DEVELOPMENT AND EVALUATION OF PROTOTYPE BACTERIOPHAGE AMPLIFICATION IMMUNOCHROMATOGRAPHY STRIPS FOR THE IDENTIFICATION OF *YERSINIA PESTIS*

by

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ABSTRACT

The slow analysis and detection processes applied by our public health infrastructure to the deliberate 2001 anthrax attacks has ignited a movement towards improving our surveillance and detection capabilities for selected biological agents. In response, the Voorhees group at the Colorado School of Mines has focused on applying the fundamental process of the bacteriophage lytic cycle to produce a natural increase in phage protein production to generate a signal or "amplification event" when in the presence of a targeted bacterial pathogen. This method exploits the specificity of phage/bacterial interactions and the normal synthesis of progeny phage proteins within the bacterial pathogen. When the bacteriophage amplification process is coupled to a simple, easy to use and inexpensive detection device such as an immunochromatography strip, the methodology provides a prototypic detection device, coined bacteriophage amplification immunochromatography strips (BAmICS), that is rapid, sensitive and selective.

Development of two prototype BAmICS systems to specifically detect *Yersinia pestis* subspecies is outlined in this thesis. One system utilizes the rapid lysing (r+) yersiniophage Y and the other employs yersiniophage ϕA1122 to produce the amplification event. Selection of the two phages for BAmICS development was a result of studies conducted on phages ϕA1122, PKR, R, Y and V in terms of their morphology, host range and cross reactivity effects.

Selected candidate(s) based on results from the above studies were analyzed for unique phage protein biomarker(s) for use in subsequent phage amplification studies using MALDI-MS for detection. Modeling was utilized in conjunction with MALDI-MS phage amplification to predict and explain the phage-derived signal and what factors significantly controlled its detection.

Bacteriophages ϕA1122 and Y were selected for coupling the amplification process to the ICS which required anti-phage antibody production, characterization and conjugation to colloidal gold for reporting. Final investigation of the prototype BAmICS devices were evaluated for performance in terms of matrix, selectivity, speed and sensitivity.

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LIST OF ABBREVIATIONS

BAmICS	Bacteriophage amplification immunochromatography strips
BCA	Bicinchoninic acid
BHI	Brain heart infusion (broth)
BSA	Bovine serum albumin
CsCl	Cesium chloride
cfu	colony forming unit
Da	Dalton
DAPI	4'-6-Diamidino-2-phenylindole
DFA	Direct fluorescent F1-antibody
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
F1	Fraction 1 antigen
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
IACUC	Institutional animal care and use committee

ICA	Immunochromatography assay
ICS	Immunochromatography strips
IgG	Immunoglobin G
kDa	kilo Dalton
KDO	keto-deoxy-octonate
Lcr	Low calcium response
LOD	limit of detection
LPS	Lipopolysaccharide
m/z	mass-to-charge ratio
MALDI	Matrix Assisted Laser Desorption Ionization
MOI	Multiplicity of infection
MPL	Monophosphoryl Lipid A
MS	Mass Spectrometry
NaCl	Sodium chloride
OD	Optical density
PAb	Polyclonal antibody
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pfu	Plaque forming unit
Phage	Bacteriophage or yersiniophage

PHA/PHI	Passive hemagglutination and inhibition assay
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TDM	Dicorynomycolate
TE	Tris-ethylenediaminetetraacetic buffer solution
TEM	Transmission electron microscopy
TMB	3,3',5,5'-Tetramethylbenzidine
TOF	Time of flight
TSA	Tryptic soy agar
TSB	Tryptic soy broth

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DEDICATION

For my son Royce, my one and only

Bubbles…I love you with all my heart and soul

CHAPTER 1

THESIS INTRODUCTION

"Let me tell you the secret that has led me to my goal:
my strength lies solely in my tenacity"
Louis Pasteur, French Chemist and Microbiologist

1.1 Aims of thesis

The Voorhees Group at the Colorado School of Mines has focused on applying the fundamental process of the bacteriophage (phage) lytic cycle or "phage reproduction cycle" to produce a natural increase in phage to generate a detectable signal or "amplification" for the identification of a targeted bacterial pathogen. The focus of this research has been to couple this fundamental process to immunochromatography strips (ICS) to detect *Yersinia pestis*, the etiological agent of plague.

The described work in this thesis had the goal of applying the most selective, sensitive and rapid lytic yersiniophage to produce a sufficient amplification event via progeny phage protein synthesis and to detect that signal with a portable immunochromatographic strip (ICS) device. Coupling these two processes will produce a prototype assay, which has been coined bacteriophage amplification immunochromatography strips (BAmICS), that is economical and portable, requiring little to no experience by the user.

Specific objectives were:

1. Investigate the selectivity of the yersiniophage candidates in terms of their morphology, host range and cross-reactivity via transmission electron microscopy (TEM) and phage lysis susceptibility testing.

2. Predict and explain the phage-derived signal with mathematical modeling and MALDI-MS detection of the selected host-phage system.

3. Develop, purify, and characterize binding of anti-phage polyclonal antibodies for coupling the bacteriophage amplification process to the immunochromatography strips.

4. Construct a prototype BAmICS device using polyclonal antibodies conjugated to colloidal gold particles for reporting.

5. Characterize the bacteriophage amplification immunochromatography strips matrix effects, selectivity, speed and sensitivity.

1.2 Format of thesis

The remainder of this chapter provides a historical perspective and background information on plague, *Yersinia pestis* and phages as well as describing the importance of developing and applying a new detection system for this pathogen. Chapters two through eight are dedicated to the specific objectives outlined above and are written in such a manner that each chapter can be submitted for publication to a peer reviewed journal, as it is the intent of this author to submit a major portion of this thesis for publication. Finally, because this work is multidisciplinary, the appendices address basic theory and fundamentals and an extensive laboratory protocol section will provide future researchers with the practical laboratory knowledge for applying this technology to other pathogens of interest.

1.3 Background

Many plague epidemics and pandemics have occurred throughout history. The three most notable pandemics include: the Justinian plague originating in Africa in 541; the Black Death which began around 1347; and the most recent pandemic occurring worldwide in the 19th and 20th centuries. Death counts reached into the millions during these plague pandemics. For example, during the Justinian pandemic, the Byzantine historian Procorpius described the plague as killing thousands of people in Constantinople every day. Reportedly as much as 40% of Constantinople's population died during this epidemic [1-5].

The Black Death, the most historically notable pandemic, killed more than a third of the European population, where it has been said that there were not enough people left alive to bury the dead [6-8]. The "Great London Plague of 1665" a subsequent epidemic following the Black Death has been frequently described as having congested streets of wooden, rat-infested houses, ideal places for plague to spread [3]. Reportedly, infested houses were closed, guarded, marked with a red cross and inscribed with the words, "*Lord have mercy on us*", while ringing of bells and calls could be heard of, "*bring out your dead*" [9].

The last major pandemic spread to Canton and Hong Kong in 1894 and India by 1898 [5]. By 1900 steamships had spread the disease throughout the world including Africa, Australia and the Americas [10-12]. Because of this pandemic, plague has established itself within well-defined geographic boundaries on every continent with the exception of Australia and Antarctica [10, 12, 13].

The etiological agent responsible for causing plague is the bacillus bacterium *Yersinia pestis*, which has also been formerly known as *Bacterium pestis* until 1900, *Bacillus pestis* until 1923 and *Pasteurella pestis* until 1970 [10]. Discovery of the bacterium occurred independently and practically simultaneously in 1894 by both Alexandre Yersin and Shibasaburo Kitasato[1.1] [14]. Following isolation of the bacterium, Yersin successfully developed an antiserum

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[1.1] Currently both Yersin and Kitasato are credited with the discovery of *Y. pestis* as independent discoveries, however current thinking is that Kitasato probably isolated a bacillus bacterium which may or may not have been *Y. pestis*.

against *Y. pestis* to treat plague patients [10, 15]. Yersin is also credited with making the epidemiological connection between rats and plague, while Masanori Ogata and Paul-Louis Simond autonomously discovered the role of the rodent flea vector in plague transmission during the 1898 Indian epidemic [10].

Plague occurs naturally in rodent populations. The main transmission vector of plague to humans is the oriental rat flea, *Xenopsylla cheopis* [5, 16]. When a flea is infected with plague, the organism colonizes and blocks proventriculus.[1,2] This blockage causes the flea to develop a voracious hunger because of its inability to digest the mass of blood and bacteria [17]. In an effort to try and clear the mass, the flea bites the nearest host and regurgitates bacteria into the bite wound, causing inoculation. If disease develops, it typically presents in the form of bubonic plague which causes swelling in the lymph nodes, referred to as buboes. Patients typically develop symptoms of fever, headache, chills, and swollen lymph nodes within 2 to 6 days after initial inoculation [16]. If left untreated bubonic plague can progress to septicemia (a bacterial infection of the blood) and the secondary pneumonic form of the disease [18]. If the bacteria deposits in the lungs, pneumonic plague may result and human to human transmission can occur through the dispersion of respiratory droplets when people cough, sneeze or exhale. Relative to other infectious diseases, natural transmission of plague to humans is typically infrequent with reported worldwide cases averaging 3,000/year [16, 19].

Regrettably, plague has also seen development and use as a biological weapon throughout history. Earliest reports of its use began in 14th century Europe as attempts were made by the Tartar Army to spread plague in the besieged city of Caffa by catapulting plague victims over the city walls [20, 21]. However catapulting these dead bodies did not likely contribute significantly to the spread of plague as rats within the city walls were more than likely already infected with the bacteria [21]. During World War II the bacterium was used by the Japanese Imperial Army biological warfare and human experimentation Unit 731. Lead by Ishii Shiro, Unit 731 dropped plague-infected fleas from aircraft over the populations of Changte and Hunan China causing outbreaks of bubonic plague [22, 23]. In addition, when

[1,2] Comparable to the human gastroesophageal area.

the Japanese surrendered in World War II, Unit 731 set free thousands of infected rats that caused widespread plague in 22 counties of the Heilungchiang and Kirin provinces that took more than 20,000 Chinese lives. More recently during the cold war, scientists in several countries, including the United States, worked on developing aerosol-based plague delivery systems [22]. Even as recently as the 1990's, the Union of Soviet Socialist Republics attempted to develop plague as an aerosol agent to cause primary pneumonic plague in large populations [23].

1.4 *Yersinia pestis*

The plague bacterium is categorized in the Enterobacteriaceae family and the genus *Yersinia*. Of the 11 species which comprise the genus there are only three which are human pathogens: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. The bacterium is characterized as a pleomorphic Gram negative facultative anaerobe that is non-motile, non-spore forming and is considered to be a "fatty rod". The coccobacillus morphology ranges from 0.5 x 1-2 μm in length and stains bipolar (closed safety pin) in appearance (Figure 1.1) [16].

Bacterial growth can occur over a temperature range of 4-40°C, while optimal growth occurs at ~28°C and a pH ranging from 7.2 to 7.6 [16]. Differential expression of proteins do occur with this bacteria at different temperatures [24]. For example, expression of the F1 protein is only efficiently expressed at temperatures greater than 33°C [25]. Average doubling time of *Y. pestis* at 37°C is ~75-90 minutes at slightly less at 28°C. Supplements of glutamic acid, glycine, isoleucine, methionine, phenylalanine, valine, biotin, pantothenate, thiamine and trace metals are required for optimal growth. Biochemically, *Y. pestis* lacks many enzyme functions typical of Enterobacteria, however strains can be differentiated into three biovars: 1) *antiqua*; 2) *mediaevalis*; and 3) *orientalis* based on the strains ability to ferment glycerol and reduce nitrate [16, 26]. Genetically, the bacterium contains a single chromosome and

typically carries three plasmids: 1) Lcr (also known as pCad or pCD1)[1.3]; 2) pFra (also known as pTox or pMT1); and 3) pPCP (pPst) which code for virulence factors. pMT and pPCP are specific to *Y. pestis* whereas, pCD1 is also common to pathogenic strains of *Y. pseudotuberculosis* and *Y. enterocolitica* and is designated pYV.

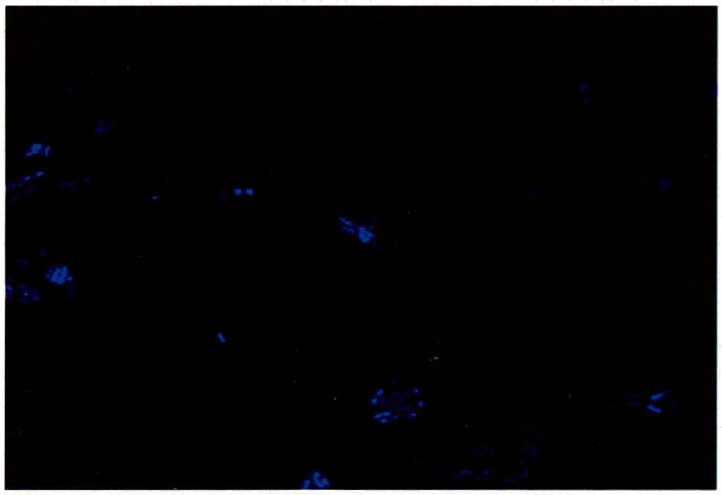

Figure 1.1 *Yersinia pestis* A1122 fluorescently stained with DAPI.

[1.3] In the *Y. pestis* strain A1122, this plasmid has been removed producing an avirulent strain acceptable for handling under BSL-2 conditions.

1.5 Yersiniophage

Fredrick Twort is considered the father of modern phage research, however, it was Felix d'Herelle in 1917 who discovered a "microbe" that was antagonistic to bacteria, resulting in their lysis in liquid cultures and created plaques or clearings in bacterial lawns when plated onto the surface of agar [27-29]. D'Herelle conjectured that these invisible microbes were "ultraviruses" that invaded bacteria and multiplied at their expense. Ultimately he coined these "microbes" bacteriophage (eaters of bacteria).

D'Herelle pioneered two important areas in phage research, phage therapy and the biological nature of phage. The latter was based on evidence that the nature of phages were obligate intracellular parasites which have antigenic properties and host-range specificity for a particular group of bacterial pathogens, thus providing simple "organisms" for genetic study [27]. The former was based on his findings that phage titers rose in patients with infectious diseases just as recovery was taking place and reasoned that phages represented natural agents of resistance to infectious diseases [30, 31]. One of the earliest reports of using yersiniophage to treat an infectious disease was D'Herelle himself while stationed in Alexandria, Egypt as he successfully treated four laboratory diagnosed plague patients using anti-plague phage preparations, which he injected directly into the infected lymph nodes or buboes [32, 33].

In addition to the use of phages to treat infectious diseases, the specificity of lytic phage/host interaction has been extensively utilized in methods for typing pathogenic bacteria [16, 34]. Several yersiniophages specific to the genus *Yersinia* have been described in the literature [35-41]. In terms of phage/*Y. pestis* typing, focus has been mainly on the isolation of phages that could specifically infect all natural isolates of *Y. pestis* [42]. Yersiniophages reported to infect and lyse *Y. pestis* strains include, but are not limited to ϕA1122, EV, P, H, PKR, R, V, and Y. Yet, information pertaining to phenotypic properties such as morphology, host range and cross reactivity of these phages remains extremely limited or unexplored [34, 39, 42-46]. For example, lysis susceptibility studies have been

conducted with *Y. pestis* and a limited number of *Escherichia coli* strains using phage Y. Rudimentary results indicate that this phage has the ability to lyse strains of both species [43]. Another study using bacterial species of *Shigella* and *Salmonella* have also been analyzed for cross reactivity with a yersiniophage producing positive results for lysis, however ambiguity exists as to what phage was actually used as the authors referred to the phage causing lysis as only the "bacteriophage" [42]. The majority of useful and detailed yersiniophage studies have been conducted by investigators in the former Soviet Union [47-51]. Yersiniophage have been divided into 4 serovars: 1 lytic and 3 lysogenic[1.4] [40, 46, 48].

The lytic yersiniophages are thought to belong to the genus and species of T7-like phages which have non-enveloped icosahedral nucleocapsids with head diameters ranging from 50-60 nm and a short conical tail which protrudes from one vertex of the capsid [52]. Including the yersiniophages, reportedly, there are 26 phages in this species and include: T7, T3, H, PTB, R, Y, V, PKR, W31, WPK, ϕYeO3-12, ϕI, ϕII, ϕA1122, EV, Kvp1, gh-1, 2, III and Bpp1 [53, 54]. The T7 phage of the T7-like species has been well characterized and is reported to be composed of 415 major and/or minor capsid proteins, while the conical tail has 6 tail fibers extending from its surface [53, 54].

1.6 Importance of developing new detection methods for plague

Due to the historical plague pandemics and its development and use as a biological weapon, this infectious disease still incites fear and mass panic in societies around the world when plague outbreaks are suspected. Tied with today's bioterrorism potential against the United States and other allied nations, *Y. pestis* is considered one of a limited number of biological agents that, if used in malicious acts, could cause disease and death in sufficient numbers to cripple a region. Thus the intentional release of plague for harming or killing

[1.4] Focus of this thesis is on the lytic yersiniophage, however examples of lysogenic yersiniophages are L-413, II and 513.

civilians warrants development of improved detection, surveillance and diagnosis by our public health infrastructure.

The Centers for Disease Control and Prevention (CDC) consider *Y. pestis* as one of six category A[1.5] critical biological threat agents based on its potential to: incite public fear, cause widespread fatalities, to be mass produced as well as its special preparedness needs due to its potential to be aerosolized. In 1999, anti-terrorism legislation generated the means for the CDC, Federal Bureau of Investigation (FBI) and the Association of Public Health Laboratories (APHL) to establish a Laboratory Response Network (LRN). The LRN is responsible for ensuring an effective laboratory response to bioterrorism by helping to improve the nation's public health laboratory infrastructure. One key initiative of the LRN has been to develop and validate assays for identification of *Y. pestis* and other critical biological threat agents [55].

In response, real time polymerase chain reaction (RT-PCR) assays based on targeting one or more virulence genes from *Y. pestis* chromosomal DNA and or plasmids have been developed [56-59]. Examples of targets used in RT-PCR have included the *Pla, caf1, Ymt, yopT*, and/or 16s rRNA genes. Results show sensitivities as low as 0.1 genome equivalents and accuracy rates approaching 100% [60]. Other diagnostics routinely employed to identify *Y. pestis* include the direct fluorescent anti-F1 antibody test, enzyme immunoassays and haemagglutination assays which are based on the F1-antigen/antibody binding response [61]. Another method used to diagnose plague, and in fact used by the CDC as a confirmatory test[1.6], is the phage lysis strip method [16, 34]. This method utilizes the lytic ϕA1122 yersiniophage to differentiate *Y. pestis* cells from those of *Y. pseudotuberculosis* and other clinical isolates [16]. This assay takes advantage of lysis differentiation due to the fact that *Y. pestis* cells are susceptible to phage lysis over a wide temperature range (20°C to 37°C), while

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[1.5] The 6 Category A threat agents are given the highest priority of the total 24 agents on the list. In addition to plague or its etiological agent *Y. pestis*, category A threat agents include: anthrax, botulism, smallpox, tularemia and viral hemorragic fevers.

[1.6] Confirmed plague is diagnosed if one of the following conditions are met 1) A bacterial isolate is lysed by specific bacteriophage or 2) two paired serum specimens demonstrate a four fold anti-F1 antigen titer difference by agglutination testing (PHA/PHI)

Y. pseudotuberculosis cells are only susceptible to phage lysis at temperatures greater than 28°C [16, 34]. This technique is performed streaking agar plates with bacterial cultures across the surface of the strip followed and placing a phage coated strip perpendicular to the streak lines followed by several hours of incubation at the desired temperature. Traditional detection of the lysis event[1.7] is by visualizing clearings next to the strip [16, 34].

Sometimes visible lysis can be ambiguous and take up to 24 hours to obtain results by traditional means. Thus, this thesis addresses coupling a modern detection device, specifically immunochromatography strips, to modernize the lysis strip method to facilitate the surveillance, detection and diagnostic capabilities of our public health infrastructure and aid in detection where other methods are limited. Coupling the phage infection, replication and lytic cycle to immunochromatography strip (ICS) makes interpretation of results much less ambiguous and speeds up the detection process. Because this method requires the use of bacterial isolates, its intended use remains as a tool for an *in vitro* laboratory testing environment.

Several phage-based diagnostics have been applied to detect bacterial pathogens [62-74]. Specifically, bacteriophage amplification technology relies on the natural receptor specificity that a wild-type phage has for its host or target pathogen replicating within it, synthesizing new progeny phage, and upon release, providing a signal for detection. The possibility of this detection system to be purposely circumvented is extremely low. The rationale for this is that not all cells share the same surface structures or biochemical groups on their cell walls and many of these cell surface structures have evolved to mediate specific cell surface recognition events, such as those involved with nutrient uptake, transduction of signals across the membrane, or binding to other cells. While other bacteria and bio-assay systems require increased incubation temperatures to differentially express a specific protein, optimal growth conditions for *Y. pestis* occurs near room temperature, making detection of the

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[1.7] The "lysis event" is also referred to as the "amplification event" or "phage amplification" and is used interchangeably throughout this thesis. The release of progeny phage or its proteins from the bacterial host denotes the onset of the event.

bacterium possible in: 1) the flea vector; 2) naturally deficient F1 strains; 3) genetically manipulated strains which can circumvent F1 detection; 4) the percentage of an infected population which tests negative to F1 based assays; and 5) antibiotic resistant strains.

CHAPTER 2

COMPARATIVE STUDY OF YERSINIOPHAGES ϕA1122, PKR, R, V, AND Y. DETERMINATION OF MORPHOLOGY, HOST RANGE AND CROSS REACTIVITY FOR A PHAGE BASED DIAGNOSTIC TO DETECT *YERSINIA PESTIS*

"I've always believed that if you put in the work, the results will come. I don't do things half-heartedly. Because I know if I do, then I can expect half-hearted results."

Michael Jordan

2.1 Abstract

Lytic phage can serve as a natural and specific probe to generate a signal or amplification to identify a targeted bacterial pathogen. Desirable phage characteristics for this type of application include the ability to infect, replicate and lyse a wide range of *Yersinia pestis* strains (host range) while maintaining minimal cross-reactivity with other bacteria to prevent false positives. This research describes the characterization of five yersiniophages: ϕA1122, PKR, R, V and Y for the identification of *Y. pestis*. Several studies which characterize these yersiniophages in terms of their morphology, host range and cross reactivity via transmission electron microscopy (TEM) and phage lysis susceptibility testing are discussed. TEM results demonstrate that all five phages show characteristic T7-like species morphology, while lysis

susceptibility testing indicates that phage ϕA1122 can lyse all host strains tested and has a low (5.6%) cross reactivity rate with other related bacteria.

2.2 Introduction

Events in recent years have heightened awareness of the risk of acts involving biological agents [22]. The need for preparedness and planning for response at multiple levels is now recognized, including surveillance and detection for high profile bio-threat agents like *Yersinia pestis (Y. pestis)*, the etiological agent of plague, thus supplicating the need for diagnostic tests which are portable, inexpensive, sensitive, selective and easy to use by field personnel.

The cornerstone of many plague diagnostics rely on the unique F1 protein produced on the cell surface of *Y. pestis*. For example one of the recommended confirmatory tests is demonstrated through seroconversion (development of antibodies) to the F1 antigen in recovering plague patients. Non-confirmatory (presumptive) plague diagnostics also rely on the direct or indirect presence of F1 antigen/antibody binding and include the direct fluorescent F1-antibody (DFA) test, passive hemagglutination and inhibition assay (PHA/PHI), enyzme immunoassays (EIA), fiber-optic biosensors and the immunochromatography dipstick assay [61, 75]. However, it has been estimated that as much as 12% of an infected population could produce false negative results based on the F1 protein [61]. This has been attributed to either poor response to F1 within a population (biological variability) or because F1-antibody levels can fall below the sensitivity of the assay during early stages of infection. Since the F1 protein is expressed only at temperatures greater than 33°C, many of these F1 assays would be ineffective if a person were to become infected with a naturally F1 deficient strain or potentially manipulated strain which circumvents F1 detection. Developing a non-F1 based diagnostic would aid in the identification of *Y. pestis* strains which are F1 negative or manipulated to circumvent F1 detection, and allow identification in flea vector as the bacteria proliferates in these species at 26°C.

Molecular techniques, such as real time polymerase chain reaction (RT-PCR), are also used for the presumptive identification of *Y. pestis* [58, 60]. This technique targets a chromosomal gene or a specific virulence gene on a plasmid. However RT-PCR is subject to false negatives if deliberate manipulation or natural deficiencies coding the target occur, therefore, the technique necessitates multiplexing [59]. In addition, even with the development of instrumentation amenable for field use, PCR still suffers the added burden of expensive equipment and reagents as well as the need for highly trained personnel, both of which are limited in developing nations where plague outbreaks typically occur.

