI = 10.0) (Figure 5.3C) did not produce any visual positives during the 3 h duration of the experiment.

Table 5.1. Starting concentrations for phage amplification experiments.

<table>
<thead>
<tr>
<th>MOI</th>
<th>Input Bacteria (cfu/mL)</th>
<th>Input Phage (pfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>$5.0 \times 10^6$</td>
<td>$5.0 \times 10^5$</td>
</tr>
<tr>
<td>1.0</td>
<td>$5.0 \times 10^5$</td>
<td>$5.0 \times 10^5$</td>
</tr>
<tr>
<td>10.0</td>
<td>$5.0 \times 10^4$</td>
<td>$5.0 \times 10^5$</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0</td>
<td>$5.0 \times 10^5$</td>
</tr>
</tbody>
</table>

Figure 5.3. SERS LFI strips for three ϕA1122 amplification (A. MOI = 0.1, B. MOI=1.0, C. MOI=10.0) monitored for formation of a visual positive (+) over 3 h (T=time in h).
Figure 5.4 shows a comparison of PA SERS LFI-based detection and plaque titer assays. Detection of $5.0 \times 10^6$ and $5.0 \times 10^5$ cfu/mL by SERS analysis was observed at 1 h (Figure 5.4A). $5.0 \times 10^6$ cfu/mL detection bordered on visual levels, explaining the faint line seen in Figure 5.3A. Positive SERS results for $5.0 \times 10^4$ cfu/mL were achieved at 3 h post infection. Negative controls did not produce any detectable SERS signal within 3 h. To confirm assay reproducibility, further triplicate amplifications were performed and analyzed by SERS LFI after 1 h for MOIs of 0.1 and 1.0 and at 3 h for MOI of 10.0 (Table 5.2). The IQR of twelve spectra collected for each amplification experiment and the averages of the first (Q1) and third quartiles (Q3), along with standard deviations, are presented in Table 5.2. The coefficient of variance (CV) for the Q1 and Q3 of MOI=0.1 were 4.7% and 5.6%, while the CVs of MOI=1.0 were 1.3% and 4.7% for Q1 and Q3, respectively. Q1 and Q3 of MOI=10.0 had CVs of 24.3% and 22.6%, respectively. The large CVs for MOI=10.0 are due to one amplification producing a lower IQR SERS signal (1.52-1.60) compared to the other sets. However, this amplification still had a SERS signal above the established LOD (1.34).

**Figure 5.4.** Comparison of visual and SERS detection of A) PA SERS LFI and B) Spot titer assay. Visual LOD, dotted line. SERS LOD, dashed line. ■ represents an MOI=0.1, ▲ represents an MOI=1.0, ♦ represents an MOI=10.0, ● represents negative control
### Table 5.2. Triplicate phage amplification detected by SERS LFI at various MOIs

<table>
<thead>
<tr>
<th>MOI</th>
<th>SERS Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1.93±0.09 – 2.14±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>2.10±0.51 – 2.17±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>SERS signal measured at 1 h phage amplification. <sup>b</sup>SERS signal measured at 3 h amplification. SERS signals are represented by the average of the first and third quartile with standard deviations.

PA results displayed in this work confirm previous findings that as the MOI increases, detection time also increases. This is because at low phage and bacterial concentrations, there is a decreased chance of phage and bacteria interacting, thus increasing time to detection.<sup>45-48</sup>

### 5.5 Conclusions

The high mortality rate associated with pneumonic plague and potential use of *Y. pestis* as an aerosolized agent of bioterrorism makes rapid detection of this pathogen of critical importance. The PA SERS LFI method described here reduces detection time by 4 days over conventional culture methods. PA SERS LFI provides similar testing time to other rapid based test, however, it does not rely on surface markers, such as F1, that may be absent on certain strains of *Y. pestis*. Additionally, the incorporation of phages as a secondary biomarker provided live/dead distinction that cannot be achieved by DNA amplification-based approaches. This is critically important because only living cells can cause disease.

Previously, PA SERS LFI was adapted for the detection of *L. monocytogenes*. The method described here utilized phage \( \phi A1122 \) because of its ability to lyse a broad range of *Y. pestis* strains. Additionally, PA SERS LFI displayed a phage LOD three orders of magnitude better sensitivity than previous PA LFI work for detection *Y. pestis*. Visual LOD was \( 6.7 \times 10^7 \) pfu/mL, while SERS LOD was \( 7.7 \times 10^6 \) pfu/mL. PA SERS LFI detected *Y. pestis* in a little as one hour and detected down to a concentration of \( 5.0 \times 10^4 \) cfu/mL in three hours. Utilization of
SERS reporters provided quantitative information, eliminating reliance on visual determination. Additionally, handheld Raman spectrometers can be utilized to enable field deployment of PA SERS LFI.