Non-F1 and non-genetic based techniques utilizing phage technology have been successfully employed for the detection of several pathogens including *Mycobacteria* and *Listeria* species [67, 73, 76-78]. More specifically, the phage amplification methodology is performed by adding a known concentration of phage to a sample containing the target bacterium. Phage specific to the target bacteria adsorb and infect the susceptible cells. The replication cycle follows, producing progeny phage and upon cell lysis, phage are released in to the surrounding milieu. The resulting increase in progeny phage generates the signal (amplification) for detection [67, 73, 77, 78]. Detection of phage is commonly carried out by visualizing lysis or clearings on a bacterial lawn after several hours of incubation. However, more novel and rapid approaches to detect the amplification event include bioluminescent reporters and MALDI-MS detection [66, 79]. Thus these technologies exploit the host/phage receptor specificity and lytic replication within susceptible cells avoiding the potential pitfalls of plasmid and F1 deficiencies.

To apply the phage amplification methodology to the detection of *Y. pestis*, characterization of yersiniophages is required. Selectivity of the phage which is to generate the signal is one parameter that is paramount. If the phage selected for amplification is not discriminatory between *Y. pestis* strains or biovars and other related bacteria, than the potential for false positives increases dramatically.

Several yersiniophage specific to the *Yersinia* genus have been described in the literature [35-41]. Yersiniophages reported to infect and lyse *Y. pestis* include ϕA1122, PKR, R, V, and

Y. Yet information pertaining to phenotypic properties such as morphology, host range and cross-reactivity of these phages remains limited or unexplored [34, 39, 42-46]. For example, lysis susceptibility studies have been conducted with *Y. pestis* and a limited number of *Escherichia coli* strains using phage Y. Rudimentary results indicate that this phage has the ability to lyse strains of both species [43]. Bacterial species of *Shigella* and *Salmonella* have also been analyzed for cross reactivity with a yersiniophage producing positive results for lysis, however ambiguity exists as to what phage was actually used as the authors referred to the phage causing lysis as only the "bacteriophage" [42].

The majority of useful detailed yersiniophage studies have been conducted by investigators in the former Soviet Union [47-51]. Yersiniophage have been divided into 4 serovars: 1 lytic and 3 lysogenic [40, 46, 48]. The lytic yersiniophages are thought to belong to the genus and species of T-7 like phages which have non-enveloped icosahedral nucleocapsids with head diameters ranging from 50 to 60 nm and a short conical tail which protrudes from one of the capsid vertices [52]. Thus, because of the lack of information (including their morphology) on these phage and the need to determine their selectivity for a phage based diagnostic, investigation into their morphology, host range and cross reactivity is presented.

2.3 Materials and methods

Morphology studies by TEM

Five phages ϕA1122, PKR, R, V, and Y were concentrated with polyethylene glycol (PEG) 8000 and differentially centrifuged 1:1 with chloroform to give a phage concentration greater than 10^{10} pfu/mL and considered to be semi-purified. Selected semi-purified phage preparations were further subjected to CsCl equilibrium gradient purification in a Beckman LM-8 ultracentrifuge at 25,000 rpm for 24 hours (SW-28 rotor) and considered to be 1x purified.

The semi-purified and purified phage preparations were negatively stained with 1% (w/v) uranyl acetate solution, excess stain was removed with filter paper. The remaining stained phages were allowed to air dry on top of a glow discharged 400 mesh copper electron microscopy grid coated with formvar and carbon film. The grid was glow discharged to make hydrophilic for sample plating. Analysis was performed using a Philips CM10 transmission electron microscope (FEI, Inc. Hillsboro, OR) operated at 80kV. Phage were digitally photographed at a magnification of x92,000. More detailed descriptions of the technique are contained in Appendix C.

Phage lysis susceptibility testing

The cross-reactivity and host range of the five phages were determined by bacterial susceptibility to phage lysis. Briefly, bacterial isolates were cultured overnight in TSB at 37°C and/or 28°C. Three-hundred μL of a log phase growth bacterial suspension was added to 3mL of soft TSA and overlaid on a Petri dish containing TSA to create the bacterial lawn. Five μL of each phage (all phage concentrations greater 10^{10} pfu/mL) were spotted onto each bacterial lawn, incubated overnight at the respective temperature and checked for lysis (clearings in the bacterial lawn) the following morning.

A total of 100 bacterial strains were tested for lysis against the 5 yersiniophage in order to determine the amount of cross reactivity. Table 2.1 lists 11 broad ranging bacterial strains tested including *Bacillus anthracis, Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolitica, Salmonella Typhimurium, Shigella flexneri, and Shigella dysentariae*, while Table 2.2 shows *Escherichia coli* (*E. coli*) specific strains and includes: the reference ECOR and EHEC collections (Michigan State University) as well as other miscellaneous *E. coli* strains [80]. In addition, forty *E. coli* environmental isolates from humans, raccoon, and swine were tested for cross reactivity with each of the 5 phages. Human strains were isolated from effluents from Boulder County CO wastewater treatment plant and Mines Park septic system, Golden CO,

while the raccoon and swine strains were isolated from fecal material collected from various sites throughout Colorado.

Host range for *Y. pestis* isolates was also determined by susceptibility to phage lysis. Since the CDC/NCID/DVBID (Ft. Collins, CO) has more than 3,000 *Y. pestis* isolates, ten representative *Y. pestis* isolates were selected and considered to be representative of the collection. The distribution panel contained 2 avirulent and 8 virulent strains representative of the three known biovars (*antiqua, mediaevalis,* and *orientalis*). The specificity of phages ϕA1122, PKR, R, V, and Y were then tested against the 10-member *Y. pestis* distribution panel listed in Table 2.4. Susceptibility testing was conducted in the BSL-3 facility at the DVBID.

2.4 Results and discussion

Morphology

T7-like morphology was confirmed by TEM for phages ϕA1122, PKR, R, V and Y (Figure 2.1A-E). All 5 phages displayed icosahedral capsid arrangements with mature head diameters of 50±5 nm and short conical shaped tails. It is reported that the capsid of the phages comprising the T7-like species is composed of ~415 major and/or minor capsid proteins, while the conical tail has 6 tail fibers extending from its surface [53, 54]. Upon enlargement of the ϕA1122 and R electron micrographs tail fibers are visible (Figure 2.2 A and B) extending from the tail. Tail and tail fibers are important for phage attachment as they interact with the lipopolysaccharide (LPS) layer on the bacterial outer membrane initiating irreversible binding and subsequent infection [81].

Of the five phages investigated, only phage ϕA1122 has had its genome sequenced and suggests that the capsid is composed of major capsid (36,586 Da) and minor capsid proteins (43,028 Da) [44]. Since all five of the phages analyzed by TEM show T7-like morphology, the probability is high that the other phages not yet sequenced will have similar molecular

masses for their respective capsid proteins (See Chapter 3 for MALDI-MS characterization of capsid molecular mass determinations).

Interestingly, small spherical structures measuring 12±3 nm with a 19±4 nm spike protruding from one end of the sphere-shaped structure is also apparent in the electron micrographs enlargements of ϕA1122 (Figure 2.1A) and R (Figure 2.1C). These structures are too large to be individual capsid proteins and too small to be mature phage and appear to be consistent with precursor structures to mature phage called procapsids (Figure 2.2). Presumably these structures are assembly points for mature capsid formation of phage having icosahedral arrangements. Models predict that the assembly of an icosahedral capsid is constructed around the head to tail connector via formation of a procapsid or inner shell. A scaffolding or assembly protein is required not only in the construction of a procapsid, but also for nucleation of the coat proteins about the inner shell to form a fully mature phage [82-34]. Once assembled, the nucleic acid material is inserted through the core protein and enclosed by portal proteins.

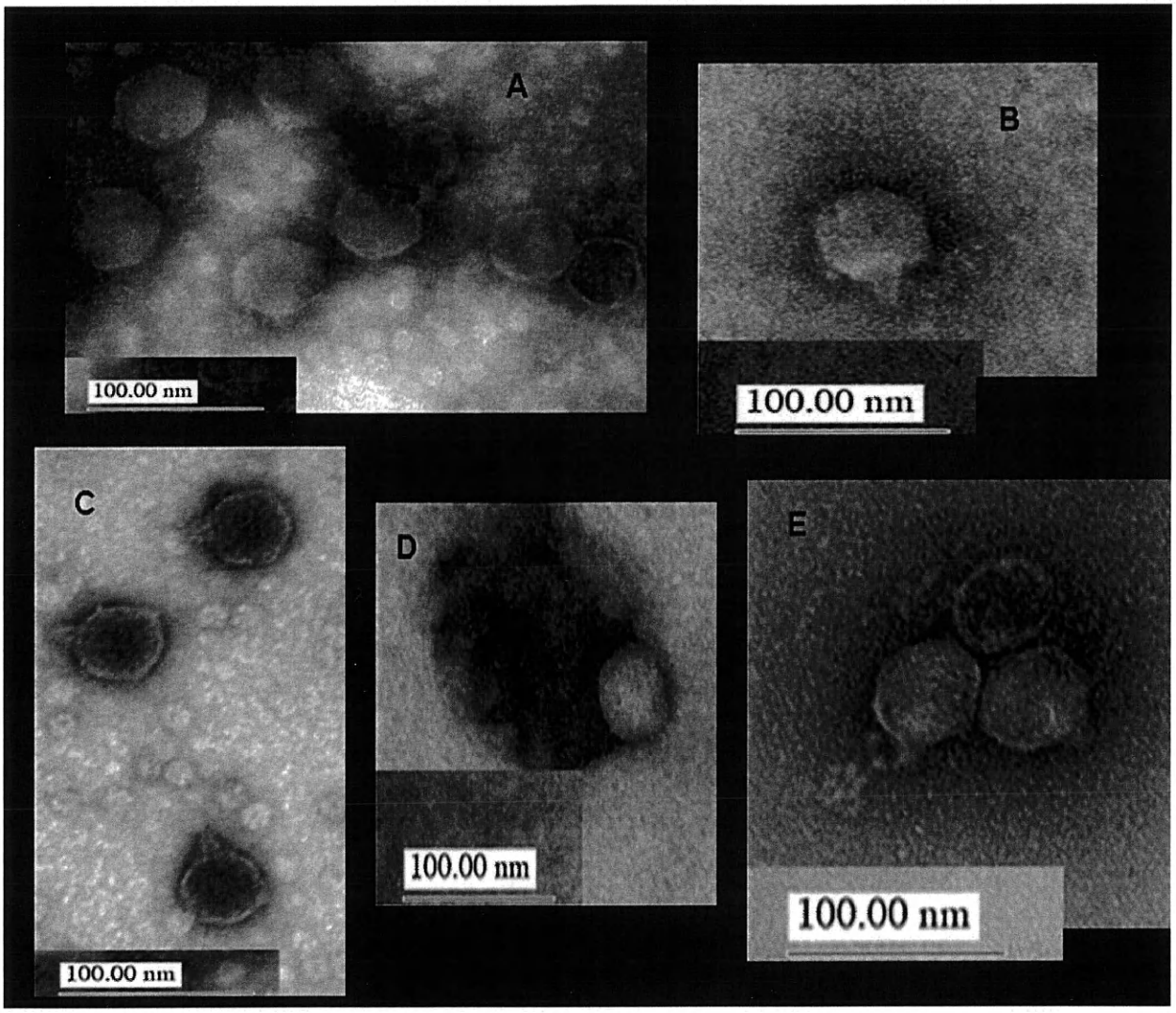

Figure 2.1 TEM of yersiniophages: (A) ϕA1122; (B) PKR; (C) R; (D) V; (E) Y.

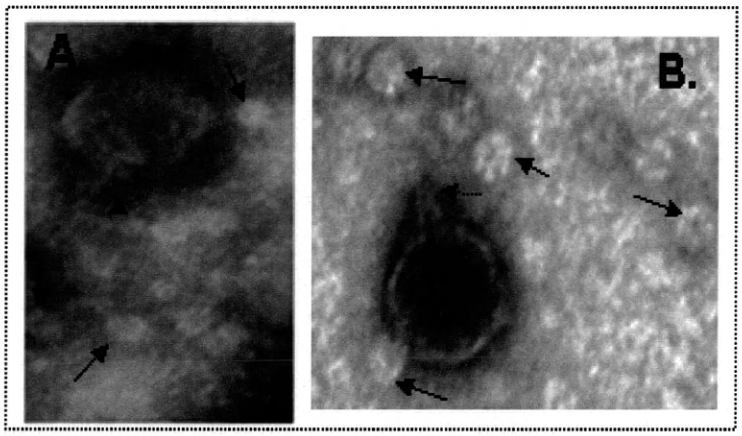

Figure 2.2 TEM enlargements of yersiniophages. Proheads and tail fibers of (A) ϕA1122 and (B) R. Solid arrows indicate proheads, while dashed arrows indicate tail fibers.

Cross reactivity and host range

The top half of Table 2.1 shows results of 6 bacterial strains outside of the Enterobacteriaceae family that were tested for lysis against the 5 yersiniophage, while the lower half gives results from 6 strains within the Enterobacteriaceae family. Of the strains tested only phage Y tested positive for lysis with *Y. enterocolitica* and *E. coli* species, while phage R tested positive for plaque formation only with the *E. coli* 15597. Interestingly, in this study no lysis was apparent with phage Y towards the *Shigella* strains tested, even though it has been reported to lyse other *Shigella* strains. Due to the positive lysis susceptibility from two of the 5 phages with *E. coli*, further cross reactivity was investigated with specific *E. coli* strains.

Of the 90 *E. coli* strains tested for cross reactivity (Table 2.2), significant phage lysis occurred for phage R (17.8%), while phage Y (10.0%) and PKR (7.7%) showed much less cross reactivity susceptibility. Phage ϕA1122 and V both exhibited the lowest cross-reactivity with *E. coli* at 5.6%. Two *E. coli* strains, ECOR 4 and ECOR 25 were lysed by all five phages, suggesting at least one common binding epitope site. While it is probable that all phage will have some cross reactivity, it is also very important to take into consideration if a particular organism will be present in significant amounts in the environment under test (e.g. samples from a bubo, blood, urine, etc.).

Forty *E. coli* environmental isolates from humans, raccoon and swine were also tested for susceptibility to phage lysis by ϕA1122, R and Y.[2.1] Lysis results were negative for all isolates with the exception of two. A swine isolate and a human isolate from the Boulder County effluent were both susceptible to lysis from all three phages tested (Table 2.3), resulting in a cross reactivity of 5% for all three phages.

Results of the susceptibility testing with *Y. pestis* isolates showed that all five phages were able to lyse each strain on the test panel with the exception of one (Table 2.4). Phage V

2.1 Phages PKR and V were not tested against the 40 environmental isolates as I obtained these phages much later in the course of investigation and after determining their very long latent periods (discussed Chapters 4-6), it was decided that further investigation was not warranted as they would not serve as useful probes.

showed resistance to lysis with *Y. pestis* strain KIM10+, which is a *mediaevalis* biovar strain that is lacking the pCD and pPCP plasmids. Phage lysis did occur, however, for all sources of the *Y. pestis* isolates tested which included human, squirrel, rodent and flea.

Conclusions

Transmission electron microscopy studies demonstrated that all five phages had similar T7 morphology. Individual procapsids are evident in both the ϕA1122 and R micrographs, which serve to help nucleate the capsid protein around the core structure to assemble fully mature phage capsids. Phage lysis susceptibility testing showed that ϕA1122 had the lowest cross reactivity while maintaining the ability to lyse all *Y. pestis* strains on the distribution panel, demonstrating that phage ϕA1122 is the most promising candidate for use in a phage based diagnostic to identify *Y. pestis*.

Table 2.1 Cross reactivity study: broad range. Bacterial strains susceptible to lysis by yersiniophages.[a,b]

Bacterial Strain	V	PKR	ϕA1122	R	Y
S. aureus, ATCC 27660	ND	ND	-	-	-
S. aureus, ATCC 27691	ND	ND	-	-	-
S. aureus, ATCC 27694	ND	ND	-	-	-
S. aureus, ATCC 27709	ND	ND	-	-	-
B. anthracis, Stern	ND	ND	-	-	-
L. monocytogene ATCC 19112	ND	ND	-	-	-
Enterobacteracea Family					
S. typhimirium ATCC 14028	ND	ND	-	-	-
S. flexneri ATCC 12022	ND	ND	-	-	-
S. dysentariae ATCC 29026	ND	ND	-	-	-
Y. enterocolitica ATCC 23715	-	-	-	-	+
Y. enterocolitica MO81-5 *lcr-*			2	ND	-
Y. pseudotuberculosis 1A	+	+	17	+	+
E. coli, ATCC 15597	-	-	-	+	+

[a] + zone of lysis or # plaque formation; - no zone of lysis or plaque formation; ND no data

[b] all bacteriophage concentrations greater than 10^{10} pfu/mL

Table 2.2 Cross reactivity: *E. coli* strains susceptible to lysis by yersiniophages[a]

E. coli strain	V	PKR	ϕA1122	R	Y	*E. coli* strain	V	PKR	ϕA1122	R	Y
ECOR 1						*ECOR 46*					
ECOR 2						*ECOR 47*					
ECOR 3						*ECOR 48*		+		+	
ECOR 4	+	+	+	+	+	*ECOR 49*					
ECOR 5						*ECOR 50*					
ECOR 6						*ECOR 51*					
ECOR 7						*ECOR 52*					
ECOR 8						*ECOR 53*					
ECOR 9						*ECOR 54*					
ECOR 10						*ECOR 55*					
ECOR 11						*ECOR 56*					
ECOR 12						*ECOR 57*				+	
ECOR 13	+	+	+	+		*ECOR 58*					
ECOR 14				+		*ECOR 59*					
ECOR 15						*ECOR 60*					
ECOR 16				+		*ECOR 61*				+	+
ECOR 17						*ECOR 62*					
ECOR 18						*ECOR 63*				+	+
ECOR 19						*ECOR 64*					
ECOR 20						*ECOR 65*					
ECOR 21						*ECOR 66*					
ECOR 22						*ECOR 67*			+		
ECOR 23						*ECOR 68*					
ECOR 24						*ECOR 69*					
ECOR 25	+	+	+	+	+	*ECOR 70*					
ECOR 26						*ECOR 71*				+	+
ECOR 27						*ECOR 72*					
ECOR 28						*XL-1Blue*				+	+
ECOR 29	+	+				*Hfr 1+*			+	+	+
ECOR 30						*ATCC 15597*				+	+
ECOR 31						*EHEC 1 0157H7*					
ECOR 32						*EHEC 2 0157H7*					
ECOR 33						*EHEC 3 0157H7*					
ECOR 34				+	+	*EHEC 4 0157H7*					
ECOR 35						*EHEC 5 O157H7*					
ECOR 36						*EHEC 6 0157*					
ECOR 37	+	+				*EHEC 7 0157*					
ECOR 38						*EHEC 8 0157*					
ECOR 39		+		+		*EHEC 9 0157*					
ECOR 40						*EHEC 10 0155H7*					
ECOR 41						*EHEC 11 AR1*				+	+
ECOR 42						*EHEC 12 0155H7*					
ECOR 43						*WSU 580 0157H7*					
ECOR 44						*WSU 400 0157H7*					
ECOR 45						*WSU 180 0157H7*					

[a]+ positive zone of lysis or individual plaque formation, - no zone of lysis or plaque formation.

Table 2.3 Cross reactivity: *E. coli* environmental isolates susceptible to lysis.[a]

Type of Environmental *E. coli* isolate, designation	ϕA1122	R	Y	Type of Environmental *E. coli* isolate, designation	ϕA1122	R	Y
Swine, SW-3-FC-041002-1	-	-	-	Human, MP-061003-3-5	-	-	-
Swine, SW-1-FC-041002-5	-	-	-	Human, MP-061003-6-3	-	-	-
Swine, SW-10-FC-070902-4	-	-	-	Human, MP-061003-2-1	-	-	-
Swine, SW-15-FC-070902-2	+	+	+	Human, MP-061003-2-2	-	-	-
Swine, SW-11-FC-070902-1	-	-	-	Human, MP-061003-2-8	-	-	-
Swine, SW-4-FC-041002-5-1	-	-	-	Human, MP-061003-3-3	-	-	-
Swine, SW-15-FC-070702-3	-	-	-	Human, MP-061003-3-1	-	-	-
Swine, SW-5-FC-041002-1	-	-	-	Human, MP-061003-3-2	-	-	-
Swine, SW-4-FC-041002-1	-	-	-	Human, MP-061003-6-1	-	-	-
Swine, SW-2-FC-041002-2	-	-	-	Human, MP-061003-5-8	-	-	-
Raccoon, Rccn-23-BC-070203-2	-	-	-	Human, Beff-28-BC-073003-1	-	-	-
Raccoon, Rccn-21-BC-070203-2	-	-	-	Human, Beff-26-BC-073003-1	-	-	-
Raccoon, Rccn-18-BC-070203-6	-	-	-	Human, Beff-21-BC-073003-5	-	-	-
Raccoon, Rccn-16-BC-070203-4	-	-	-	Human, Beff-14-BC-061203-1	-	-	-
Raccoon, Rccn-14-BC-041303-2	-	-	-	Human, Beff-15-BC-061203-3	-	-	-
Raccoon, Rccn-12-BC-041303-1	-	-	-	Human, Beff-02-BC-032703-4	-	-	-
Raccoon, Rccn-09-BC-041303-5	-	-	-	Human, Beff-06-BC-032703-1	-	-	-
Raccoon, Rccn-08-BC-041304-5	-	-	-	Human, Beff-16-BC-061203-3	-	-	-
Raccoon, Rccn-01-BC-041303-2	-	-	-	Human, Beff-21-BC-073003-2	+	+	+
Raccoon, Rccn-19-BC-070203-3	-	-	-	Human, Beff-18-BC-061203-1	-	-	-

[a]+ positive zone of lysis or individual plaque formation, - no zone of lysis or plaque formation.

Table 2.4 Natural host range study: *Y. pestis* isolates susceptibility to lysis by yersiniophages

Y. pestis strains	Source	Biovar	Phage V	Phage PKR	Phage ϕA1122	Phage R	Phage Y
ZE94	Human	*Orientalis*	+	+	+	+	+
CO92	Human	*Orientalis*	+	+	+	+	+
KIM 10+	Human	*Mediaevalis*	-	+	+	+	+
A1122	Squirrel	*Orientalis*	+	+	+	+	+
Nepal 516	Human	*Antiqua*	+	+	+	+	+
Harbin 35	Human	*Mediaevalis*	+	+	+	+	+
PB6	Flea	*Orientalis*	+	+	+	+	+
PE xu 2	Rodent	*Orientalis*	+	+	+	+	+
UG05-0454	Human	*Orientalis*	+	+	+	+	+
MG05-1020	Human	*Orientalis*	+	+	+	+	+

[a] + zone of lysis or # plaque formation; - no zone of lysis or plaque formation; ND no data

[b] all bacteriophage concentrations greater than 10^{10} pfu/mL

CHAPTER 3
IDENTIFICATION AND SELECTION OF A YERSINIOPHAGE PROTEIN BIOMARKER BY MALDI-MS DETECTION

"One sometimes finds what one is not looking for"

Alexander Fleming

3.1 Abstract

Detection of the progeny phage produced during phage amplification by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), requires that a protein biomarker be chosen and monitored over the course of the event. Since phage genomes are relatively small and many of the genes are allocated for coding non-structural functions, phage synthesize a few structural proteins in large numbers rather than producing a small number of many different proteins. Structural proteins produced in high copy numbers make them the reasonable choice for monitoring amplification by MALDI-MS. The aim of this investigation was to identify and compare selected MALDI-MS protein profiles of semi-purified and purified yersiniophages ϕA1122, PKR, R, V and Y in order to select an appropriate biomarker to monitor the phage amplification event. Results indicate that phage ϕA1122 produces a very intense mass to charge signal at 15.8 kDa, known from the

literature as being due to the head assembly protein. The head assembly protein has been found to occur at a distinct molecular mass when compared to *Y. pestis* A1122 bacterial proteins, providing an excellent biomarker for monitoring the amplification event by MALDI-MS detection.

3.2 Introduction

A major challenge in analyzing biomolecules with matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) is sample preparation. Many sample preparation methods include various detergents and buffers as well as organic and inorganic salts which can be detrimental to the MALDI-MS signal. Several MALDI-MS studies have been conducted in the presence of detergents [85-88]. For example, Zhang and Li [88] compared the quality of the MALDI signal using the detergents sodium dodecyl sulfate (SDS) and ammonium dodecyl sulfate (ADS) on several proteins with molecular weights ranging from 5-80kDa. Results showed that the protein samples containing up to a 1% SDS concentration could be analyzed by MALDI, but the signals were severely degraded due to peak broadening and adduct formation. ADS also showed detrimental effects on the MALDI signal in terms of peak broadening and mass accuracy, but to a lesser extent than SDS.

In addition to the difficulty of choosing an appropriate type of sample preparation method for analysis of biomolecules, MALDI analysis of phages presents a unique challenge because viruses (including phage) replicate at the expense of host cells from plants, animals, or bacteria creating a mixture of proteins or lysate. Therefore in order to detect progeny phage proteins produced during an amplification event by MALDI-MS detection, a phage protein biomarker that is distinctly different from bacterial proteins must be chosen and monitored over time.

Since phage are structurally limited in the amount of DNA that they can contain, theoretically structural proteins such as assembly or capsid proteins that are produced in high

copy numbers are excellent biomarker candidates for monitoring the amplification process. For instance, T7-like bacteriophage are structurally composed of an icosahedral capsid, short conical tail and a connecting region at one of the vertices called a head to tail connector [84]. The capsid protein of the T7 phage has a molecular mass of 36,647 Da [84, 89], while the major capsid protein for phage ϕA1122 has a molecular mass of 36,588 Da [44]. Since a fully mature phage requires ~415 capsid proteins for full assembly of a mature nuecleocapsid [89-91] and MALDI can produce singly ionized high molecular masses, the ionization of the capsid proteins should be easily detected by MALDI-MS [92].

This research focused on identifying and comparing selected MALDI-MS protein profile spectra of phages ϕA1122, PKR, R, V and Y. Comparison of semi-purified and purified phage preparations is discussed to determine which structural protein will produce a useful biomarker for monitoring the bacteriophage amplification process by MALDI-MS.

3.3 Materials and methods

Phage semi-purification and purification

A modified procedure of that used by Sambrook [93] for polyethylene glycol (PEG) precipitation and cesium chloride (CsCl) gradient purification of phage samples was used. To 500 mL of 0.22 micron filtered phage stock 50 g of PEG 8000 (10% w/v) and 30 g of NaCl were dissolved at room temperature with stirring or shaking. The sodium chloride was added to promote the dissociation of phage particles from any nucleic acids remaining in solution. The mixture was then iced for no less than one hour or refrigerated overnight to precipitate the phage particles. Precipitated phage were pelletized by centrifugation (11,000 x g for 15 min at 4°C) and resuspended in 8 mL of 0.85% normal saline solution. An equal amount of chloroform was added and the solution was vortexed for 30 seconds and centrifuged at 4,000

x g for 15 minutes at 4°C. The aqueous phase (top layer) containing phage particles was collected and considered as semi-purified.[3.1]

To obtain a purified preparation, PEG-precipitated phage samples were subjected to a CsCl equilibrium gradient. To a 35 mL polyallomar tube containing 5-7 mL of phage, a 4 step CsCl:TE gradient was prepared and underlaid in the following order: 1:2; 1:1, 2:1, 1:0. The gradients were ultra-centrifuged for 24 hours at 25,000 rpm using a SW-28 rotor and LM-8 Beckman Ultracentrifuge. Purified phage bands were collected above the 2:1 layer, dialyzed against 0.85% saline and 0.2 μm filter sterilized before analysis by MALDI-MS.

MALDI preparation

Ferulic acid (15 mg/mL) in a 17:33:50 mixture of 88% formic acid, acetonitrile and de-ionized water solution was utilized as the matrix [94]. Mass spectra were obtained with a 337 nm N_2 laser in linear mode using a PerSeptive Biosystems Voyager-DE STR+ MALDI-TOF-MS (Applied Biosystems, Inc. Framingham MA, USA). Samples were applied to a hydrophobic target plate using the dried droplet method in a sandwich fashion as follows: 0.5 μL of matrix: 0.5 μL of sample: 0.5 μL of matrix.

The following parameters were used to collect spectra: accelerating voltage 25kV; grid voltage 75%; delayed extraction time of 100 ns; and 2 kDa low mass ion gate. Mass spectra were acquired as an average of 150 laser shots taken from 3 replicate sample spots (50 shots per spectrum). Raw data from Data Explorer (Applied Biosystems, Inc. Framingham, MA, USA) was exported into SigmaPlot 7.0 (Point Richmond, CA USA) for spectral comparison.

3.4 Results and discussion

The MALDI-MS spectral profile comparison for phage ϕA1122 is shown for the semi-purified preparation in Figure 3.1A and the purified preparation in Figure 3.1B. The singly

[3.1] Polyethylene glycol precipitated samples after differential centrifugation are considered semi-purified preparation since it is possible that bacterial debris could precipitate in this preparation as well.

charged major capsid protein is obtained at a mass of 36.6kDa in both spectra correlating extremely well to the calculated sequenced mass of the major capsid protein for this phage [44]. The gradient-purified preparation clearly shows the presence of a doubly charged capsid protein at a mass of 18.3kDa. The signal intensity is consistently stronger for the capsid protein in the gradient purified preparation than the semi-purified preparation. Interestingly, the singly charged capsid protein and its doubly charged species almost disappear relative to a large ionizing signal obtained at 15.8kDa in the semi-purified phage preparation (Figure 3.1A), while the purified phage spectrum clearly lacks the 15.8kDa signal. An SDS-PAGE protein banding profile of the semi-purified ϕA1122 sample preparation (Lane 4, Figure 3.2) also indicates a large quantity of the capsid protein present at ~36kDa, while only a faint band is apparent between 15-16kDa.

One explanation for the reduced signal intensity at the capsid molecular mass can be attributed to an occurrence known as competitive ionization [92]. This phenomenon commonly known as preferential ionization is characterized by the suppression of a protein's molecular ion when ionized in the presence of other proteins in a mixture. Protein signals, like the capsid protein signal, may almost disappear or not even be detectable in the presence of a preferentially ionizing protein. However when the capsid protein is analyzed without the preferential ionizing species present or analyzed individually, the protein is easily ionized and detectable.

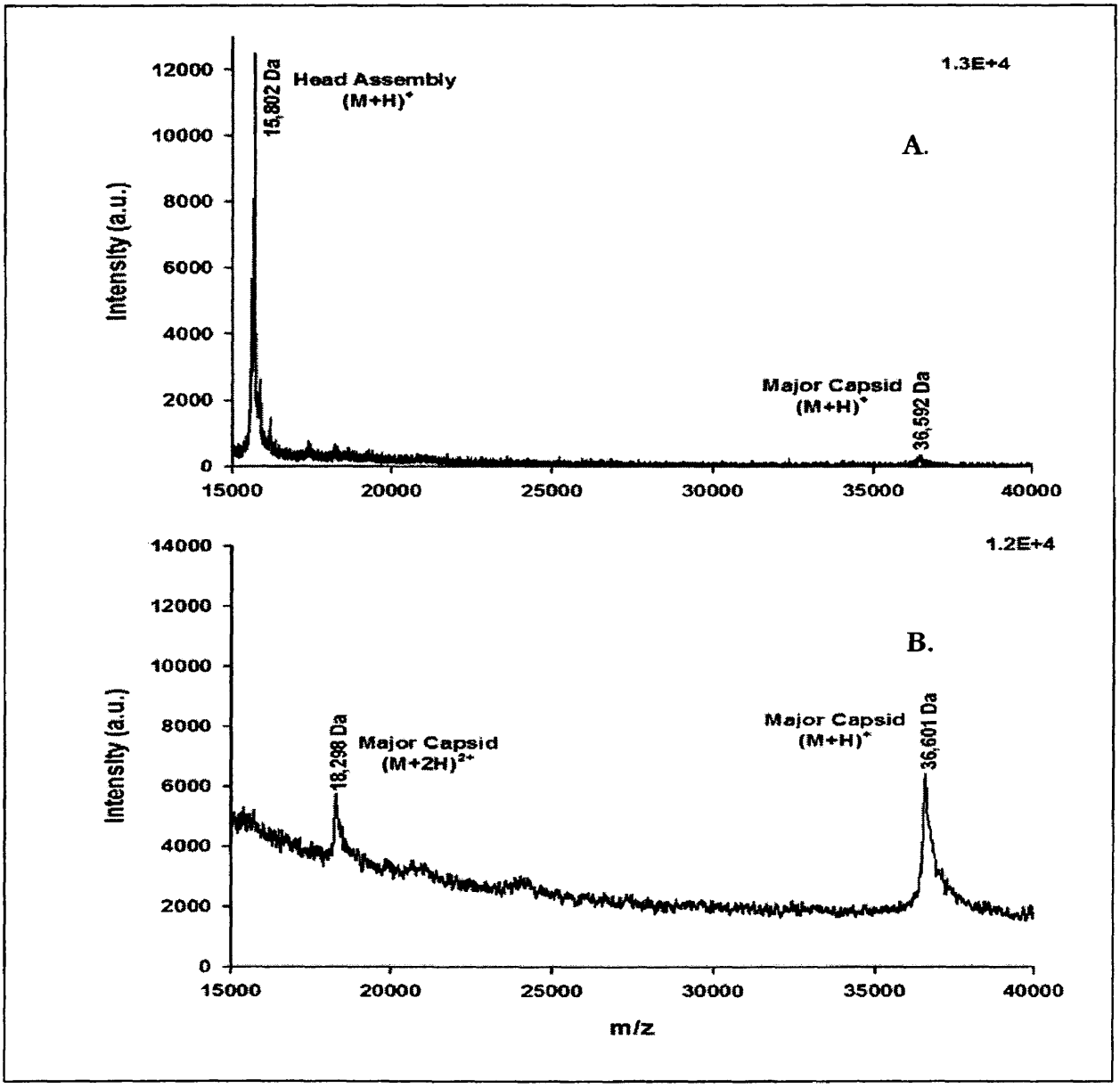

Figure 3.1 MALDI-MS spectra of phage ϕA1122. A) PEG semi-purification and B) CsCl equilibrium gradient purified in the mass range of 15-40kDa.

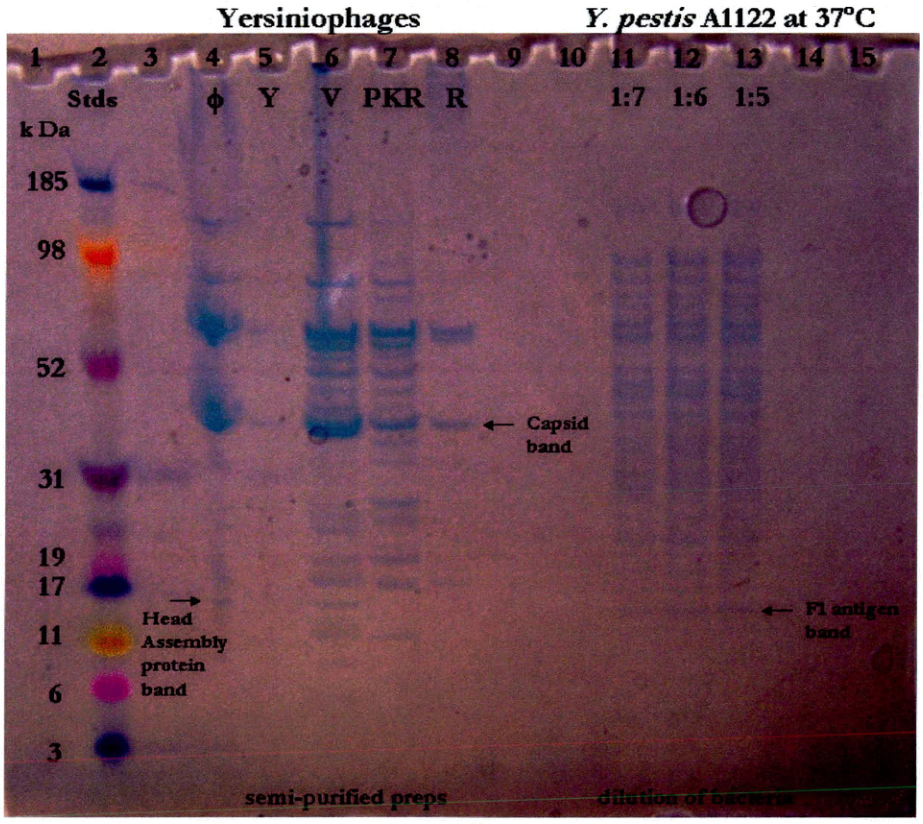

Figure 3.2 SDS-PAGE protein profiles of phage preparations and *Y. pestis* A1122 grown at 37°C. Lane 2 molecular weight standards; lane 4 through 8 PEG semi-purified of phages ϕA1122, Y, V, PKR, and R . Lanes 11-13 show different dilution of *Y. pestis* A1122 incubated at a temperature of 37°C.

While preferential ionization is a reasonable explanation for the reduced signal intensity of the capsid protein, another possibility maybe that the 15.8kDa protein co-precipitated with the phage and is bacterial in origin. In an attempt to establish this premise, the SDS-PAGE protein banding from *Y. pestis* A1122 grown at 37°C (Lanes 11-13) was compared to the ϕA1122 phage banding profiles (Figure 3.2). The semi-purified phage preparation profile shows a faint migration band apparent at ~15-16 kDa (lanes 3 and 4), while the *Y. pestis* A1122 grown at 37°C (lanes 12-14) band in the same region indicates a slightly faster migration rate suggesting these two bands are different proteins with similar molecular masses. While the low resolution of the gel makes it nearly impossible to draw any definitive conclusions, MALDI-MS spectra clearly distinguish the differences between bacterial and phage molecular masses. The mass spectrum obtained for *Y. pestis* A1122 at 37°C (Figure 3.3) clearly indicates the presence of a 15.5kDa protein. Signals obtained from several independent *Y. pestis* A1122 bacterial samples show that the 15.5kDa signal is consistently obtained within ±50 Da, while the 15.8kDa signal obtained from the ϕA1122 phage is consistent within ±10 Da. It is well known that at temperatures greater than 33°C *Y. pestis* expresses the F1 capsular protein and the 15.5kDa signal is a result of that expression [24]. A MALDI-MS spectrum of *Y. pestis* A1122 grown at 28°C is shown in Figure 3.4 and clearly establishes that the 15.8kDa signal is of phage origin. Sequencing data shows that this signal correlates to the host assembly protein from the ϕA1122 phage [44]. Refer to Appendix C for the ϕA1122 phage host assembly protein sequence.

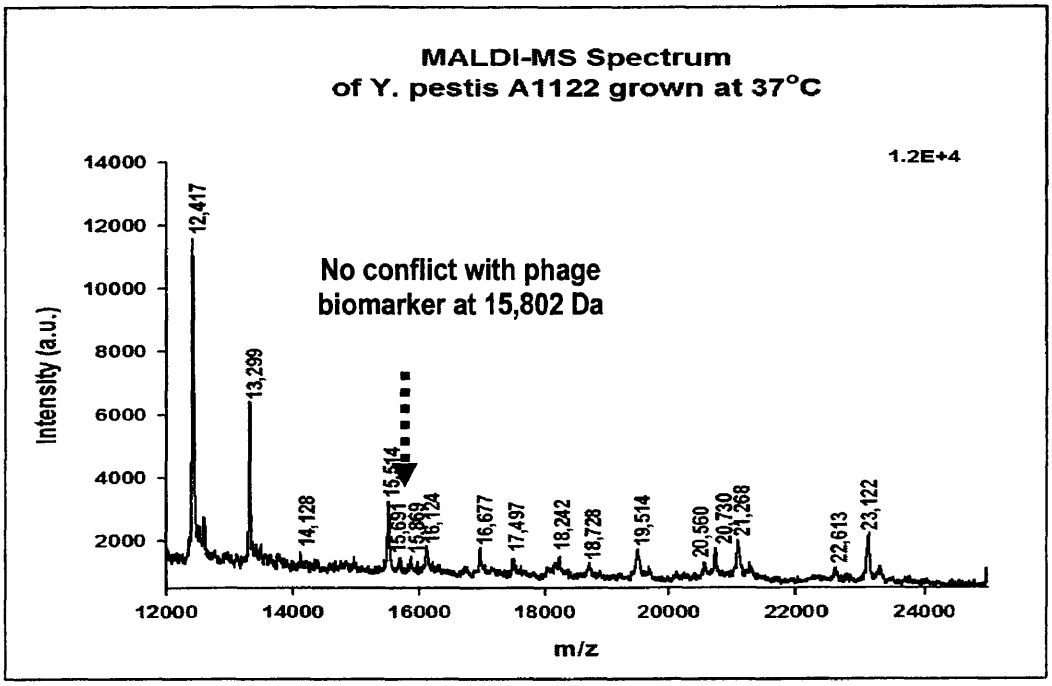

Figure 3.3 MALDI-MS control spectrum of *Y. pestis* A1122 bacterial cells grown at 37°C, in the mass range of 12-20kDa. Cells were taken from a liquid culture at an OD = 0.4 , washed twice in PBS, pelleted and re-suspended in 0.85% normal saline solution for analysis.

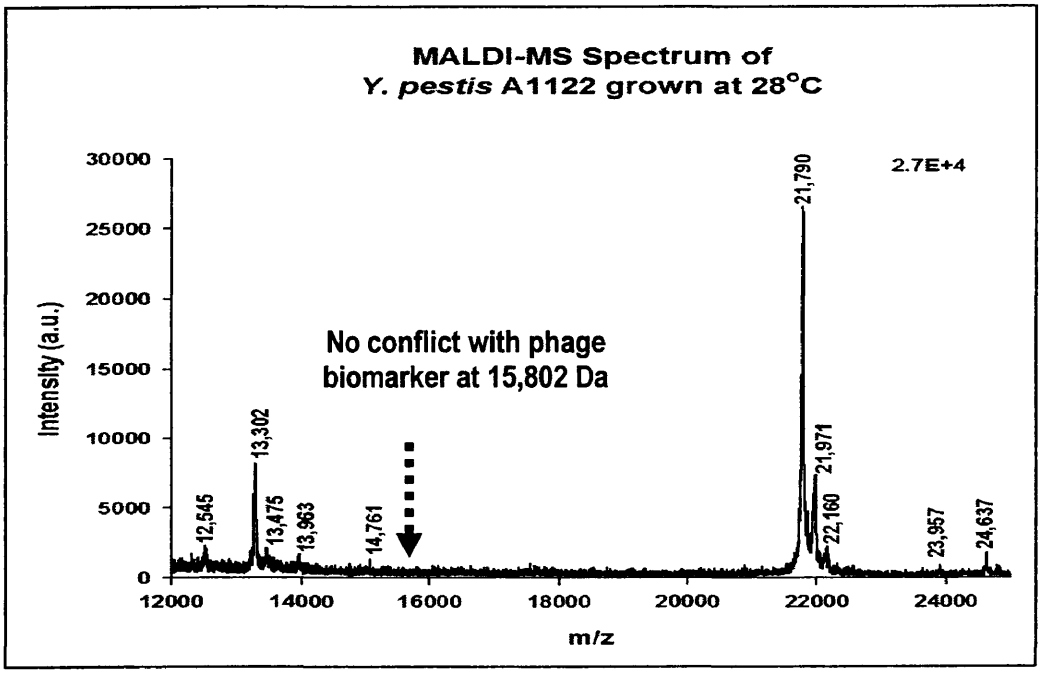

Figure 3.4 MALDI-MS control spectrum of *Y. pestis* A1122 bacterial cells grown at 28°C, in the mass range of 12-20kDa. Cells were taken from a liquid culture at an OD = 0.4 , washed twice in PBS, pelleted and re-suspended in 0.85% normal saline solution for analysis.

An additional question exists as to why this 15.8kDa head assembly protein signal is not apparent in the purified phage MALDI-MS spectrum. Speculation that this protein may be soluble and/or is degraded via proteases over time is a possibility. If the protein is freely soluble, purification based on density gradients would cause this protein to band out at a lighter density, likely with incomplete phage components, rather than at the heavier density band of the fully assembled phage particles collected for analysis. On the other hand proteases cannot be ruled out as re-analysis of the semi-purified phage preparations by MALDI-MS over the course of several months indicates a decrease and eventual absence of the 15.8kDa signal. However because no loss in phage infectivity was observed over the same time period (phage titers remained stable) degradation by proteases is considered a more remote justification.

The most plausible explanation for the absence of the 15.8kDa signal in the purified MALDI-MS spectrum suggests that the head assembly protein becomes internalized or degraded upon completion of assembling a fully mature capsid [90, 91, 95, 96]. The assembly of an icosahedral capsid is known to be constructed around the head to tail connector via formation of a procapsid or inner shell. A scaffolding or assembly protein, like the head assembly protein of phage ϕA1122, is not only required in the construction of a procapsid, but also for nucleation of the coat proteins about the inner shell to form a fully mature phage. Assembly proteins are typically not found as part of mature phage and may be internalized as the assembly of mature capsid proceeds via nucleation of ~415 capsid proteins [97] on the outside and assembly proteins on the inside of the inner shell, shielding them from ionization. Evidence supporting this theory is shown in TEM micrographs of the two different ϕA1122 phage preparations (Figure 3.5). The semi-purified micrograph exhibits a high concentration of apparent intermediate-sized procapsid structures (Figure 3.5A), while the micrograph of the purified phage preparation lacks these small structures (Figure 3.5B).

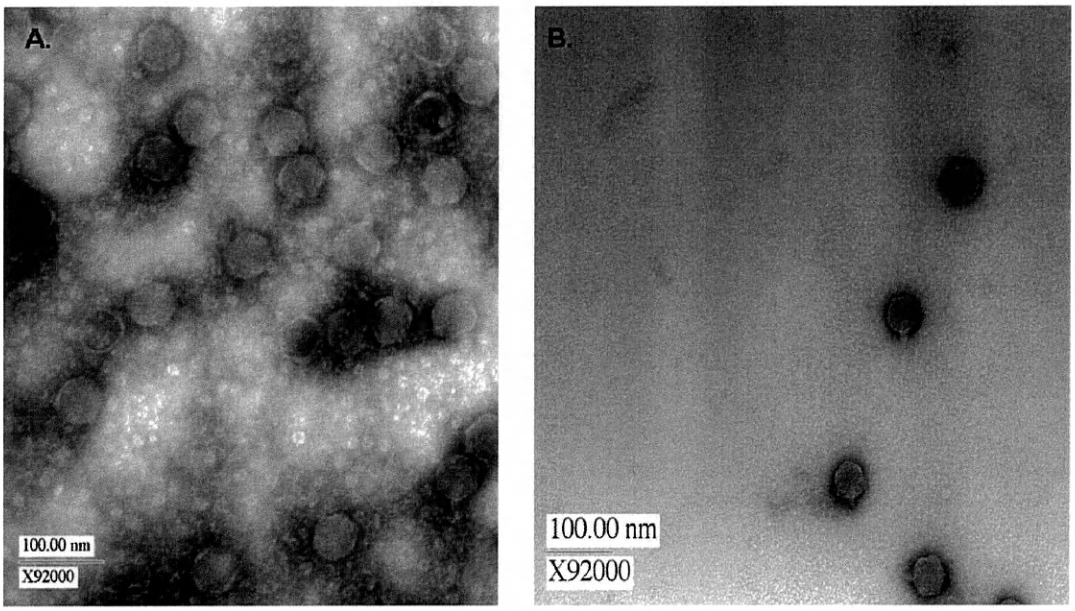

Figure 3.5 TEM of phage ϕA1122 preparations of A) semi-purification; B) CsCl equilibrium gradient purification.

The MALDI-MS spectral profile comparison for phage Y is also shown for the semi-purified preparation (Figure 3.6A) and the equilibrium gradient purified preparation (Figure 3.6B). Like the ϕA1122 semi-purified phage, the semi-purified phage Y preparation may preferentially ionize a similar peak at 15.9kDa or be the result of a high concentration of procapsids as the singly charged capsid protein intensity becomes severely diminished and the doubly charged species is completely inhibited (Figure 3.6A). Phage Y, when gradient purified and analyzed autonomously from other proteins, also easily ionizes the singly and doubly charged capsid protein at 36.6kDa and 18.3kDa respectively (Figure 3.6B). However the purified phage also ionizes a 15.5kDa protein which cannot be distinguished from that of the expressed F1 antigen protein of *Y. pestis* at temperatures greater than 33°C and therefore makes it an unlikely biomarker candidate for producing the phage amplification event with MALDI-MS detection.

Additional analysis of purified phages V (Figure 3.7) and PKR (Figure 3.8) as well as the semi-purified phage R (Figure 3.9) preparations were also analyzed by MALDI-MS as potential candidates for producing the amplification event. However, because of other studies which showed high cross reactivity rates via phage lysis susceptibility testing or extremely long latent periods, these phages were not investigated further as biomarker candidates.