5.6 Acknowledgements

We wish to thank the Armed Forces Institute of Pathology and the Defense Threat Reduction Agency for funding this work (W81XWH-07-C-0061). The authors also thank Dr. Scott Bearden of the Centers for Disease Control, Division of Vector-Borne Diseases for kindly providing *Yersinia pestis* A1122 and bacteriophage φA1122. We would also like to acknowledge Leah Luna and Stephanie Matyi for their preliminary studies.

5.7 References cited


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CHAPTER 6

CONCLUSIONS

This dissertation included a review of phage amplification assays and discussed a phage amplification method applied to three different projects, utilizing lateral flow immunochromatography to detect phage amplification. The following is a summary of the major results and conclusions from the review and the three projects as well as a brief discussion of future work.

6.1 Bacteriophage amplification as a bacterial detection method

A summary of the advantages of phage-based techniques and strategies was presented in Chapter 2. This chapter discussed the many phage-mediated bacterial detection approaches; however, the main focus was on the phage amplification assay. Phage amplification assays involved the use of input phage to infect and target host. The production of progeny phages indicated the presence of the target host. Three main approaches to detecting progeny phages by phage amplification assays were 1) input phage concentrations below the detection limit of the chosen analytical detector and an increase in phage concentrations above instrumental LOD indicated the presence of the target bacteria, 2) differentiation of input phage proteins from wild-type progeny phage proteins and detection by mass spectrometry and 3) inactivation of exogenous input phages after initial infection and subsequent release of progeny phages detected. The phage amplification strategies discussed in this chapter were: plaque assay, high-performance liquid chromatography (HPLC), optical density (OD) measurements, PCR-based assays, mass spectrometry, lateral flow immunochromatography, staining and colorimetric assays and multichannel series piezoelectric quartz crystal (MSPQC) sensors.
6.2 Rapid detection of *Listeria* by bacteriophage amplification and SERS-lateral flow immunochromatography

Chapter 3 discussed the development of an A511 phage amplification method to detect *Listeria monocytogenes*. Progeny phages were detected by LFI devices that utilized SERS reporters covalently linked with anti-A511 antibodies. Much of the work described in this chapter focused on development and optimization of the SERS LFI devices. Ideal SERS LFI devices produced a minimal negative control signal while still providing high sensitivity. Various parameters were investigated that led to the successful development of SERS LFI devices. These included optimization of the conjugation of antibody onto SERS reporters, blocking of thiol groups on SERS reporters, optimization of capture antibody on the test line of the LFI, membrane flow rate and composition of running buffer. The ideal molar ratio of antibody to SERS reporters was reported to be 350-500; higher ratios resulted in the production of false positives due to agglomeration of SERS reporters. Additionally, free thiol groups on the SERS reporters were blocked with NEM to prevent nonspecific binding. It was found that the application of a concentration of 2 mg/mL of capture antibody produced the highest sensitivity, while higher concentrations caused a decrease in sensitivity due to the blocking of binding sites on the antibodies. Slower membrane flow rates provided higher sensitivity compared to faster membrane flow rates by allowing more time for analyte-reporter complexes to bind to the capture antibody at the test line. The optimal running buffer composition for SERS LFI devices consisted of a 0.1 M borate, 3% BSA, 1% Tween20 solution. A running buffer consisting of 0.05M HEPES, 0.05% Tween previously used for polystyrene-based LFI development did not facilitate proper flow of SERS reporters along nitrocellulose membranes.
Visual limit of detection was observed to be $6 \times 10^7$ pfu/mL, while SERS LOD was $6 \times 10^6$ pfu/mL. Next, two sets of phage amplification experiments were conducted. The first set consisted of 4 amplification experiments performed at an MOI of 0.1 with decreasing phage and bacterial concentrations. Detection of \textit{L. monocytogenes} concentrations of $1 \times 10^7$ cfu/mL, $5 \times 10^6$ cfu/mL, $5 \times 10^5$ cfu/mL and $5 \times 10^4$ cfu/mL was achieved in 2, 2, 6, and 8 h, respectively. The second set of amplifications consisted of a constant input phage concentration ($5 \times 10^5$ pfu/mL) and decreasing bacterial concentrations. MOIs of 1.0, 2.5 and 5.0 and were detected in 2, 4, and 5 h, respectively.