Conclusions

A summary in Table 3.1 lists the proteins ionized by MALDI-MS for each phage sample preparation in the mass range of 15-40kDa. Of the five phages analyzed by MALDI-MS, phage ϕA1122 produced the strongest ionizing protein biomarker signal at 15.8 kDa. Phage gene sequencing data for ϕA1122 shows that the 15.8kDa biomarker corresponds to the phage head assembly protein [44]. MALDI-MS spectra indicate that the head assembly protein preferentially ionizes in the presence of other proteins, while TEM suggests that a high concentration of proheads may be attributed to the intense signal at this molecular mass in the semi-purified phage preparations. It is possible that even a synergistic combination of

both phenomena is producing the intense signal at this molecular mass in the ϕA1122 mass spectrum. Differences in SDS-PAGE migration rates and MALDI-MS mass-to-charge ratios show that the host assembly protein can be differentiated from that of the host bacteria F1 capsular protein when the bacteria is grown at temperatures greater than 33°C and demonstrates that this biomarker is unique to the phage and the most appropriate choice for monitoring the phage amplification process by MALDI-MS.

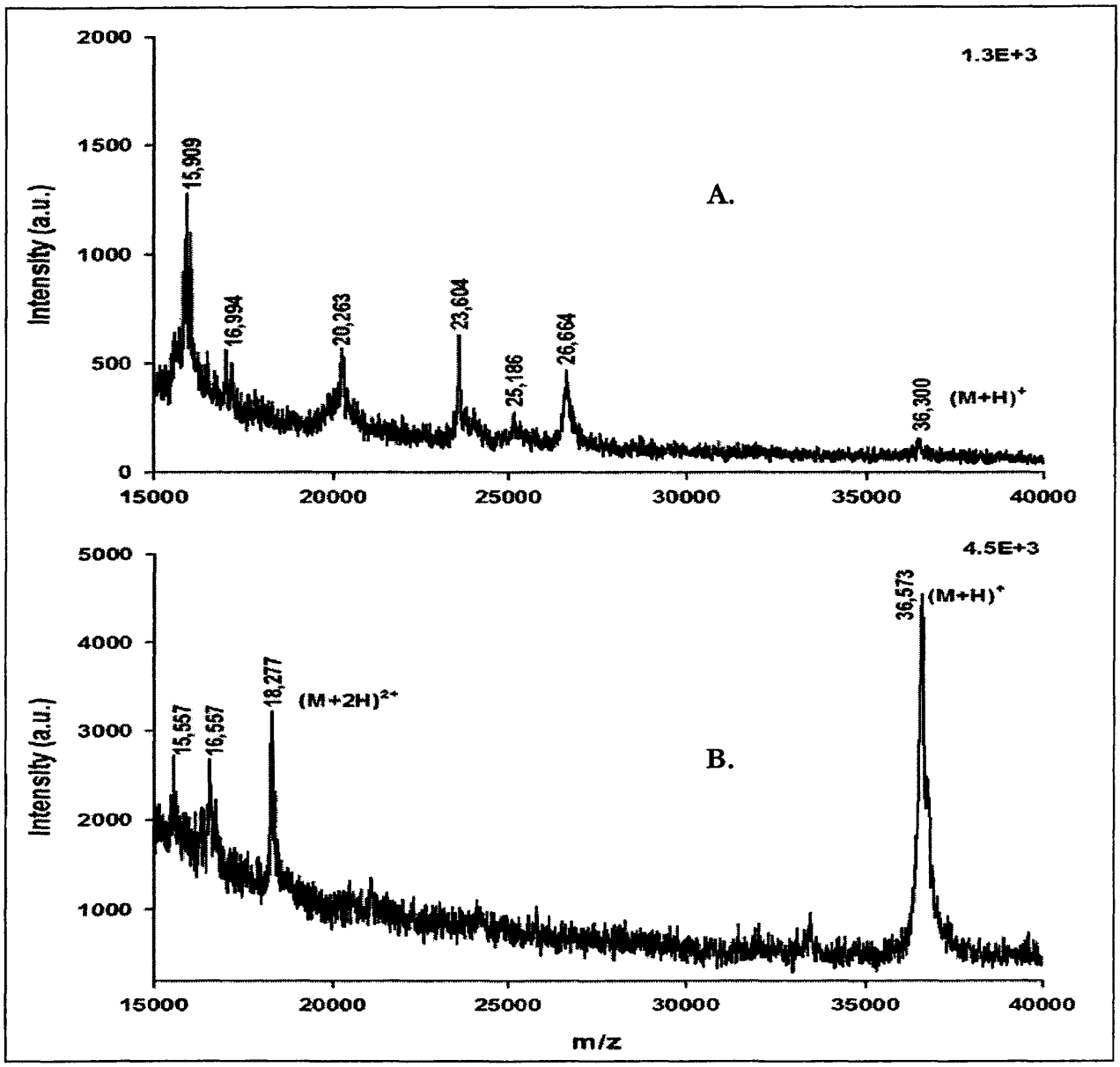

Figure 3.6 MALDI-MS spectra of phage Y. A) PEG semi-purification and B) CsCl equilibrium gradient purified in the mass range of 15-40kDa.

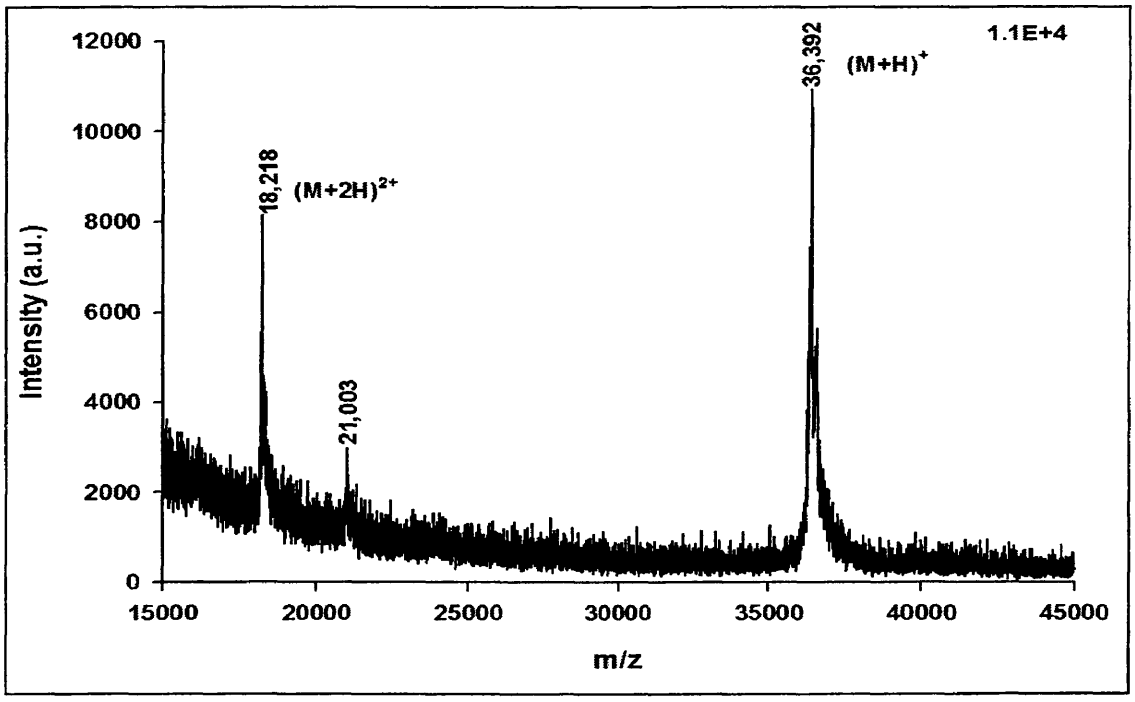

Figure 3.7 MALDI-MS spectrum phage V in the mass range of 15-45kDa, phage purified by CsCl equilibrium gradient.

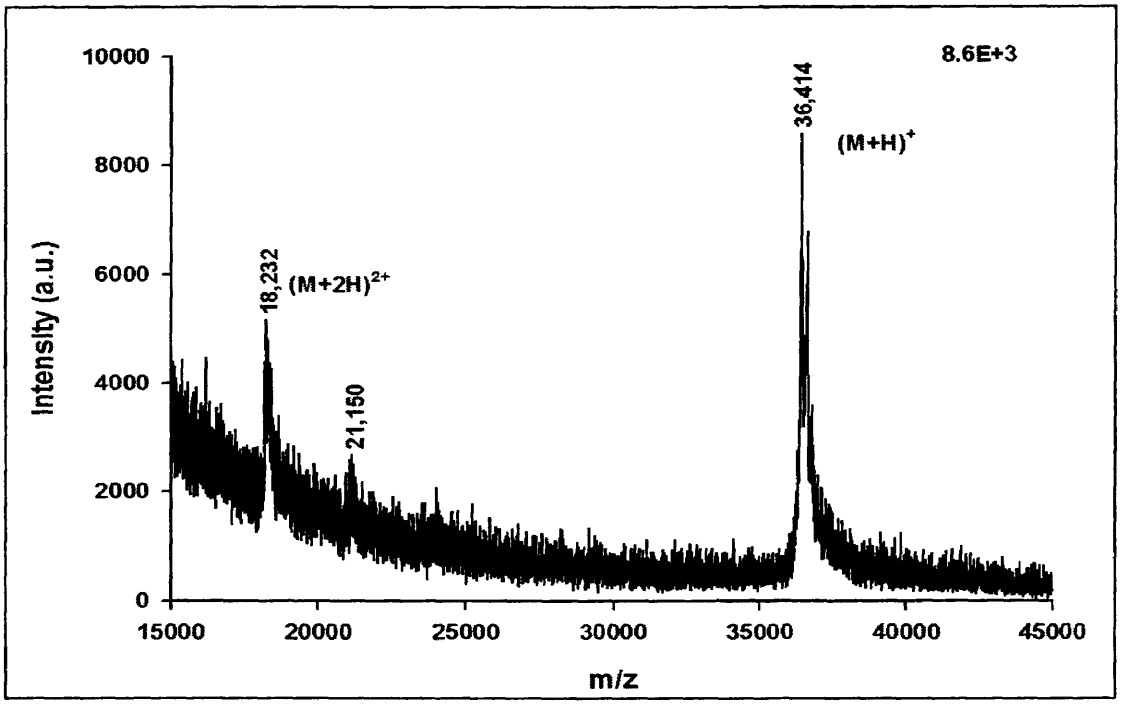

Figure 3.8 MALDI-MS spectrum of phage PKR, in the mass range of 15-45kDa, phage purified by CsCl equilibrium gradient.

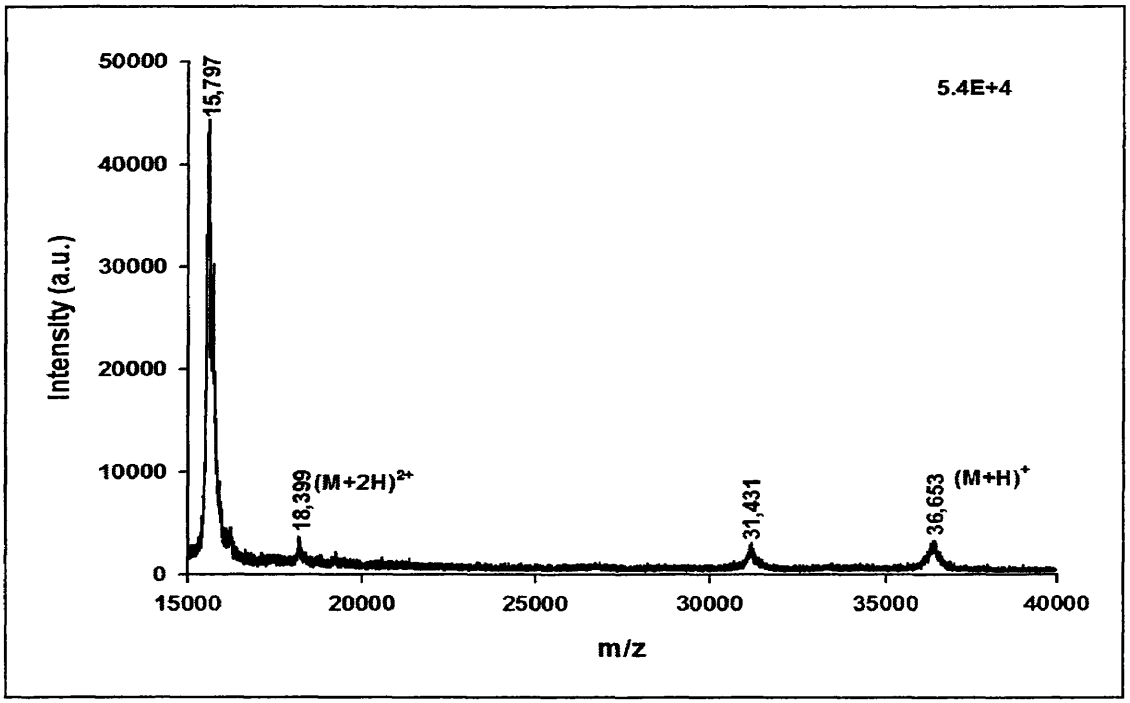

Figure 3.9 MALDI-MS spectrum of phage R, in the mass range of 15-40kDa, semi-purified by PEG.

Table 3.1 Summary of major protein peaks ionized by MALDI-MS.

Phage	Major Proteins Peaks* Observed From Purified Phage Preparation (kDa)	Major Protein Peaks* Observed From Semi-purified Phage Preparation (kDa)
ϕA1122	36,601 18,298	36,592 18,296 15,802
Y	36,573 18,277 16,557 15,557	36,300 26,664 23,604 20,263 16,994 15,909
V**	36,392 21,003 18,218	28,311 23,627 15,914
PKR**	36,414 21,150 18,232	28,298 23,616 15,915
R	NA	36,653 31,431 18,399 15,597

* Proteins peaks listed are restricted to the mass-to-charge range between 15-40 kDa. NA not analyzed

**Note that semi-purified spectra of phages V and PKR are not included in the results and discussion

CHAPTER 4
ONE STEP GROWTH EXPERIMENTS TO DETERMINE BURST SIZE FOR THREE YERSINIOPHAGES WITH *YERSINIA PESTIS* STRAIN A1122

"Numquam ponenda est pluritas sine necessitate"
William of Ockham (1285–1349)

Translation...

Given two equally predictive theories choose the simpler one,
the one with fewer unnecessary assumptions...Ockham's Razor

4.1 Abstract

Sensitivity of the phage amplification signal is linked to the number of overall progeny phage produced. Since a detectable phage signal is inherently dependent upon the threshold of detection of a given detection system, whether that be matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) or immunochromatography strips (ICS), one parameter which can potentially decrease the detection threshold is the amount of phage produced per bacterium (burst size) during the infection, replication and lytic cycle. It has been hypothesized that a large burst size is ideal in decreasing the detection limit. Thus an investigations using one-step growth experiments was conducted to determine the effects of

inducing large burst sizes as a function of the multiplicity of infection. Results using *Yersinia pestis* A1122 and three different yersiniophages (ϕA1122, R and Y) show that high multiplicity of infection which apparently give larger burst sizes when assayed by one step growth experiment actually induces a phenomenon referred to as *lysis from without*, producing overall less phage per bacterium.

4.2 Introduction

It is well known that a single bacterium can adsorb many phage particles and this is referred to as the multiplicity of infection (MOI). Ellis and Delbrück were the first to describe a one-step growth experiment which allowed study of phage proliferation within individually infected bacteria, rather than in mass cultures [98]. Their classic one-step growth experiment is defined by the latent period, rise time, and the plateau period (Figure 4.1), where the termination of the latent period indicates the onset of cell lysis, rise time refers to the increase in number of progeny phage, and the plateau period refers to the maximum number of free phages [99]. The process begins when a phage adsorbs to its target bacterium and injects its DNA. Over the course of the next several minutes (15-20 minutes), enzymes are synthesized for DNA replication followed by production of newly synthesized DNA. Once DNA is synthesized, the next 20 minutes (or longer) is dedicated to synthesizing structural phage components and assembly of those components into fully mature phage. Upon completion, the bacteria begin to burst or release progeny phage into the surrounding milieu. Once the bacterium has completed lysis, a plateau is reached and denotes maximum progeny phage released from the bacterium. The difference in plaque formation between the plateau of infectivity and the onset of lysis is known as the burst size.

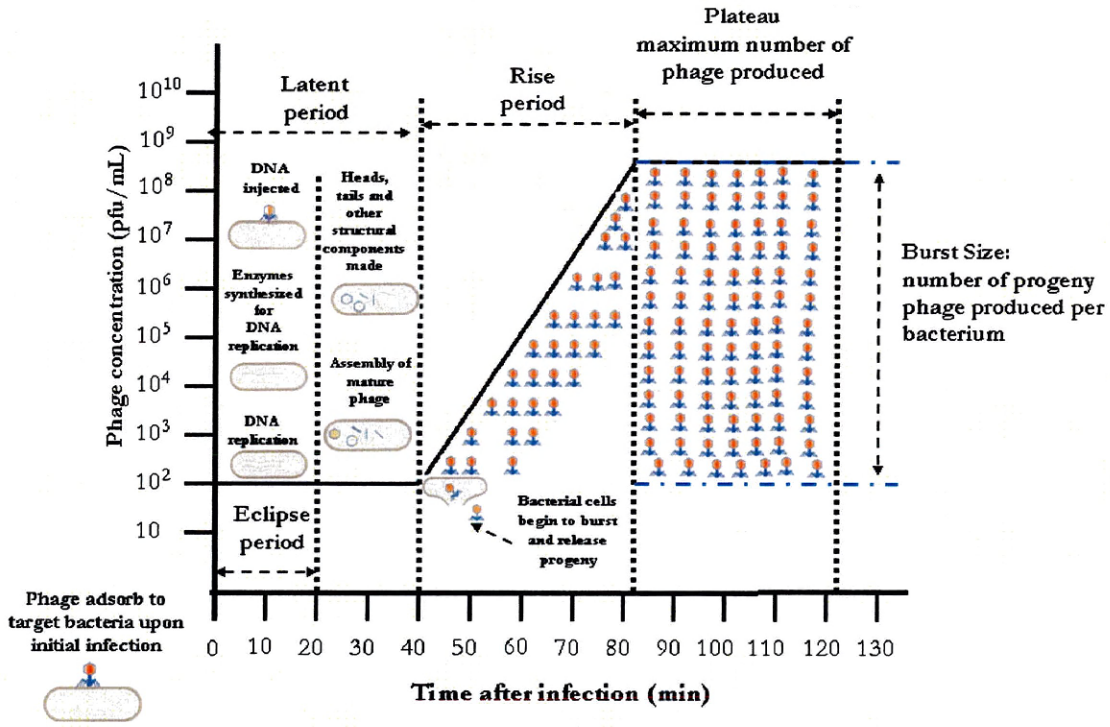

Figure 4.1 Stages in one-step growth experiments.

Furthermore, Delbrück was able to show that there exists completely different mechanisms by which phage can lyse a susceptible bacterial cell [100, 101]. One of these mechanisms is referred to as *lysis from without* and is often encountered when the MOI is much greater than one phage per bacterium. Loss of input phage due to adsorption, rather than progeny phage proliferation occurs from this form of lysis. The other mechanism of lysis, which is referred to as *lysis from within*, is the form of lysis which is properly connected with intracellular progeny phage proliferation, and its onset is signaled by the end of the latent period (also referred to as the onset of the lysis event).

Experimentally in one-step growth experiments, a culture of bacterial cells is mixed with a phage suspension at a low phage MOI. Subsequently initial infections are diluted substantially to assure no secondary adsorption events can occur. Samples are withdrawn periodically over the course of the event and plated onto a lawn of sensitive bacteria. After overnight incubation the plaques were counted to determine burst size. Results from one-step growth experiments using *Y. pestis* A1122 and three different yersiniophages (ϕA1122, R and Y) show that the use of large amounts of phage upon initial infection induces the phenomenon referred to as *lysis from without* which proliferates very few or no progeny phage whatsoever.

4.3 Material and methods

One-step growth curves were generated in order to determine the burst size. Optical density measurements (λ=620 nm) were correlated to viable cell counts to determine bacterial concentrations at time of initial infections. To perform burst size experiments, 2 mL of *Y. pestis* culture and 0.1mL of phage stock were added to a culture tube, incubated at 37°C with shaking and time recorded as "time zero" to indicate initial adsorption of the phage to the bacteria. After 5 minutes, 0.1mL of the phage/bacterial mixture was transferred to a second adsorption culture tube containing 9.9 mL of fresh media and incubated at 37°C with shaking for an additional 10 minutes. After the cumulative 15 minute adsorption

period, 0.1 mL of the mixture in the second adsorption tube was transferred to another culture tube containing the 20 μL of chloroform and 9.9 mL of additional fresh media broth to ensure complete cell lysis. Next, 0.1mL of the mixture was transferred and further diluted in another tube containing 9.9 mL of growth media. Subsequently, 0.1mL of this diluted mixture was transferred to a tube containing 300 μL of *Y. pestis* (OD_{620} = 0.4) in 3 mL of soft agar and plated onto a Petri dish, allowed to harden, and incubated at 37°C overnight. This procedure was performed at 5, 10, 15, or 30 minute intervals over a period of 1-4 hours [102]. Plaques from each plate were counted and plotted as a function of time the following day. The resulting data was input into Sigma Plot 7.0 (SPSS Inc., Chicago, Ill.) to compare the MOI effects.

4.4 Results and discussion

Figures 4.2 – 4.4 show results of a 65 minute timed study that was conducted with 3 different yersiniophages (ϕA1122, R and Y) during which the concentration of phage was varied in order to give the appropriate MOI. At first glance interpretation of the results from the one-step infection experiments may lead to the conclusion that an increased burst size can be obtained by increasing the amount of phage relative to the amount of bacteria used upon initial infection. However this would only be true if one assumes that only one phage particle had caused the increase and the others progeny particles released upon lysis during the event were essentially "inactivated" and played no role in the infection process. However this is truly not the case. In fact what is occurring is a classic case of the *lysis from without* mechanism.

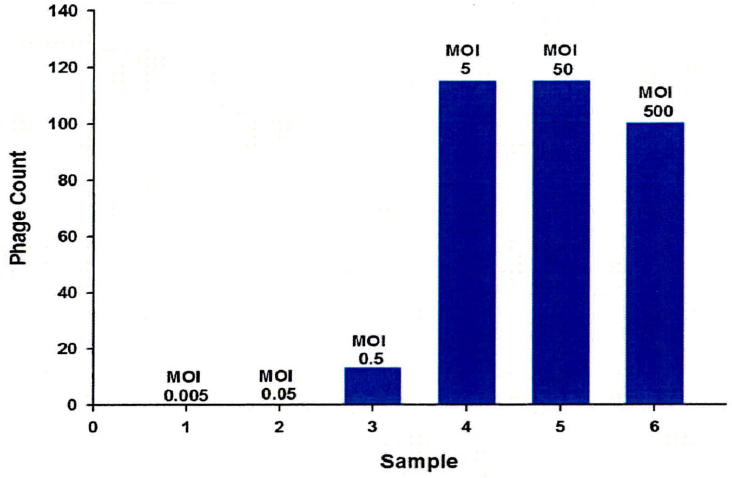

Figure 4.2 Multiplicity of infection effects on phage ϕA1122. Phage count units are in pfu/mL and results are shown at 65 minutes post infection for each MOI.

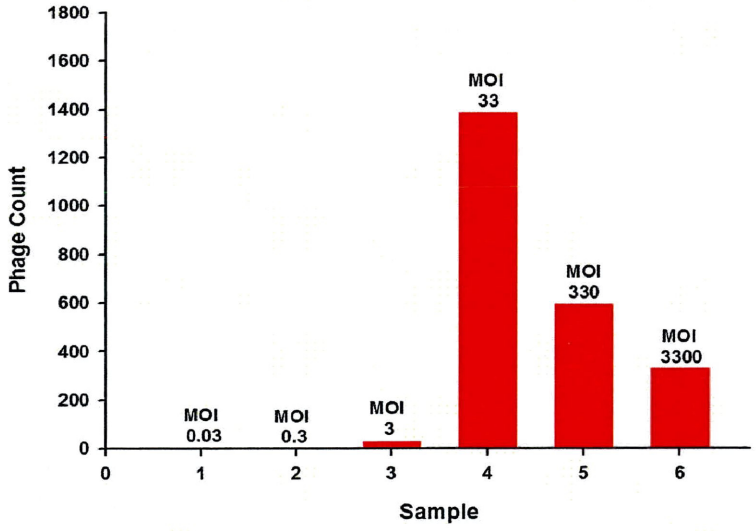

Figure 4.3 Multiplicity of infection effects on phage R. Phage count units are in pfu/mL mL and results are shown at 65 minutes post infection for each MOI.

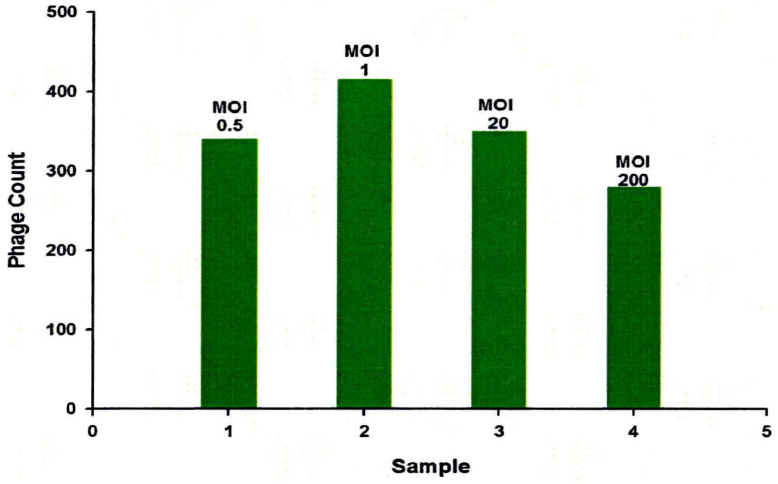

Figure 4.4 Multiplicity of infection effects on phage Y. Phage count units are in pfu/mL mL and results are shown at 65 minutes post infection for each MOI.

When the phage concentration exceeds that of the bacterial concentration upon initial infection, a decrease in the burst size is observed at 65 minutes post infection using one-step growth experiments. Figures 4.2 – 4.4 show this effect graphically at all MOI greater than 1. The reason for this is that multiple adsorptions of many phages on a single bacterium induce the mechanism of *lysis from without* to occur. *Lysis from without* is induced when almost instantaneous adsorption of phage occurs at the adsorption capacity of the bacteria. Thus no progeny phages are actually liberated, but rather the phages which adsorbed to the bacteria are lost (lose their infectivity) and this is experimentally seen as a reduction in burst size.

If however, an initial concentration of phage is approximately equal to that of the bacterial concentration used upon initial infection, theoretically 100% of the culture should cause almost simultaneously infection and subsequent lysis of the entire culture. Burst sizes representing this scenario are shown in Figure 4.2 at an MOI of 5, Figure 4.3 at an MOI of 3 and Figure 4.4 at an MOI of 0.5. Experimentally this translates into an apparent larger burst size in the one-step growth experiment at 65 minutes post infection.

When a low MOI is utilized upon initial infection, this provides time for the uninfected bacteria population to continue growing and dividing, until such a time that the phage concentration is large enough to infect and subsequently lyse all of the bacterial cells. What this translates to experimentally using a one step growth experiment is that when low MOI are employed, few to no phage are produced when sampling early in the phage infection and replication process.[4.1] This is demonstrated in Figures 4.2 at MOI of 0.005 and 0.05 and in Figure 4.3 at MOIs of 0.03 and 0.3 whereby hardly any progeny phage are produced per bacterium at 65 minutes post infection.

It is well known that when infecting mass cultures, it is advantageous to use a low MOI to produce the largest amount of progeny phage. Table 4.1 shows generalized results that are typically obtained at a given MOI when lysis occurs in a mass bacterial culture.

[4.1] Sixty-five minutes is considered "early" in the one-step growth experiment as it takes on average (at least for most yersiniophage/*Y. pestis* systems) 40 minutes to complete intracellular activities in the replication cycle. I say most because phage Y is an exception as it is considered to be a rapid lysing (r+) phage.

Table 4.1 MOI effects on lysis of liquid bacterial cultures.

Multiplicity of Infection* (MOI)	Average Phage Titer** (pfu/mL)
Low <1	$>10^{10}$
Equal ratio	10^8-10^9
Phage exceeds bacterial concentration	$<10^7$

*Note rapid lysing phages are an exception to this rule and are predicted to result in titers which resemble those obtained with equal or high MOIs.

**Final average titers depend upon the initial starting bacterial concentration, these numbers were obtained using 10^7 and 10^8 cfu/mL, however relative order changes in magnitude is what is significant here.

So why is there a discrepancy between one step growth experiment results and the lysis of mass bacterial cultures? The answer lies in the assumption of the one-step growth experiment, which prevents secondary adsorption from occurring and thus the reason why samples must go through a series of several large dilutions to prevent further adsorption of progeny phage upon the onset of lysis. This technique does not account for subsequent secondary infections of the uninfected bacterial population. In infection experiments at low MOIs using mass bacterial cultures, whereby the culture is not diluted to prevent this effect, the release of progeny phage can and do subsequently adsorb and infect the uninfected bacterial population.[4.2] Therefore at low MOI, phage are able to infect a larger population of bacteria through "rounds" of progeny phage release and adsorption events, until the point where the entire bacterial population is infected and subsequently lysed.

[4.2] Based on statistical probability, it is just as likely that multiple phage adsorb to the same bacterium resulting in an overall phage concentration that is lower due to secondary adsorption events and mimics overall production seen at high MOI phage concentrations. These secondary adsorptions cause loss of free phage and are also seen as a reduction in overall phage production.

One might wonder then why such a large burst size is obtained at an MOI of 0.5 for phage Y, but relatively reduced when compared to the burst size obtained using an MOI of 1. The answer lies in the fact that phage Y is considered to be a rapid lysing (r+) phage, meaning little time is required for intracellular reproduction and release of progeny phage. The apparent increase in burst size for phage Y is related to the fact that the release of progeny phage occurs very quickly and secondary adsorption of these phages to the uninfected bacterial population occurs very quickly. In turn this rapid increase in phage can subsequently lyse the entire culture very rapidly, giving the appearance of an increased burst size at lower MOIs.

Admittedly, this chapter is very esoteric and can be confusing to understand unless a thorough knowledge of the infection, replication and lytic cycle is understood. However it does makes some very key observations and points out the applicability of one-step growth experiments to obtain the burst size parameter and applying this methodology to lyse a mass bacterial culture. Observations have been summarized as follows:

1) Under one-step growth experiment conditions, *lysis from within* occurs at low MOI and *lysis from without* at high MOI.
2) One step growth experiments are limited in predicting the outcome of the overall progeny phage produced from the lysis of undiluted bacterial cultures (which is the foundation of the phage amplification event). The reason for this is that the one-step growth experiment utilizes a large number of dilutions to ensure no secondary adsorption events occur when progeny phage are released into solution.
3) Burst sizes obtained by one-step growth experiments are only valid when low MOI are used such that dilutions prevent secondary adsorption events from occurring.
4) Burst sizes obtained from one-step growth experiments cannot be simply multiplied by the number of uninfected bacteria upon initial infection to provide a final number of progeny phage that will be produced in mass bacterial cultures. The reason for this is that secondary adsorption and infection events are critically important whereby they

change the overall phage concentration as a function of time until the entire culture is completely lysed.

5) Larger burst sizes are not advantageous to lowering the detection limit, and in fact have the opposite effect as larger burst sizes cause *lysis from without.*
6) Clearly obvious is that if low MOI are to be utilized in the phage amplification process, the speed at which this event takes place is ultimately sacrificed.
7) Since the one-step growth experiment cannot account for secondary effects a different model must be utilized in order to predict overall progeny phage production which can take into account secondary release of progeny phage and subsequent infections of the uninfected bacterial population.
8) One-step growth experiments do provide valid parameters of burst sizes, when determined under low MOIs[4.3] which can be utilized in other models to determine overall progeny phage production under a given set of environmental conditions.
9) Under low MOI conditions, burst sizes for all phage appear to range from as few as 20 to as many as a few hundred. A summary of burst sizes under at different MOI for ϕA1122 are listed in Table 4.2.

[4.3] Burst sizes obtained at low MOI via one-step growth experiments for rapid lysing phages do not apply.

Table 4.2 Summary of apparent burst size at different MOIs at 65 minutes post-infection.

Phage	MOI	Burst Size (pfu/mL)
ϕA1122	0.005	0
	0.05	0
	0.5	17
	5	120
	50	117
	500	115
R	0.03	0
	0.3	0
	3	40
	33	1,369
	330	590
	3,300	370
Y	0.5	325
	1	400
	20	335
	200	280

CHAPTER 5

MODELING CELL DENSITY EFFECTS ON THE ϕA1122 PHAGE AMPLIFICATION SIGNAL FOR USE AS AN *IN VITRO* DIAGNOSTIC TO IDENTIFY *YERSINIA PESTIS*

"Know where to find the information and how to use it - That's the secret of success"

Albert Einstein

5.1 Abstract

The specificity of the interaction of a bacteriophage with its host cell lends itself to methods for the identification of bacterial pathogens, while sensitivity and rapidity of the infection and replication process (referred to as the bacteriophage amplification signal) are linked to the number of overall progeny phage produced. Maximizing this signal for detection is advantageous for decreasing not only the number of detectable bacteria, but also for minimizing the time at which the signaling event is detectable. Since the phage infection, replication and lytic process has been shown to be primarily dependent upon the doubling time of the bacteria and in turn dependent upon physiological parameters of the host bacteria, the aim of this investigation is to determine, through modeling, how the doubling time affects the maximum obtainable phage-derived signal at various bacterial cell densities for the *Yersinia pestis*/ϕA1122 host phage system. A modified phage therapy model developed by Payne and Jansen, was employed for *in vitro* modeling of the phage, infection, replication, and lytic cycle. The model utilizes a series of three differential equations to

predict the progeny phage generated signal as a function of time. Modeling demonstrates that the overall amount of phage produced is primarily dependent upon the doubling time parameter (a) of the uninfected bacterial population.

5.2 Introduction

The specificity of the interaction of a phage with its host cell immediately lends itself to methods for the identification of bacteria. While many other procedures, such as enzyme linked immunosorbent assays (ELISA) or nucleic acid amplification techniques such as polymerase chain reaction (PCR), have been artificially developed to allow differentiation of bacterial cell structures, the use of bacteriophage provide a naturally evolved system in which bacteriophage specifically recognize and bind only to its host cells. This interaction has been exploited in a number of different methods for the specific detection and differentiation of individual host bacteria. One particular method which has been established to detect pathogens is phage amplification technology [78]. This detection technique does not use any modified phage and the endpoint of detection is the formation of plaques or clearing of a liquid culture (visual indication of lysis of cells). Various assays have been developed using this technology and have been coined bacteriophage amplification assays. For example, bacteriophage amplification has been successfully employed to detect pathogens such as *Listeria, Campylobacter, Pseudomonas, Salmonella, Escherichia coli* and *Mycobacterium tuberculosis* [67, 73, 77, 78, 103-105].

The term *bacteriophage amplification* refers to the fact that the initial infection of the target pathogen is allowed to replicate and subsequently lyse the bacterial cells. Bacteriophage amplification is performed by adding a known concentration of phage to a sample containing the target bacterium. Phage specific to the target bacteria adsorb to and infect the susceptible cells. The replication cycle follows producing progeny phage within the infected cells and upon cell lysis, progeny phage are released in to the surrounding milieu. The resulting increase in progeny phage generates the signal (amplification) for detection. Since sensitivity of the phage amplification signal is directly linked to the overall concentration of

progeny phage produced, maximizing the progeny phage production for signal detection is advantageous for decreasing the number of detectable bacterial cells, while a fast lysis time is beneficial for rapid signal proliferation and subsequent detection.

Typically phage amplification (also referred to as the infection, replication and lytic cycle or just lytic cycle) is measured quantitatively by the classical one-step growth experiments and is defined by the latent, rise time, and plateau periods. Where the termination of the latent period indicates the onset of the of cell lysis; rise time refers to the increase in number of progeny phage; and the plateau period is the maximum number of free progeny phages [99]. In addition, it is well documented that the phage lytic cycle is dependent upon intracellular resources of their bacterial hosts and several studies utilizing the one step growth experiment have demonstrated this dependence [83, 106-113]. Different stages of phage growth, including the attachment of the phage particle to its host, the penetration of phage DNA into the host, and the synthesis of the phage components within the host have been found to be sensitive to the physiological state of the bacteria which is subsequently dependent upon parameters such as growth medium, temperature and pH [107, 108, 111, 112]. These studies have shown that the faster the host grows at the time of phage infection, the faster that the phage will replicate, corresponding to a shorter latent period, increased rates of progeny protein production, and larger burst sizes within infected bacteria.

However, experiments using this classical model assume that the latent period ends prior to cell burst and that the different numbers of plaque forming units (PFU) per bacterium obtained by titration as a function of time are only due to the different burst times of individual cells. As shown in Chapter 4, while the one-step growth experiment is adequate to obtain burst size at low multiplicities of infection, it is an inadequate model to explain liquid lysis of cultures, since it cannot account for subsequent re-adsorption events of newly released progeny phage to the uninfected bacterial population left in the culture at low multiplicities of infection. Rabinovitch and coworkers [114] subsequently showed through modeling data from infection experiments with phage T4 and *E. coli* that all one-step growth

parameters are reliant solely upon the dependency of the culture doubling time at the time of initial infection [114, 115].

To investigate the doubling time dependency on ϕA1122 progeny phage production, temperature is a useful physiological parameter which affects the replication (doubling) time of *Y. pestis* bacteria. At a temperature of 37°C the doubling time of *Y. pestis* cells is approximately 90 minutes, while the doubling time hovers around 60 minutes at optimal growth temperature (28°C).[5.1]

A mathematical model described by Payne and Jansen [116] was modified and employed to simulate the infection and proliferation process for use in an *in vitro* environment. The modified model utilizes a system of 3 differential equations to explain the phage infection process:

$$dx/dt = ax - bvx \qquad \text{(Equation 1)}$$

$$dy/dt = bvx - ky \qquad \text{(Equation 2)}$$

$$dv/dt = kLy - bvx \qquad \text{(Equation 3)}$$

Equation 1 describes the change in the density of the uninfected bacteria (x); Equation 2 describes the change in the density of the infected bacteria (y); and Equation 3 describes the change in the free phage density outside the cell (v). Variables utilized within the three equations are: the replication rate of the bacteria (a); the transmission coefficient (how readily a phage enters a bacterial cell) (b); the lysis rate of the infected bacteria (k); and the

[5.1] A 60 minute doubling time can be obtained for *Y. pestis* at temperatures of 28°C, however this time can varying depending upon the growth medium employed. The important point here is that at 28°C, *Y. pestis* growth is significantly faster than when the cells are grown at 37°C.

burst time (number of phages released during lysis) (L). The modified model reflects an *in vitro* environment, thus parameters originally described by Payne and Jansen model for human host effects on the bacteria H(t)x and h(t)y, the replication rate for infected bacteria (ay), and the phage decay rate were set to zero as it is assumed that the infected bacteria do not replicate and phage do not decay in a test tube as they would in a natural environment. Detailed modeling syntax is provided in Appendix C.

5.3 Material and methods

Initial infection experiments were modeled for bacterial densities of 10^5 and 10^6 cfu/mL using a multiplicity of infection (MOI) of 0.3 to generate the mathematically derived curves for 60 and 90 minute doubling times, parameter (a), respectively. Equations and parameters were input into Mathematica 5.1 (Wolfram Research, Inc., Champaign, IL) and evaluated by interpolating between 0 and 12 hours. Graphical representations of the resulting interpolations were generated to plot the change in phage concentration as a function of time (v vs. t) and the change in the infected bacterial concentration as a function of time (y vs. t).

5.4 Results and discussion

The doubling time parameter at 28°C was obtained for *Y. pestis* A1122 from experimentally determined bacterial growth curves, while the 90 minute doubling for *Y. pestis* at 37°C is well reported in the literature [5, 16]. The parameters for lysis time (k) and burst size (L) were also obtained experimentally for *Y. pestis* A1122 and phage ϕA1122 host phage system via one-step growth experiments [83] and determined to be 40 minutes and 180 phage/bacterium, respectively.

Figure 5.1 models maximum phage production with *Y. pestis* doubling times of 60 and 90 minutes respectively. At an initial infecting bacterial concentration of 10^6 cfu/mL almost an order of magnitude is gained in progeny phage generated when the bacteria have a 60 minute

doubling time, while only about half an order is gained when initial infection utilizes 10^5 cfu/mL at 60 minutes. Figure 6.2 shows the rate of change in the infected bacterial population as a function of time when an MOI of 0.3 is used to initially infect 10^6 cfu/mL and 10^5 cfu/mL at each doubling time respectively. With a faster bacterial doubling time and a lower initial infecting phage concentration, the uninfected bacterial population has additional time to replicate thereby producing more bacteria for subsequent phage infection. However this increase in phage production does not come without the sacrifice of time. While the rate of infection is similar for both doubling times, it takes more time to lyse the entire culture with a faster doubling time due to subsequent secondary infections.

The importance of demonstrating the results for two bacterial populations upon initial infection is to show that the probability decreases significantly in liquid cultures as the initial cell density is decreased. However when time is provided for the uninfected cell population to double and a critical cell density[5.2] is reached, replication and lysis will ensue in sufficient numbers to infect the rest of the uninfected bacterial population. The critical cell density can be seen in Figures 5.1 and 5.2 for both modeled doubling times. Figure 5.1 demonstrates the critical cell density in terms of the latent period and the time required before the onset of lysis, while Figure 5.2 shows this as the time required before the infected cell population begins to increase.

What does all of this modeling translate to in terms of producing a phage signal for the indirect identification of *Y. pestis*? First, the doubling time of the bacteria is directly related to the number of progeny phage produced. A slow bacterial doubling time does not produce as many progeny phage at a faster bacterial doubling time. Thus, a growth temperature of 28°C is preferential for the *Y. pestis*/ϕA1122 system to produce faster growth of the bacteria. Second, time is sacrificed at the expense of increasing the sensitivity in order to reach maximum progeny phage production. If sensitivity is of utmost concern in a diagnostic method utilizing this technique, then it would be advantageous to optimize physiological

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5.2 Critical cell density corresponds to the cell density (concentration) at which the phage has a good probability of coming into contact and infecting a bacterial cell.

parameters to create conditions such that the bacteria will replicate as fast as possible and use a low MOI, such as 0.001. As discussed in Chapter 4, the phage concentration should be lower than the bacterial concentration at initial infection to maximize progeny production. Thus, if one were also trying to detect a low density of bacterial cells there is also a time delay due to the fact that a critical cell density must be met before a phage has any significant probability of infecting a bacterial cell in a liquid culture. Third, if speed of the assay is of utmost concern and sensitivity secondary, then an MOI of 0.1 or 0.001 would be more advantageous to generate a signal. Finally, this modeling demonstrates that the phage lytic cycle inherently sacrifices speed at the expense of sensitivity when trying to detect a low density of bacterial cells and likely limits its application to an *in vitro* diagnostic use.

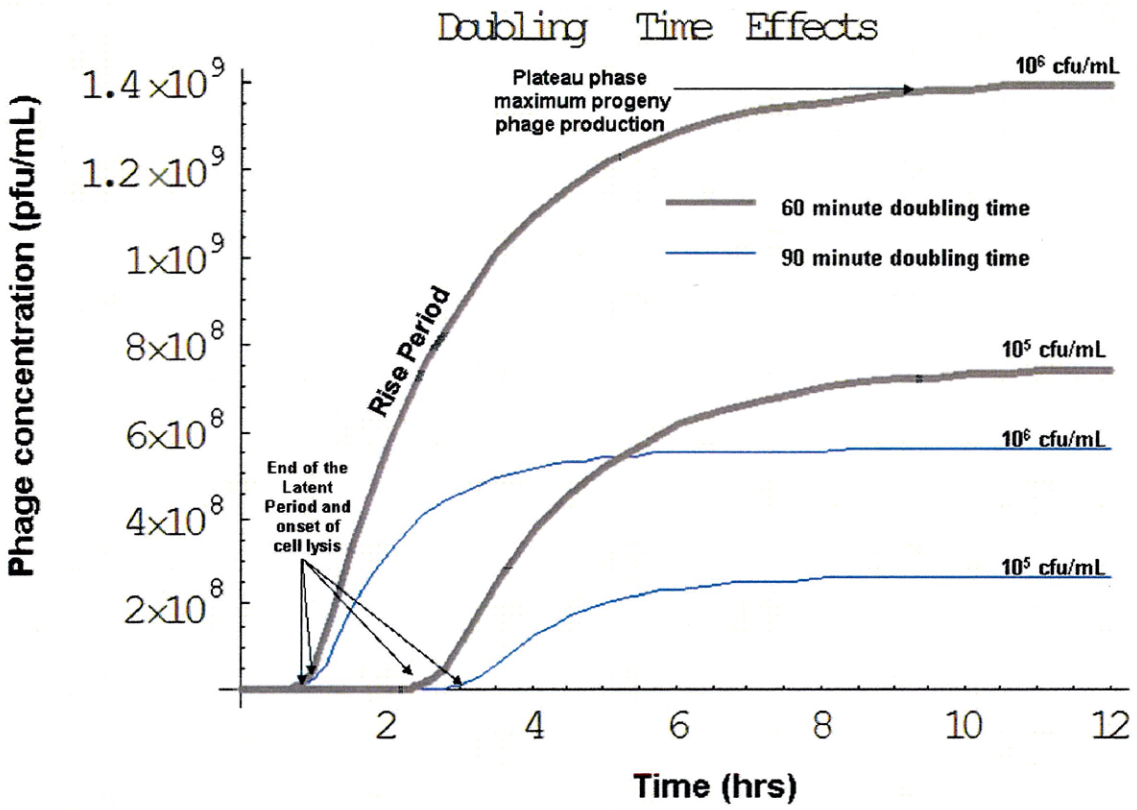

Figure 5.1 Modeling phage infections: *Y. pestis* doubling time effects. The rate of change in ϕA1122 phage concentration as a function of *Y. pestis* doubling times of 60 and 90 minutes respectively. Thick lines model maximum phage production for two different bacterial concentrations using a doubling time of 60 minutes (a = 1 hr), while thin lines represent the same two bacterial concentrations with a doubling time of 90 minutes (a = 0.67 hr). A MOI of 0.3 was used to model each initial infection of the bacterial concentration (x) and ranged from 1 x 10^5 - 1x10^6 cfu/mL. All curves were generated with parameters set to: transmission coefficient of 10^{-7} (b); lysis time (k) of 1.5 hrs and burst size (L) 180.

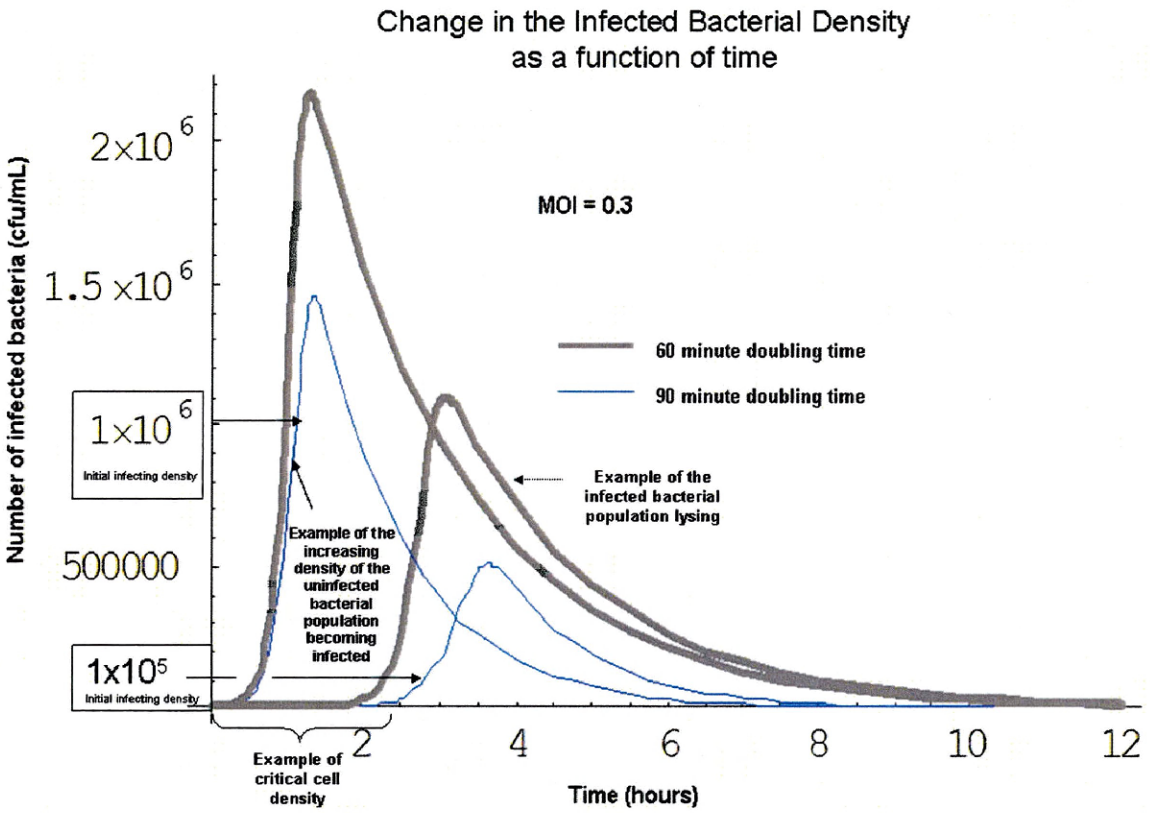

Figure 5.2 Modeling phage infections: density effects of infected *Y. pestis* cells. Density of infected *Y. pestis* cells as a function of the culture doubling time. Infected bacterial concentration (y) modeled with the same parameters as used in Figure 5.1. Left slope indicates number of infected bacteria as a function of time, while the right side of slope indicates lysing infected bacterial cells as a function of time.