6.3 Detection of \textit{Listeria monocytogenes} in inoculated food by bacteriophage amplification and surface-enhanced Raman spectroscopy coupled to lateral flow immunochromatography

The PA SERS LFI strategy was further extended in Chapter 4 for detection of \textit{L. monocytogenes} in different food matrices. Cantaloupe, ice cream, queso fresco and Camembert cheese were selected because of recent \textit{L. monocytogenes} outbreaks in these food types. An enrichment protocol was needed to elevate low levels of \textit{L. monocytogenes} to detectable levels for PA SERS LFI analysis. To this end, the FDA enrichment media and procedure were applied prior to PA SERS LFI analysis. PA SERS LFI analysis successfully and reproducibly detected 1 cfu/g of \textit{L. monocytogenes} in all food types tested after 24 h enrichment. Negative controls did not produce detectable signals. An overall coefficient of variance of 9.0% was achieved for all positive food samples.
6.4 Rapid detection of *Yersinia pestis* by bacteriophage amplification and SERS lateral flow immunochromatography

Chapter 5 discussed the utilization of protocols developed for SERS LFI detection of *Listeria* phage A511, a SERS LFI system was constructed for the detection of ϕA1122, a *Y. pestis* phage. *Y. pestis* is the etiological agent of plague and present day concerns are related to its high mortality and morbidity rates and its ability to be aerosolized, making it a potential biowarfare agent. Thus, a need exists for rapid detection of *Y. pestis*. Visual and SERS LODs were $6.7 \times 10^7$ and $7.7 \times 10^6$ pfu/mL, respectively. Using an input concentration of $5 \times 10^5$ pfu/mL, phage amplification experiments were carried out at MOIs of 0.1, 1.0, and 10.0, providing positive *Y. pestis* detection in 1 h, 1 h, and 3 h respectively.

6.5 Future work

Rapid bacterial diagnostics are essential to directing early treatment of infections and PA SERS LFI offers a potentially valuable tool to this end. One possible extension to the work presented here is the evaluation of PA SERS LFI to detect drug-resistant strains. An approach would be taken similar to the Microphage KeyPath ® MRSA/MSSA detection system for antibiotic resistance determination. A sample would be added to two tubes containing 1) media and phage and 2) media, phage and antibiotic of choice. A positive by PA SERS LFI in tube 1 confirms the identity of the target bacteria, while a positive in tube 2 would determine resistance. To this end, the next target for PA SERS LFI could be *Klebsiella pneumoniae*. A *K. pneumoniae* infection that was resistant to all known antibiotics in the U.S. was recently reported in the death of a Nevada woman.†

Lastly, as a potential way to improve SERS LFI, the use of aptamers instead of antibodies could be considered. Aptamers are oligonucleotides that bind to specific target molecules.
Purified phage can be screened against a library of aptamers to select ideal targets that can be used in conjugation to SERS reporters and as capture agents at the LFI test line. Aptamers offer the advantages of being sequenced and being accurately and reproducibly synthesized.²

6.6 Paper published and to be published

The review present in Chapter 2 is an in-progress manuscript to be submitted to *Microbiology and Molecular Biology Reviews*. Chapter 3 is work that has been published in *Viruses* (DOI:10.3390/v7122962). Chapters 4 and 5 are manuscripts that have been submitted to *Letters in Applied Microbiology* and *Journal of Medical Microbiology*, respectively. These works may appear slightly modified after reviewer’s comments are addressed. It should also be noted that for each of these works, I have designed experiments, troubleshooted problems, collected, analyzed and interpreted data, wrote manuscript drafts, incorporated revisions by co-authors, submitted manuscripts, and revised manuscripts per reviewer suggestions.

Published papers


Submitted papers

[1] Stambach, N. R.; Cox, C. R.; Voorhees, K. J., Detection of *Listeria monocytogenes* in inoculated food by bacteriophage amplification and surface-enhanced Raman


**Papers to be submitted**


### 6.7 References Cited


APPENDIX A

MATLAB INSTRUCTIONS FOR ANALYSIS OF RAMAN SPECTRA

To load reference files

- Name of file = dlmread('filename');
- X(:,1) = Name of file (:,2);
- 1-for SERS, 2-for nitrocellulose
- Ex.
  - SERSReference = dlmread('440 reference.prn');
  - X(:,1) = SERSReference(:,2);
  - NitroReference = dlmread('nitrocellulose reference.prn');
  - X(:,2) = NitroReference(:,2);

To load test spectrum

- Open ASCII file and delete words at top (displaying instrument conditions)
- Name of file = dlmread('filename');
- Y = name(:,2);
- B = (X’*X)(X’*Y);
APPENDIX B

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