CHAPTER 6
INDIRECT IDENTIFICATION OF *YERSINIA PESTIS* BY BACTERIOPHAGE AMPLIFICATION MATRIX ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

6.1 Abstract

A methodology called bacteriophage amplification, based on the phage infection and replication process, can be used to indirectly identify *Yersinia pestis*, the etiological agent of plague. Since the sensitivity and speed of the phage infection and replication process is linked to the number of overall progeny phage produced, maximizing the phage signal for detection is advantageous for decreasing not only the number of detectable bacteria, but also for minimizing the time required to generate a detectable signal. Therefore, the aim of this investigation is to determine the concentration of phage and the period of time required to generate a detectable signal as well as parameter(s) where upon the phage-derived signal is dependent for the *Yersinia pestis*/ϕA1122 host phage system. A phage therapy model developed by Payne and Jansen, which utilizes a series of three differential equations, has been modified and employed to model *in vitro* phage amplification. Comparisons between the model and experimental results obtained by MALDI-MS show that increased phage

amplified signals can be obtained at low initial infecting phage concentrations and the magnitude of this phage-derived signal is dependent upon the uninfected bacterial population within the culture.

6.2 Introduction

The cornerstone of many plague diagnostics is the unique F1 antigen produced on the cell surface of *Y. pestis*. For example, one of the recommended confirmatory tests in recovering plague patients is seroconversion to the F1 antigen [16]. In addition, other presumptive plague diagnostics rely on the direct or indirect presence of the F1 antigen/antibody binding response including the direct fluorescent F1-antibody (DFA) test, passive hemagglutination and inhibition assay (PHA/PHI), enzyme immunoassays (EIA), fiber-optic biosensors, and the immunochromatography dipstick assay [16, 61, 75]. It has been estimated that as much as 12% of an infected population can produce false negatives based on the F1 antigen [61]. This has been attributed to either poor response to the F1 antigen within a population or because F1-antibody levels can fall below the sensitivity of the assay during early stages of infection. Because the F1 antigen is produced only at temperatures greater than 33°C, many of these assays would be ineffective against naturally occurring or potentially manipulated F1 negative strains. In addition, detection based on F1 antigen is nearly impossible within the main transmission vector, the flea, as the bacteria proliferates in the gut of these species at 26°C.

However, a methodology called bacteriophage amplification based on bacterial lysis to a specific phage can be used to identify *Y. pestis* where F1 is limited. Various bacteriophage amplification techniques have been successfully employed for the detection of several pathogens including *Mycobacteria*, *Listeria* and *E. coli* species [66, 67, 73, 74, 77, 78]. This technology exploits the host/phage receptor specificity and lytic replication within susceptible bacterial cells, avoiding the potential pitfalls of plasmid deficiencies or losses associated with genetic techniques such as polymerase chain reaction (PCR) [56, 58-61].

In general, phage amplification is performed by adding a known concentration of phage to a sample containing the target bacterium. Phage specific to the target bacteria adsorb to and infect the susceptible cells. The replication cycle follows thereby producing progeny phage within the infected cells and upon cell lysis, releasing phage into the surrounding milieu. The resulting increase in progeny phage generates the signal or "amplification" for detection. Detection is commonly carried out by visualizing plaques on a bacterial lawn after several hours of incubation. However, more novel and rapid approaches to detect the amplification event include bioluminescent reporters and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) detection [66, 74, 103, 104]. For example, Madonna and coworkers [103] utilized this technique with MALDI-MS detection to identify *E. coli* with an MS2 phage.

A mathematical model described by Payne and Jansen [116] was modified and employed to simulate the infection and proliferation process for use in an *in vitro* environment. The modified model utilizes a system of 3 differential equations to explain the phage infection process:

$$dx/dt = ax - bvx \qquad \text{(Equation 6.1)}$$

$$dy/dt = bvx - ky \qquad \text{(Equation 6.2)}$$

$$dv/dt = kLy - bvx \qquad \text{(Equation 6.3)}$$

Equation 1 describes the change in density of the uninfected bacteria (x); Equation 2 describes the change in density of the infected bacterial (y); and Equation 3 describes the change in the free phage density outside the cell (v). Variables utilized within the three equations are: the replication rate (doubling time) of the uninfected bacteria (a); the

transmission coefficient (how readily a phage enters a bacterial cell) (b); the lysis rate of the infected bacteria (k); and the burst time (number of phages released during lysis) (L).

6.3 Material and methods

Bacteriophage amplification:

After determining the ϕA1122 phage MALDI-MS detection limit of to be 2(±2) x 10^8 pfu/mL, amplification experiments were performed with initial bacterial densities ranging from 1 x 10^8 to 1 x 10^5 cfu/mL which were infected with 3(±2) x 10^6 pfu/mL of ϕA1122 phage. One mL of bacteria samples were infected with 100μL of phage and incubated with shaking (150 rpm/min) at 28°C for 1-3 hours. Sample clean-up prior to MALDI analysis consisted of pelletizing bacterial debris (10,000 x g for 5 minutes), followed by filtration through a standard 0.22 μm cellulose acetate membrane. Samples were polyethylene glycol (PEG) precipitated and re-suspended in an equal volume of 0.85% saline solution. To remove the PEG, differential centrifugation (11,000 x g for 15 min) was employed with an equal amount of chloroform. The aqueous phase (top layer) containing phage particles were collected and considered semi-purified for MALDI-MS analysis.

MALDI-MS

Ferulic acid (15 mg/mL) in a 17:33:50 mixture of 88% formic acid: acetonitrile: de-ionized water was utilized as the matrix [94]. Mass spectra were obtained with a 337 nm N_2 laser in linear mode using a PerSeptive Biosystems Voyager-DE STR+ MALDI-TOF-MS, (Applied Biosystems, Inc., Framingham, MA, USA). Samples were applied to the hydrophobic target plate in a sandwich fashion as follows: 0.5 μL of matrix: 0.5 μL of sample: 0.5 μL of matrix. The following parameters were used to collect spectra: accelerating voltage 25kV; grid voltage 80%; delayed extraction time 100 ns; and a to 2 kDa low mass ion gate. Mass spectra were acquired as an average of 150 laser shots taken from 3 replicate

sample spots (50 shots per spectrum). Raw data from Data Explorer (Applied Biosystems, Inc. Framingham, MA, USA) was exported into SigmaPlot 7.0 (Point Richmond, CA USA) for spectral comparison.

Modeling

The doubling time parameter (a) was obtained for *Y. pestis* A1122 from experimentally determined bacterial growth curves (reference chapters 4 and 5). The lysis time (k) and burst size (L) were obtained experimentally for *Y. pestis* A1122 and phage ϕA1122 host phage system via one-step growth experiments [83]. Equations and parameters were input into Mathematica 5.1 (Wolfram Research, Inc., Champaign, IL) and evaluated via interpolation between 0 and 60 hours. Graphical representations of results plot phage concentrations as a function of time.

6.4 Results and discussion

A summary of MALDI-MS results and modeling experiments is presented in Table 6.1. MALDI-MS spectra from the actual 3-hour phage amplification experiments for the different bacterial densities are presented in Figures 6.1-6.3. For all three densities, initial infection is shown in the bottom spectrum in each Figure and indicates no detectable ϕA1122 phage signal when an infecting phage concentration of phage of $3x10^6$ pfu/mL was utilized. The culture was deliberately infected well below the detectable threshold (LOD = 2 x 10^8 pfu/mL) of MALDI-MS so that any detectable change is the result of progeny phage from the amplification event.

Figures 6.1 and 6.2 demonstrate that at bacterial densities of 10^8 and 10^7 cfu/mL a detectable phage signal is apparent at 1-hour post infection. Comparison of these signals to the modified model for the number of progeny phage produced after 1-hour shows that the phage concentration far exceeds that of the MALDI-MS limit of detection of $2x10^8$ pfu/mL, producing phage concentrations of $1x10^{10}$ and $1x10^9$ pfu/mL respectively. Subsequent

sampling at these densities 2 and 3-hours post infection show increased signal intensities. The corresponding modeling in Figures 6.1 and 6.2 predict that phage production has not yet reached its maximum plateau for either bacterial density after 1 hour, thus accounting for the increase in signal intensities in the MALDI-MS spectra 2 and 3 hours post infection.

Figure 6.3 demonstrates that as the bacterial concentration is decreased to 10^6 cfu/mL a MALDI-MS signal is not apparent until 2 hours post-infection. The corresponding model predicts that the phage concentration after only 1 hour is $1x10^8$ pfu/mL. This phage concentration has not met or exceeded the MALDI-MS detection threshold and therefore accounts for the lack of signal after this time period. However, allowing the infection process to proceed for 2 hours, the phage concentration increases to 5×10^8 pfu/mL, producing a phage concentration that now exceeds the detection threshold as evidenced by the presence of the phage biomarker at 15.8k Da in the mass spectrum. When the bacterial concentration was decreased another order of magnitude, no apparent MALDI-MS signal was obtained at 10^5 cfu/mL after 3-hours post infection when an MOI of 30 was utilized.

Table 6.1 Summary of MALDI-MS and modeling on the phage amplified signal.

Bacterial Concentration at initial infection (cfu/mL)	Phage Concentration at initial infection (pfu/mL)	MALDI-MS Phage Signal Present 1-Hr Post Infection	MALDI-MS Phage Signal Present 2-Hr Post Infection	MALDI-MS Phage Signal Present 3-Hr Post Infection
1.0×10^8	3.0×10^6	YES	YES	YES
1.0×10^7	3.0×10^6	YES	YES	YES
1.0×10^6	3.0×10^6	NO	YES	YES
1.0×10^5	3.0×10^6	NO	NO	NO
Bacterial Concentration at initial infection (cfu/mL)	**Phage Concentration at initial infection (pfu/mL)**	**Model Final Phage Concentration (pfu/mL) 1-Hr Post Infection**	**Model Final Phage Concentration (pfu/mL) 2-Hr Post Infection**	**Model Final Phage Concentration (pfu/mL) 3-Hr Post Infection**
1.0×10^8	3.0×10^6	2.0×10^{10}	3.5×10^{10}	4.5×10^{10}
1.0×10^7	3.0×10^6	2.0×10^9	4.0×10^9	5.0×10^9
1.0×10^6	3.0×10^6	1.5×10^8	4.8×10^8	6.8×10^8
1.0×10^5	3.0×10^6	2.0×10^8	2.0×10^8	2.0×10^8

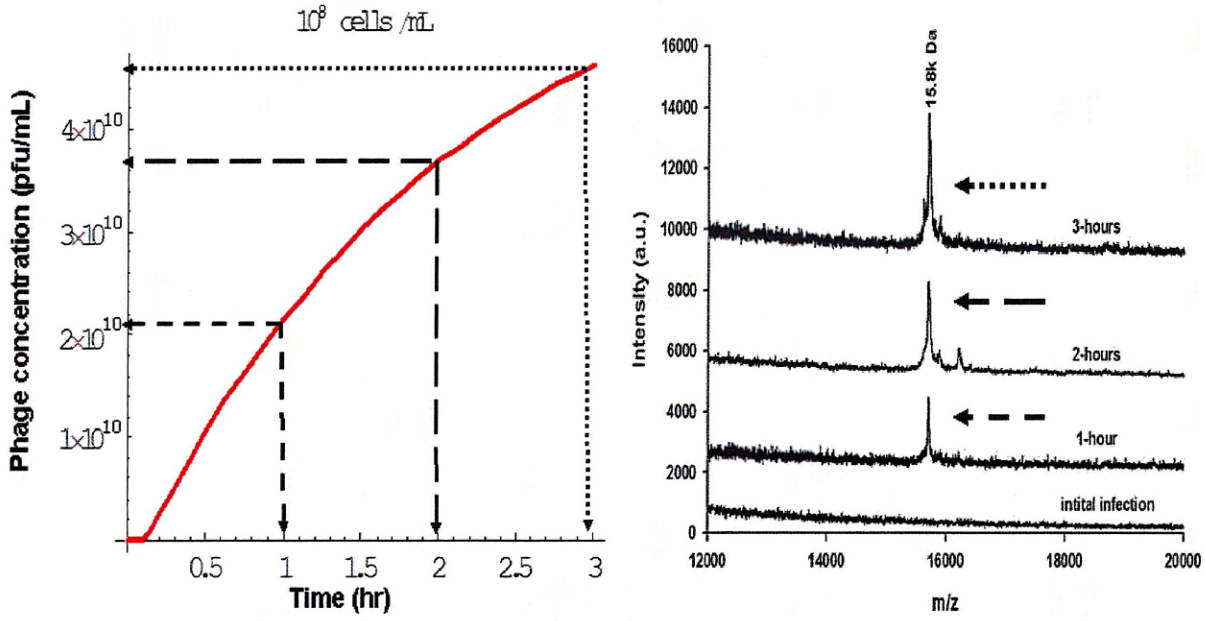

Figure 6.1 Modeling/MALDI-MS phage amplified signal at 10^8 cfu/mL using a modified Payne and Jansen model (left) comparison to MALDI-MS signals (right) obtained from 3 hour amplification experiments with initial bacterial concentration of 10^8 cells/mL. Modeling parameters: x =$1x10^8$ pfu/mL v = $3x10^6$ pfu/mL; a = 1 ; b = 10^{-7}; L = 180 phage/bacteria; k = 1.5 (40 min). Multiplicity of infection (MOI) = 0.03. MALDI-MS spectra of ϕA1122 phage amplified in *Y. pestis* cells in the mass range of 12-20 kDa.

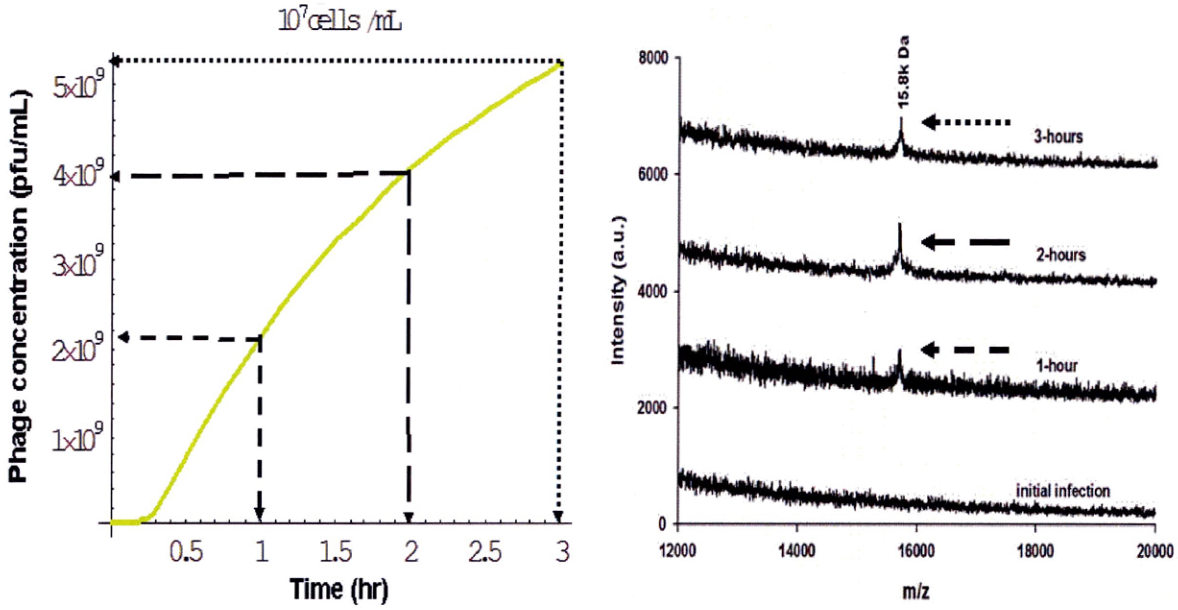

Figure 6.2 Modeling/MALDI-MS phage amplified signal at 10^7 cfu/mL using a modified Payne and Jansen model (left) comparison to MALDI-MS signals (right) obtained from 3 hour amplification experiments with initial bacterial concentration of 10^7 cells/mL. Modeling parameters: initial bacterial concentration (x) of 10^7 cells/mL.v = $3x10^6$ pfu/mL; a = 1 hours; b = 10^{-7}; L = 180 phage/bacteria; k = 1.5 (40 min). Multiplicity of infection (MOI) = 0.3. MALDI-MS spectra of ϕA1122 phage amplified in *Y. pestis* cells in the mass range of 12-20 kDa.

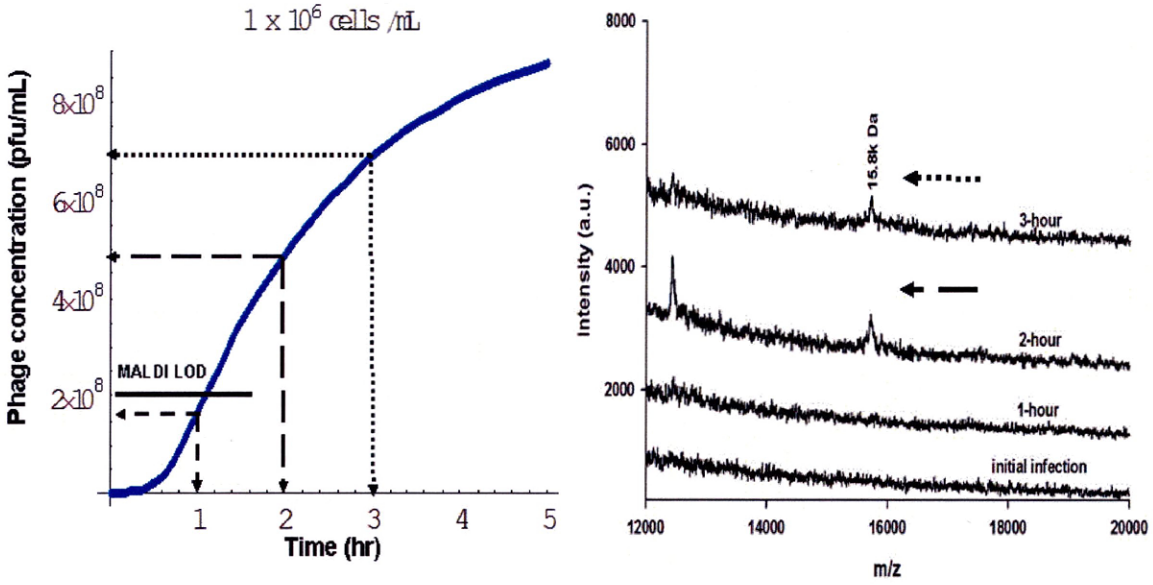

Figure 6.3 Modeling/MALDI-MS phage amplified signal at 10^6 cfu/mL using a modified Payne and Jansen model (left) comparison to MALDI-MS signals (right) obtained from 3 hour amplification experiments with initial bacterial concentration of 10^6 cells/mL. Modeling parameters: x = $1x10^6$ pfu/mL; v = $3x10^6$ pfu/mL; a = 1 hours; b = 10^{-7}; L = 180 phage/bacteria; k = 1.5 (40 min). Multiplicity of infection (MOI) = 3. MALDI-MS spectra of ϕA1122 phage amplified in 1 x 10^6 cfu/mL of *Y. pestis* cells in the mass range of 12-20 kDa.

Additional infection experiments using 10^5 cfu/mL were conducted whereby the phage concentration was varied from 10^6 pfu/mL to 10^4 pfu/mL (MOI = 30, 3 and 0.3) to investigate the MOI effects on the progeny phage signal. A summary of results for this experiment is presented in Table 6.2 Results also show that no apparent MALDI-MS signal could be obtained after 19 hours post-infection using MOI of 30 and 3 (Figure 6.4). The corresponding model at these two multiplicities predicts that the phage concentration is barely meeting or exceeding the MALDI limit of detection. Since the MALDI limit of detection signal inherently has noise and deviation associated with it, the signal can vary as much as $\pm 2 \times 10^8$ pfu/mL in magnitude and therefore accounts for the lack of signal at these MOIs.

However, when the initial infecting phage concentration is decreased to 10^4 pfu/mL (MOI = 0.3) the phage biomarker signal becomes evident at 15.8k Da in the MALDI-MS spectrum after 19 hours of phage amplification. The corresponding model predicts maximum phage production is reached in approximately 9-10 hours producing a corresponding phage concentration of 8×10^8 pfu/mL. This predicted final concentration of phage is well above the MALDI limit of detection and the noise associated with the signal. The model demonstrates that a detectable signal could have been generated in about 6.5 hours, thus sampling could have been performed far sooner than 19 hours.

More importantly, however is overall number of progeny phage produced. Varying the MOI demonstrates that low initial infecting phage concentrations increases progeny phage production. The reason for this increase is the fact that at low initial phage concentrations, not all of the uninfected bacterial cells are immediately infected and therefore gives time for the uninfected bacterial population to continue replication until such time that subsequent secondary infections cause the entire population of uninfected bacteria to become infected. Since changes occur in the uninfected bacterial population as a function of the positive rate at which the uninfected bacteria replicate (ax) and the rate at which those cells become infected (-bvx), phage production is solely dependent upon the bacterial doubling time

parameter (a). Thus the faster the uninfected bacterial population doubles when infecting at low initial phage concentrations, the higher the concentration of progeny phage produced because more uninfected cells are available to produce those progeny phage.

Table 6.2 Summary of MALDI-MS and modeling of MOI effects on the phage amplified signal.

Bacterial Concentration at initial infection (cfu/mL)	Phage Concentration at initial infection (pfu/mL)	MALDI-MS Phage Signal Present 19-Hr Post Infection	Model Final Phage concentration (pfu/mL) 19-Hr Post Infection
1.0×10^5	3.0×10^6	NO	2.0×10^8
1.0×10^5	3.0×10^5	NO	4.0×10^8
1.0×10^5	3.0×10^4	YES	6.5×10^8

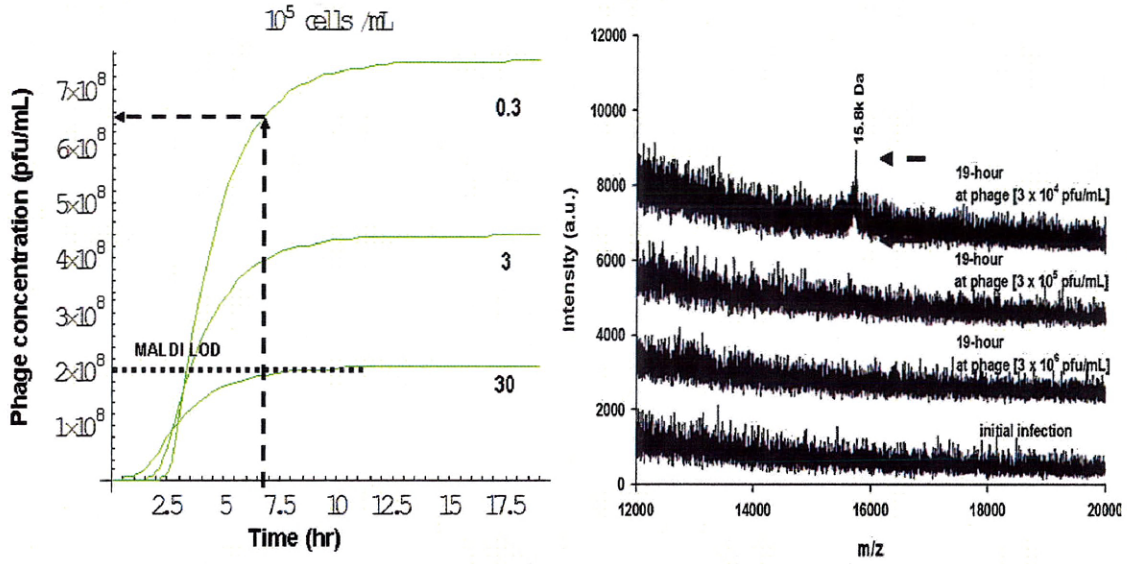

Figure 6.4 Modeling/MALDI-MS MOI effects on amplified signal at 10^5 cfu/mL using a modified Payne and Jansen model (left) comparison to MALDI-MS signals (right) obtained from 19-hour amplification experiments with initial bacterial concentration of 10^5 cells/mL and varying the initial phage concentration from $3x10^4$ – $3x10^6$ pfu/mL. Modeling parameters: x = $1x10^6$ pfu/mL; v = $3x10^4$ - $3x10^6$ pfu/mL; a = 1 hours; b = 10^{-7}; L = 180 phage/bacteria; k = 1.5 (40 min). MOIs ranging from 30 to 0.3. MALDI-MS spectra of ϕA1122 phage amplified in 1 x 10^5 cfu/mL of Y. pestis cells in the mass range of 12-20 kDa.

Conclusions

Matrix assisted laser desorption ionization mass spectrometry is not a quantitative technique, thus employment of the modified Payne and Jansen was necessary to quantitatively predict progeny phage production from the phage lytic cycle in order to predict progeny phage signal generation. The model, when compared to MALDI-MS data, under a defined set of parameters can accurately predict the amount of time it takes to generate a signal as well as if the concentration of progeny phage will exceed the predetermined MALDI-MS limit of detection. The model also demonstrates that the number of phage produced during the lytic cycle is ultimately depends upon the uninfected bacterial concentration and implementing a low phage concentration upon initial infection is advantageous to producing increased concentrations of progeny phage. Progeny phage production, as discussed in the chapter 5, is dependent on only one parameter, the doubling time of the uninfected bacterial population. Therefore it is advantageous to optimize physiological parameters, such as growth media, pH and temperature to induce a faster bacterial doubling time in order to maximize progeny phage production for signal detection.

CHAPTER 7
POLYCLONAL ANTIBODY DEVELOPMENT AND COLLOIDAL GOLD CONJUGATION TO COUPLE BACTERIOPHAGE AMPLIFICATION TO IMMUNOCHROMATOGRAPHY STRIPS FOR THE INDIRECT IDENTIFICATION OF *YERSINIA PESTIS*

"The first rule in intelligent tinkering is to save all the parts"

Paul Ehrlich

7.1 Abstract

Bacteriophage amplification immunochromatrography strips for the indirect detection of *Yersinia pestis*, the etiological agent of plague, requires conjugation of anti-bacteriophage antibodies to colloidal gold particles. Performance of the strip depends on the chemical properties of the gold particles as well as the affinity (binding capacity) of the detection antibodies. This research reports on the production and characterization of anti-ϕA1122 and anti-Y polyclonal antibodies in mice and their subsequent conjugation to colloidal gold reporter particles for development of two prototype bacteriophage amplification immunochromatography strips. Results of performance testing demonstrates that the anti-ϕA1122 colloidal gold conjugate has excellent specificity, while the anti-Y conjugate produces a weak signal from background matrices.

7.2 Introduction

One method used to diagnose plague, and in fact is used by the Centers for Disease Control and Prevention (CDC) as one of several confirmatory tests[7.1], is the lysis strip method [16, 34]. This method utilizes the lytic ϕA1122 yersiniophage to differentiate *Yersinia pestis* (*Y. pestis*) cells from those of *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*) and other clinical isolates [16]. This assay takes advantage of lysis differentiation due to the fact that *Y. pestis* cells are susceptible to phage lysis over a wide temperature range (20°C to 37°C), while *Y. pseudotuberculosis* cells are only susceptible to phage lysis at temperatures greater than 28°C [16, 34]. The technique is performed by placing a phage-coated strip on the surface of an agar plate, streaking cultures across the surface of the strip followed by several hours of incubation. Subsequent positive detection is made by means of visual inspection of a lysis event which causes clearing around the phage-coated strip [16, 34].

Because visible lysis can be ambiguous and take up to 24 hours to obtain results, this research addresses coupling a modern detection device, specifically immunochromatography strips, to modernize the detection platform of the lysis strip method. This modernization will facilitate the surveillance, detection, and diagnostic capabilities of our public health infrastructure. In addition it can aid in detection where other methods are limited as well as provide an easy to use platform for third world countries which cannot afford more expensive diagnostics requiring specialized equipment and training to perform assays.

In the last ten years, many immunochromatography assays (ICA) have been reported for the detection of infectious diseases [75, 117-138], cancer [139, 140], and illicit drugs [141, 142]. Immunochromatography strips (ICS) are rapid (<20 minutes) detection platforms which require that small amounts of sample (<100 μl) to be applied to the test strip. Immunochromatography strips employ colloidal gold-labeled antibodies dried onto a

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[7.1] Confirmed plague is diagnosed if one of the following conditions are met: 1) a bacterial isolate is lysed by specific bacteriophage or 2) two serum specimens demonstrate a four fold anti-F1 antigen titer difference by agglutination testing (PHA/PHI).

conjugate filter pad affixed to a nitrocellulose membrane (Figure 7.1). An immobilized capture antibody is applied downstream on the test line. Typically a control line is also employed downstream with species-specific antibodies to validate that the assay is working properly. When a sample containing the pathogen of interest is applied to the sample pad, binding will occur to antibody/colloidal gold conjugates, flow through the nitrocellulose membrane via capillary action to the capture zone. If two red lines develop in the capture zone this indicates a positive identification, while one red line indicates a negative response to the pathogen. If no red lines appear in the capture zone, the test is deemed invalid.

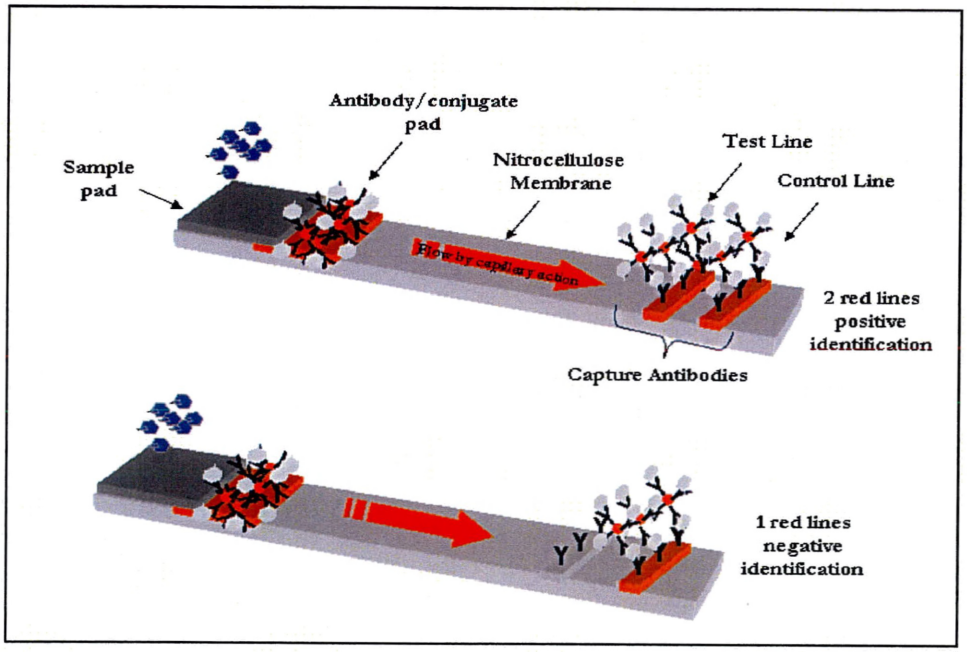

Figure 7.1 ICS general detection scheme. Top ICS pictorial shows a positive response to a pathogen of interest, while the bottom representation indicates a negative response.

While other reporter particles[7.2] have been used for antibody conjugation, colloidal gold has found application as a label in these immunoassays techniques because its absorbance is in the visible spectrum [143-146]. Unlike fluorescence or enzyme-detection systems, gold probes are more stable and easier to use as there is no need for steps such as washing or enzymatic reactions during signal detection.

In order to couple ICS detection to the phage amplification method, a predetermined phage concentration is added which is below the detection threshold of the ICS. Subsequently, this concentration of phage (or lower) is used to adsorb and infect the susceptible bacterial cells. The replication cycle follows producing progeny phage within the susceptible cells and upon lysis, progeny phage are released into the surrounding milieu. This increase in progeny phage brings the concentration of phage/conjugated antibody complex above the ICS detection threshold providing a positive result and indirectly indicating that the bacteria of interest is present. If susceptible bacterial cells are not present, replication will not occur, thereby giving a negative result on the ICS detection device for the presence of the target bacteria. Since the performance of the ICS strips depends on the chemical properties of the gold particles as well as the affinity of the detection antibody, this investigation presents details into the binding characteristics of the antibody-colloidal gold conjugates specific to phages ϕA1122 and Y as well as their binding to relevant bacteria. Thus the goal of this chapter is to characterize the performance of anti-ϕA1122 and anti-Y colloidal gold conjugates on various nitrocellulose membranes to characterize performance with background matrices, and to determine the limit of detection of each phage antigen.

7.2 In addition to colloidal gold reporter molecules, examples of other reporters are latex, fluorescent, phosphorescence and magnetic particles.

7.3 Methods and materials

Antigen purification and preparation for immunizations

Purified preparations of wild type ϕA1122 phage antigen were obtained by polyethylene glycol (PEG) precipitation and subjected to a CsCl equilibrium gradient [93]. To a 35 mL polyallomar tube containing 5-7 mL of PEG precipitated phage, a 4 step CsCl:TE gradient was prepared and under laid in the following order: 1:2; 1:1, 2:1, 1:0. The gradients were ultracentrifuged for 24 hours at 25,000 rpm using a SW-28 rotor in a Beckman LM-8 Ultracentrifuge (Beckman-Coulter, Inc. Fullerton, CA). Purified phage bands were collected above the 2:1 layer, dialyzed against 0.85% normal saline and 0.2 μm filter sterilized.

Two mL of purified phage antigen (50μg/mL or ca. $2x10^{12}$ pfu/mL) was subsequently added to an adjuvant. The adjuvant system, recommended for use in mice is composed of a 2% squalene oil-in-water emulsion containing monophosphoryl lipid A (MPL) and synthetic Trehalose dicorynomycolate (TDM) (M6536 Sigma-Aldrich, St. Louis MO).

Polyclonal antibody production

An 8 week protocol was employed for each antibody production utilizing 24 female specific pathogen free (SPF) Swiss Webster mice, from the colony maintained at the CDC/DVBID (Ft. Collins, CO). Pre-immunization serum samples (~200-500 μL/mouse) were collected from 6 mice prior to immunization and pooled. For each production, mice were immunized with 200μL of the ϕA1122 phage/adjuvant or the Y/adjuvant emulsion by subcutaneous injection at two sites (100μL each site) on the dorsal side of each mouse. Two additional booster immunizations were performed at weeks 5 and 7 by injecting 200μL of the phage/adjuvant emulsion as described previously. The humoral immune responses were monitored by analyzing serum samples by ELISA for specific antibodies during the 4^{th} and 6^{th} week respectively. Mice were sacrificed 8-10 days following final boost and blood collected using approved institutional animal care and use committee (IACUC) care

protocols [147, 148]. Anti-sera was separated from the whole blood using sera separation tubes via low speed centrifugation and pooled for each respective production.

Detection of specific anti-Y and anti-ϕA1122 antibodies by ELISA

Mouse sera was tested for anti-Y and anti-ϕA1122 activity using enzyme linked immunosorbent assay (ELISA). High binding 96-well microtiter plates (Immulon HB2) were coated with phage Y and ϕA1122 antigen respectively and allowed to bind overnight at 4°C. The plates were then blocked with a 3% BSA/PBS (Sigma Aldrich P3688 St. Louis, MO) buffer solution for 4-6 hours at room temperature. Serial dilutions from 10 to 10^3 of mouse serum were made in horseradish peroxidase (HRP) diluent (Pierce Biotechnology cat no. 37548 Rockford, IL) and incubated with the coated plates for 30 minutes at room temperature. After three washes with wash buffer solution (Pierce cat no. N503), HRP conjugated secondary antibodies (mouse antibodies raised in goat) (Bethyl Laboratories cat no. A90-116P Montgomery, TX) were diluted 1:100,000 in HRP diluent. One-hundred μL was added per well and incubated for an additional 30 minutes at room temperature. After 4 additional washes (200 μL/each well) with buffer solution, assays were developed with 100μL per well of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma Aldrich cat no. TO440 St. Louis, MO) substrate and allowed to react for 15 minutes at room temperature. The reaction was stopped using an acidic TMB stop solution (Sigma Aldrich cat no. S5814), which turned the reaction product from blue to yellow. Absorbance values were read at 450 nm with a ELX 808 IU Microplate Reader (Biotek Instruments Winooski, VT). A response was considered to bind as long as the signal was above that of the pre-bleed sera and blanks.

Affinity purification of anti-Y and anti-ϕA1122 IgG

Protein G affinity purification of anti-phage IgG from the mouse antiserum was employed[7.3]. Briefly, a 1mL Hi-Trap Protein G (GE Healthcare cat no. 17-0404-03 Piscataway, NJ) affinity column pre-packed with Protein G Sepharose was pre-equilibrated with 5 mL of binding buffer (20 mM sodium phosphate, pH 7.0). The anti-phage mouse serum was cut 1:1 with binding buffer and 2 mL applied to the column. The column was subsequently washed with an additional 5-10 mL of binding buffer. Three mL of elution buffer (0.1M glycine-HCl, pH 2.7) was applied and fractions collected in 1mL increments. Sixty μL/mL of neutralization buffer (1M Tris-HCl, pH 9.0) was added to each fraction to bring the pH of the IgG back to neutral. Purified samples were analyzed by ELISA in the same method stated above for whole sera using dilutions ranging from 10-10^6 to determine the binding response.

Quantification of purified anti-Y and anti-ϕA1122 IgG

Quantification of total protein content was performed using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, cat. No. 23227) [149, 150] in 96-well polystyrene microtiter plates (Nunc). Briefly, serial dilutions of 2 mg/mL of bovine serum albumin (BSA) ranging from 1.5 mg/mL – 0.25 mg/mL were made and implemented as calibration standards. Twenty-five μL per well of antibody sample or standard was added to the BCA working solution to give a 1:8 ratio. Microplates were covered and incubated at 37°C for 30 minutes. Absorbance values were read at 550 nm with the same plate reader as stated above.

7.3 Antigen specific columns are very hard to produce due to the sheer quantity of phage antigen required for column implementation, therefore a protein G Sepharose affinity column was employed. This column utilizes recombinant protein G (produced in . *coli*) having molecular mass of 17,000 Da and contains two IgG binding regions. The albumin binding region of the native protein G has been genetically deleted, thereby avoiding undesirable cross-reactions with the albumin.

Antibody/gold conjugation

Millenia Diagnostics, Inc. (San Diego, CA) was contracted to conjugate the phage antibodies to the colloidal gold particles. Anti-Y and anti-ϕA1122 antibodies were separately dialyzed in 10mM PBS at 1.0 mg/mL protein. Various antibody concentrations listed in Table 7.1 were conjugated to 1.0 mL of 40nm colloidal gold particles (pH 7.8, see pH determination next paragraph). Titrations were performed with 10% sodium chloride followed by incubation at 37°C to determine conjugate stability. Unstable conjugates were observed with a color change from red to purple upon incubation. Twenty percent sucrose and 5% tetrahalose (w/v) were added to conjugates prior to applying to conjugate padding.

The gold was adjusted to various pH units using 100mM potassium carbonate. Fifty mL of colloidal gold was placed into a clean beaker. Potassium carbonate was added to adjust the pH in 0.2 unit increments. Two mL of gold were removed when the gold was at a pH of 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4 and 8.6. The anti-Y and anti-ϕA1122 antibodies were then conjugated to the gold particles at each pH. Liquid testing was performed to determine the isoelectric point (pI)[7.4] of the conjugates to determine the optimal pH. Conjugates prepared at pH of 7.8 were chosen for further evaluation for each antibody.

Membrane and conjugate pad preparation

Purified antibodies were immobilized onto the various nitrocellulose membranes (Millipore Corporation, Bedford MA, and Whatman Inc., Florham Park, NJ) at concentrations ranging from 1.0-1.4 mg/mL. The antibodies were diluted to the final concentrations using 25mM (pH 7.4) potassium phosphate buffer solution (PBS). The test line and control line reagents were dispensed onto the nitrocellulose at a rate of 1.0μL/cm. After striping, the membranes were then stored overnight in a desiccator at room temperature. The membranes were subsequently blocked using 3 mL of lateral flow block

[7.4] The pI is the pH at which a molecule carries no net electrical charge, see appendix for protocol on liquid testing procedure.

solution (Millenia Diagnostics, Inc. San Diego, CA) to the bottom of each membrane and allowed to wick up the membrane, followed by a 2 minute incubation period. Membranes were subsequently dried on a heat plate for 30 minutes.

Eight thirty mm glass fiber conjugate pads were blocked as stated above for the membranes, removed from blocking solution, lined with absorbent paper and allowed to dry for 1 hour. Conjugates were dispensed onto the pretreated conjugate pad at 6 and 8 μL/cm. The pads were dried at 37°C for 1 hour and stored in a desiccant chamber until further use.

7.4 Results and discussion

Antibody Specificity and Binding Response

Initially, antibody binding responses after purification showed mean binding titers (titer is the dilution factor or measure of the concentration of specific antibodies which binds to selected bacteria or phage particles) for each of their respective phage on the order of $1:10^5$-10^6. However upon further testing the antibodies for cross reactivity (binding responses) to selected bacteria, both antibodies showed high levels of non-specific binding. This is typically observed with polyclonal antibodies because they are not all produced from the same cell line. Therefore, in order to increase selectivity of the antibodies, non-specific antibodies were bound to *Y. pseudotuberculosis* 1A.[7.5] Figure 7.2 shows binding titer responses to selected bacteria post-adsorption. Results show no significant binding occurred relative to the blank or the pre-bleed sera (see Figure 7.3) with any of the bacteria tested[7.6], indicating that non-specific antibody adsorption was effective.

After adsorption experiments were performed to remove the non-specific binding antibodies, Figure 7.3 shows final anti-ϕA1122 binding response titers to phage ϕA1122 and

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[7.5] Adsorption experiments were performed by heat inactivating *Y. pseudotuberculosis* 1A cells and adsorbing antibodies in a 1:1 ratio with the bacteria overnight at 4°C with shaking.

[7.6] While only *E. coli* ECOR 67 is shown in Figure 7.1, *E. coli* strains: ECOR 4, ECOR 13, ECOR 25, Hfr1+ were also tested for binding to the respective antibodies and also showed no significant binding responses.

Y. pestis A1122 (Figure 7.3A) and anti-Y binding response titers to phage Y and *Y. pestis* A1122 (Figure 7.3B). Results indicate that a $1:10^2$ binding response to the *Y. pestis* bacteria was observed with the anti-ϕA1122 antibodies, while very little to no binding response was noted with the anti-Y antibodies to the *Y. pestis* bacteria. Results indicate that a binding response of $1:10^3$ was obtained when the anti-ϕA1122 antibodies were allowed to react with phage ϕA1122 (Figure 7.3A), while a binding response of $1:10^4$ was observed with anti-Y antibodies and phage Y (Figure 7.3B). The binding response for each respective antibody was reduced ~33% from that obtained prior to pre-adsorption responses, indicating a fairly weak humoral immune response (an immune response that is mediated by B cells which make the antibodies) to the phages. Since the binding responses were mediocre at best, it can be concluded that the adjuvant system employed during the respective productions, to heighten the humoral response in the laboratory mice, was fairly ineffective. Frequently, in commercial antibody productions Freund's adjuvant (complete or incomplete) is used as the means of inducing the humoral antibody response in animals through the sustained release of antigen from the oily deposit. Admittedly Freund's adjuvant is the superior adjuvant on the market, however, its use may result in painful inflammation and other undesirable effects in the animal and is prohibited not only in government facilities, but by many other institutional animal care and use committees.

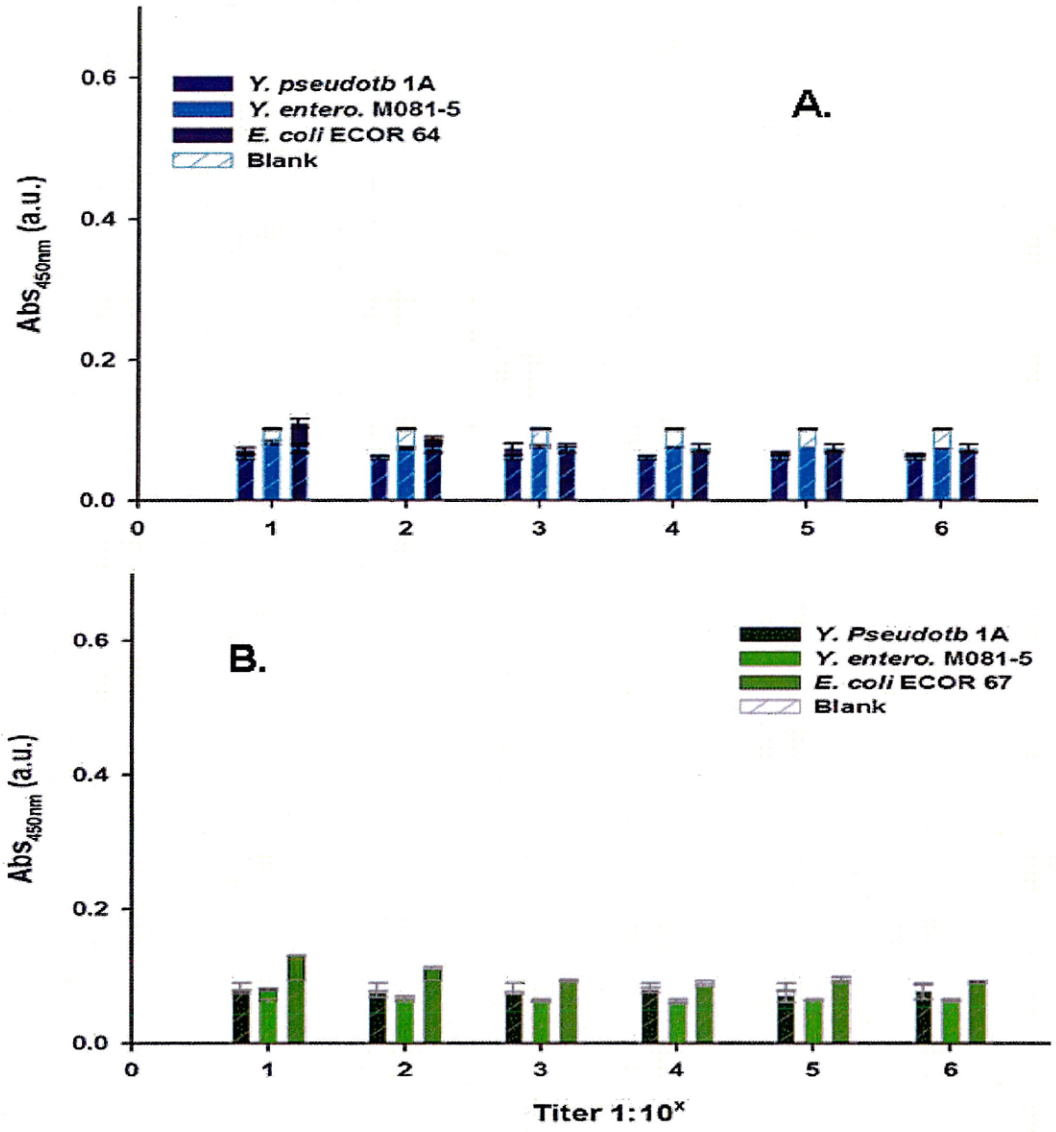

Figure 7.2 ELISA antibody binding response to selected bacteria. A) anti-ϕA1122 binding responses; B) anti-Y binding response. x = power of the titer response, i.e. $1:10^2$. Ten-fold serial dilutions of a 25μL aliquot of stock anti-ϕA1122 IgG (concentration of 1.06 ±0.02 mg/mL) and anti-Y IgG (concentration of 0.97 ±0.01 mg/mL) was utilized in the assay. HRP/2° conjugate ratio 1:100,000. Abbreviations in key above correspond to : *Y. pesudotuberculosis* 1A; *Y. enterocolitica* M081-5; and *Escherichia coli* reference strain.

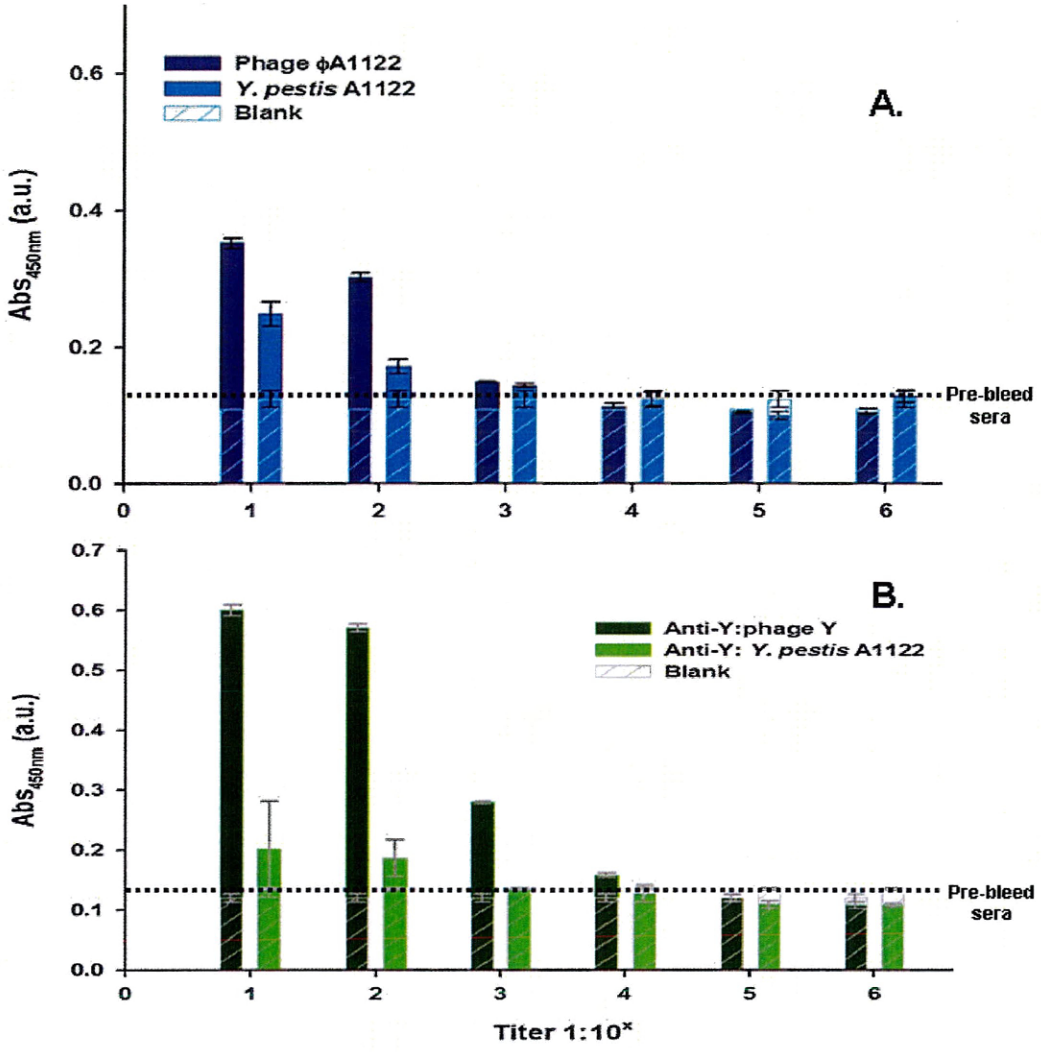

Figure 7.3 ELISA antibody binding response to phages and *Y. pestis*. A) anti-ϕA1122 binding responses to phage ϕA1122 and *Y. pestis* A1122, B) anti-Y binding responses to phage Y and Y. pestis A1122. x = power of the titer response, i.e. $1:10^2$. Ten-fold serial dilutions of a 25μL aliquot of stock anti-ϕA1122 IgG (concentration of 1.06 ±0.02 mg/mL) and anti-Y IgG (concentration of 0.97 ±0.01 mg/mL) was utilized in the assay. HRP/2° conjugate ratio 1:100,000.

Conjugation and testing

Table 7.1 shows the results of titration testing in order to determine the appropriate amount of anti-phage antibody needed to stabilize 1mL of the 40nm colloidal gold particles at a pH of 7.8.[7.7] Results indicate that 10μg of both antibodies were able to produce stable antibody conjugates, however, 14μg of each respective antibody was chosen for subsequent conjugations to assure sufficient antibody coating of the gold particles.

Binding responses to anti-ϕA1122 antibody conjugate were checked for background (false positives) on several different nitrocellulose membranes (Table 7.2) used for the ICS strips. This was performed by running 75μL of straight running buffer or with 50μL of PBS or TSB followed by 25μL of running buffer. Results shown in Table 7.2 indicate negative responses were obtained on all membranes with the anti-ϕA1122 antibody conjugate. Next, the various membranes with the anti-ϕA1122 antibody conjugate were tested for sensitivity by applying 50μL of 2 and 10-fold serial dilutions of phage ϕA1122 followed by 25μL of running buffer. Results (Table 7.2) indicate that the most sensitive membrane, HF 240 (15-20 minute assay time), showed a slight response at $2x10^9$ pfu/mL (Figure 7.4), corresponding to a ϕA1122 phage protein concentration of approximately 50ng/mL. The measured detection limit appears to be approximately 50 times higher than that of typical ICS assays where detection limits are reported around 1 ng/mL.[7.8]

The high detection limit is likely a function of the mediocre antibody response obtained during production as discussed above. Literature has reported the use of silver enhancement that could potentially decrease the detection limit 10-100 fold [151]. Most likely, however the best way to improve the limit of detection would be to use an alternative adjuvant to the one used in these productions to obtain an increased response to the specific phage. Another alternative would be to develop high quality monoclonal antibodies for use in conjugation to

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7.7 Refer to appendix C for protocol.

7.8 One ng/mL would correspond to a ϕA1122 phage concentration of ~ $4x10^7$ phage/mL

the colloidal gold particles. Monoclonals are advantageous since this would eliminate the need to adsorb out non-specific binding commonly performed with polyclonal antibodies.

Table 7.1 Anti-phage colloidal gold conjugate stabilities

Anti-ϕA1122 Antibody µg	Results at Colloidal Gold pH 7.8*	Anti-Y Antibody µg	Results at Colloidal Gold pH 7.8*
0	Control	0	Control
2.50	unstable	2.00	unstable
5.00	unstable	5.00	unstable
7.50	unstable	9.00	unstable
10.00	stable	10.00	stable
12.50	stable	12.00	stable
15.00	stable	15.00	stable

*A pH study ranging from 7.0 – 8.6 was also conducted with the 40 nm gold particles and each respective antibody and subsequently determined that a pH of 7.8 produced the most stable antibody/reporter conjugate.

Table 7.2 Nitrocellulose membrane performance with anti-ϕA1122/colloidal gold conjugate.

Membrane → with the following applied:	HF 240	HF 180	HF135	FF85/100
PBS	-	-	-	-
TSB	-	-	-	-
Running Buffer	-	-	-	-
Phage ϕA1122 $2.0x10^{11}$ pfu/mL	2+	2+	1+	2+
Phage ϕA1122 at $2.0x10^{10}$ pfu/mL	+	+		+
Phage ϕA1122 at $2.0x10^{9}$ pfu/mL	+/-			
Phage ϕA1122 at $1.0x10^{9}$ pfu/mL		NT	NT	NT

*2+ strong response, + medium response, +/- slight response, - no response, NT not tested.

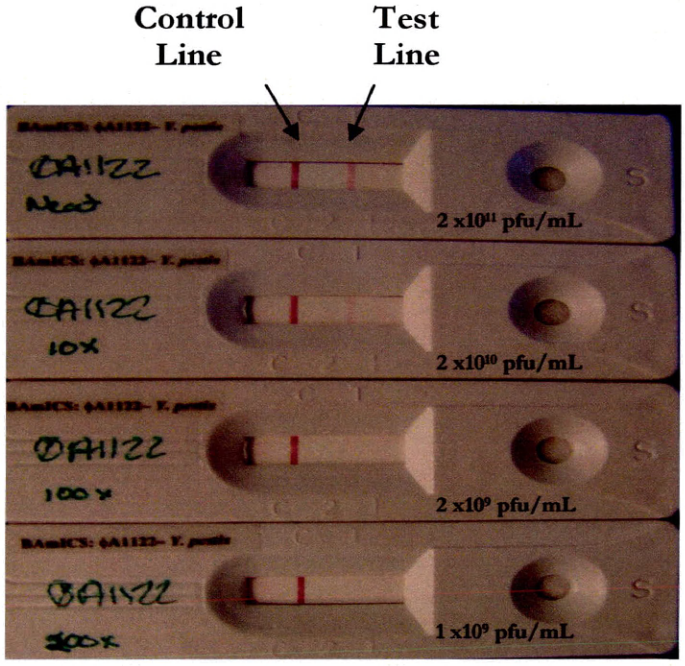

Figure 7.4 Phage ϕA1122 LOD on BAmICS. Bacteriophage ϕA1122 binding response to anti-ϕA1122/colloidal gold conjugate on the HF-240 nitrocellulose membrane.

Table 7.3 shows results of performing the same binding experiments as above with the anti-Y conjugate and membranes. Results indicate that slight responses to either TSB and or the running buffer were produced on all membranes, thus creating background and potentially false positives. Binding response to the phage Y antigen produced about the same limit of detection as the anti-ϕA1122 conjugates of $1.5x10^9$ pfu/mL, but some of the signal is likely a contribution from the background produced from the running buffer. In an attempt to reduce background with the anti-Y conjugates, the final prototype assay utilized the FF85/100 membrane which showed the least amount of background response. However the usefulness of this detector is questionable at best with the negative controls and running buffer producing false positives. Thus the cause should be investigated before further development with this conjugate.

Table 7.3 Nitrocellulose membrane performance with anti-Y/colloidal gold conjugate.

Membranes → HF 240 with the following applied:	HF 180	HF135	FF85/100
PBS	-	-	-
TSB	+/-	+/-	+/-	-
Running Buffer	-	-	+/-	+/-
Phage Y at $1.5x10^{11}$ pfu/mL	2+	2+	2+	3+
Phage Y at $1.5x10^{10}$ pfu/mL	+	+	+	+
Phage Y at $1.5x10^9$ pfu/mL	+/-		+/-	
Phage Y at $1.5x10^8$ pfu/mL		NT		NT

*2+ strong response, + medium response, +/- slight response, - no response, NT not tested.

Conclusions

While the detection limits are extremely high and unlikely to produce any significant signals at low bacterial concentrations, the anti-ϕA1122 BAmICS prototype does have the potential to perform well under *in vitro* laboratory conditions whereby the concentration of viable bacterial cells are on the order of 10^7 or 10^8 cfu/mL. These bacterial concentrations are typical of plating concentrations with which the lysis strip method is utilized, thus implementing this detection system could still potentially decrease overall assay time when the lysis strip method is utilized to confirm plague results.

CHAPTER 8

BACTERIOPHAGE AMPLIFICATION IMMUNOCHROMATOGRAPHY STRIP CHARACTERIZATION FOR THE IDENTIFICATION OF *YERSINIA PESTIS* ISOLATES: SELECTIVITY, SPEED AND SENSITIVITY

"You can't always get what you want, but if you try sometimes, you might find, you get what you need"

The Rolling Stones

M. Jagger/K.Richards

The Glimmer Twins

8.1 Abstract

The lysis strip method is one of several *in vitro* laboratory diagnostics used to confirm the diagnosis of plague. Immunochromatography strips (ICS) coupled with bacteriophage amplification is a methodology which can be used to modernize the lysis strip method of the detection. Bacteriophage amplification with ICS detection is performed by adding a known concentration of phage to a sample containing the target bacterium with the initial phage concentration below the detection threshold of the ICS. Phage specific to the target bacteria adsorb and infect the susceptible bacterial cells. The replication cycle follows producing progeny phage within the cells and upon cell lysis, phage are released into the surrounding milieu. The resulting increase in progeny phage generates the signal or "amplification" for

detection. This research reports on matrix effects, selectivity, speed and sensitivity of two prototype bacteriophage amplification immunochromatography strips to detect *Yersinia pestis*, the etiological agent of plague. Results indicate that anti-Y produced a slight background signal towards selected matrices, while the anti-ϕA1122 prototype showed 100% positive identification towards the *Yersinia pestis* isolates tested at viable cell concentrations of 10^8 and 10^7 cfu/mL and could be positively identified with the strip 2 and 3 hours post infection respectively. Sensitivity testing showed that the anti-ϕA1122 strip could positively identify *Yersinia pestis* isolate concentrations ranging from 10^8-10^6 cfu/mL of viable bacterial cells.

8.2 Introduction

One method used to diagnose plague, and in fact one that is used by the Centers for Disease Control and Prevention (CDC) as one of several confirmatory tests[8.1], is the phage lysis strip method [16, 34, 152]. This method utilizes the lytic ϕA1122 yersiniophage to differentiate *Yersinia pestis* (*Y. pestis*) cells from those of *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*) and other clinical isolates [16]. This assay takes advantage of lysis differentiation due to the fact that *Y. pestis* cells are susceptible to phage lysis over a wide temperature range (20°C to 37°C), while *Y. pseudotuberculosis* cells are only susceptible to phage lysis at temperatures greater than 28°C [16, 34]. The technique is performed by placing a phage coated strip on the surface of an agar plate, streaking cultures across the surface of the strip followed by several hours of incubation. Subsequent positive detection is by visual inspection of a lysis event which causes clearing next to the phage coated strip [16, 34].

Because visible lysis can be ambiguous and take up to 24 hours to obtain results, this research addresses coupling a modern detection device, specifically bacteriophage

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[8.1] Confirmed plague is diagnosed if one of the following conditions are met: 1) a bacterial isolate is lysed by specific bacteriophage or 2) two serum specimens demonstrate a four fold anti-F1 antigen titer difference by agglutination testing (PHA/PHI).

amplification immunochromatography strips (BAmICS), to modernize the detection platform of the lysis strip method. This modernization will facilitate the surveillance, detection and diagnostic capabilities of our public health infrastructure, aid in detection where other methods are limited, as well as provide an easy and inexpensive modernized detection platform for third world countries which cannot afford more expensive diagnostics assays [61].

Figure 8.1 shows actual BAmICS prototypes, while Figure 8.2 shows a diagram of how the prototype methodology works. To employ the BAmICS methodology, a predetermined phage concentration is added which is below the detection threshold of the ICS. Subsequently, this concentration of phage (or lower) is used to adsorb and infect the susceptible bacterial cells. The replication cycle follows producing progeny phage within the susceptible cells and upon lysis, progeny phage are released into the surrounding milieu. This increase in progeny phage brings the concentration of phage/conjugated antibody complex above the ICS detection threshold providing a positive result and indirectly indicates that the bacteria of interest is present. If susceptible bacterial cells are not present, replication will not occur, thereby giving a negative result on the ICS detection device for the presence of the target bacteria. A species specific antibody, in this case mouse antibody raised in goat, is used as a control line to validate that the assay is working properly.

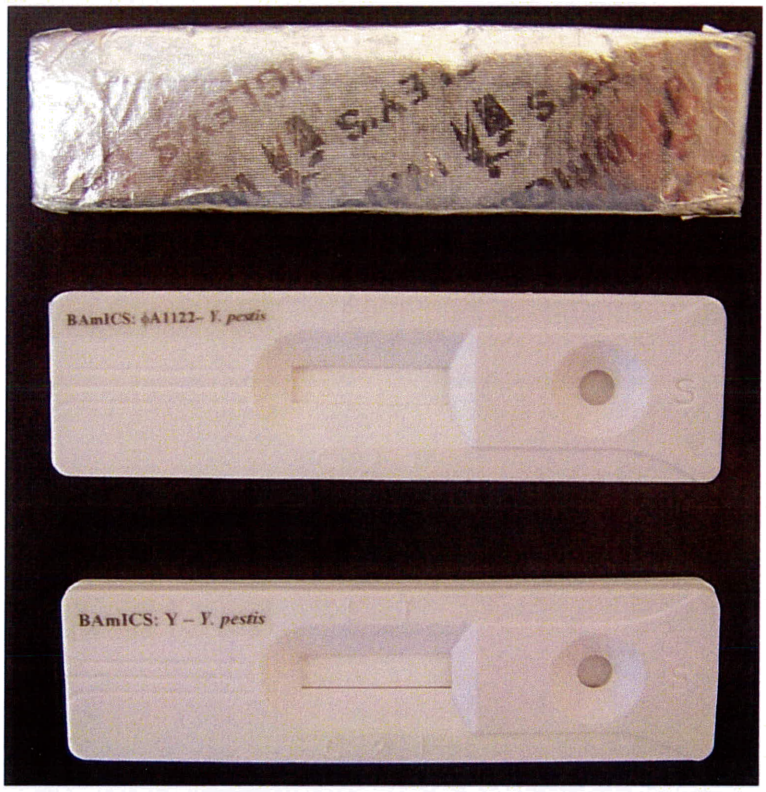

Figure 8.1 Prototype BAmICS system for the identification of *Y. pestis*. Middle detector is a picture of the anti-ϕA1122 antibody conjugated prototype, while the bottom detector is the anti-Y conjugated prototype. Development and construction was a collaborative effort between the Colorado School of Mines and the Centers for Disease Control Division of Vector-Borne Infectious Diseases.

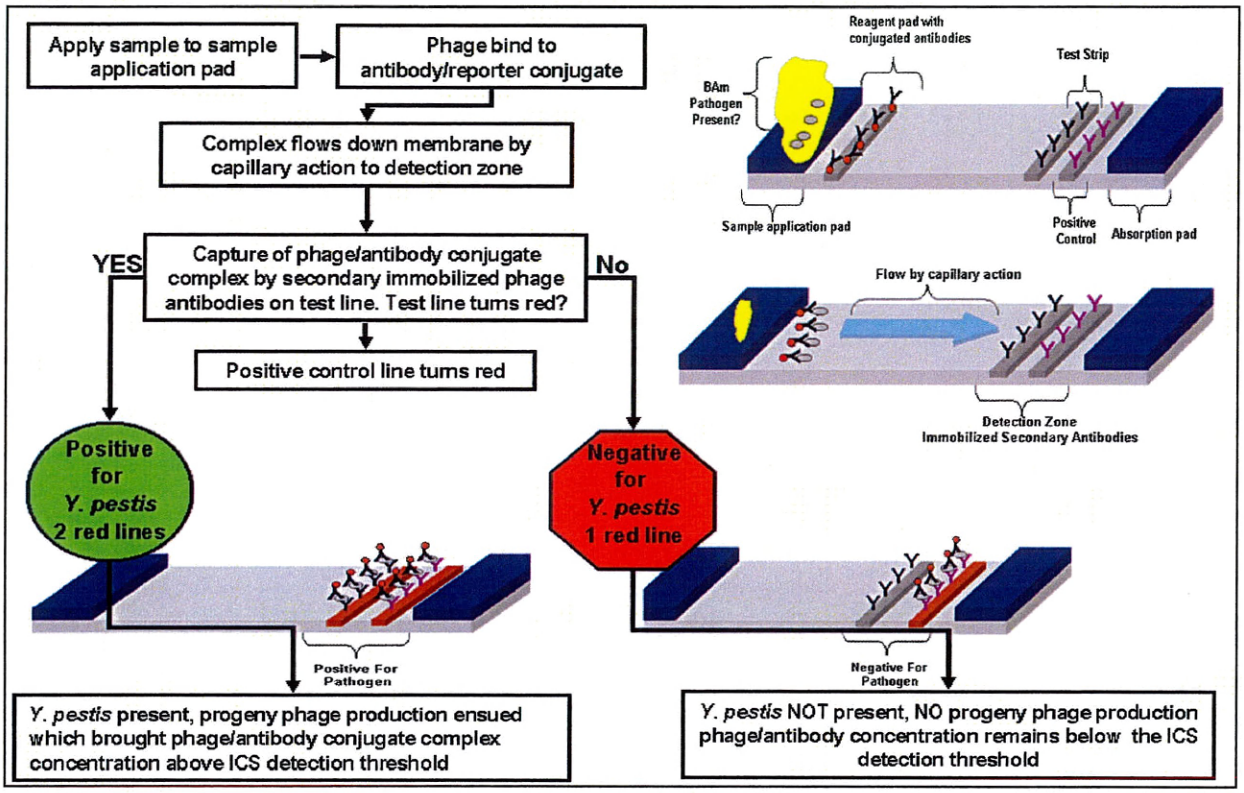

Figure 8.2 Diagram of BAmICS methodology for the identification of *Y. pestis*. Note that for diagram simplicity, only 1 antibody is shown to be attached per colloidal gold particle, however ~8 antibodies are actually conjugated per colloidal gold particle.

8.3 Methods and materials

Table 8.1 lists the bacterial isolates and the corresponding growth temperatures which were tested by the prototype BAmICS systems.[8.2] Infections were performed by adding 100μL of 10^8 pfu/mL of phage (or less) to 1mL of log phase growth bacteria at a given turbidity. Viable bacterial cell counts were correlated to turbidity measurements by the plate count method and were plated in triplicate to obtain a mean and standard deviation. Various infections experiments were allowed to amplify from 1 to 15.5 hours before applying to the BAmICS. Fifty μL of the amplified sample followed by 25 μL of running buffer (Millenia Diagnostics, Inc. San Diego, CA) was applied to the BAmICS conjugated with either anti-ϕA1122 or anti-Y conjugated colloidal gold particles. The BAmICS anti-ϕA1122 strip implements a HF-240 nitrocellulose membrane (Millipore Corporation, Bedford MA) while the anti-Y platform utilizes a FF/85-100 nitrocellulose membrane (Whatman Inc., Florham Park, NJ). Results were determined at 20 and 15 minutes respectively.

Table 8.1 Bacterial isolates tested by BAmICS.

Bacterial Strain	Growth Temperature (°C)	Biovar	Isolate type*
Y. pestis A1122	28°	*Orientalis*	Squirrel
Y. pestis KIM 6+	28°	*Mediaevalis*	Human
Y. pestis Nepal 516	28°	*Antiqua*	Human
Y. pestis CO 92 3015	28°	*Orientalis*	Human
Y. pestis CO 96 3188	28°	*Orientalis*	Cat
Y. pseuduotuberculosis 1A	28°	-	UK
Y. pesudotuberculosis 1A	37°	-	UK
E. coli ECOR 4	37°	-	Human
E. coli ECOR 25	37°	-	Dog

*UK is unknown

[8.2] A subset of bacterial strains were selected and tested from a larger group listed in Chapter 2 and were based on their biovar or the strains positive results from lysis susceptibility.

8.4 Results and discussion

Matrix Effects

Several matrices were tested with the BAmICS system to ensure no false positives resulted. Results shown in Table 8.2 indicate that the growth media, normal saline, potassium phosphate buffer and urine matrices produced negative results when applied to the anti-ϕA1122 and anti-Y conjugated strips.[8.3] Sheep's blood result in a positive response or gave an invalid result on both conjugated strips when the red blood cells either flowed through the membrane and interacted with the test line or obscured it, otherwise the results for this matrix showed negative results. This problem can be easily remedied on future manufacturing of strips by applying a sample pad with smaller pore sizes which can retain the larger red blood cells and other particulates. Saliva also gave a positive response on both test strips and further investigation is warranted into this phenomenon. However, conjecturing on the reason why saliva produced a positive response leads to salivary amylase, which is an enzyme that functions to breaks down the starches and carbohydrates found in such foods as breads and sugars. Salivary amylase contains 5 disulfide bonds in its structure and the primary binding mechanism of the colloidal gold particles to the antibodies is through dative bonding which creates sulfur bridges with cysteine residues of the antibodies, potential colloidal gold conjugation to this salivary is enzyme is a high probability [153, 154]. While the current application of this detection is to be performed primarily with a growth media, extended applications may include saliva from mouth or throat swab, thus potentially requiring a change in the conjugate reporter molecule to something such as latex where the primary binding mechanism is different than that of the colloidal gold particles.[8.4]

8.3 The anti-Y conjugated ICS detector did show a slight positive response to the TSB growth media when 25μL of running buffer was applied to the strip (see chapter 7) causing ambiguity in results, thus only limited testing was conducted on the anti-Y ICS detector. The rest of the performance testing was performed with only the anti-ϕA1122 conjugated ICS system.

8.4 While this author is very aware that there are several ICS detections systems currently available which can handle samples obtained via mouth and throat swabs, the extent to which these ICS systems utilize colloidal gold particles for reporting for this specific application has not been investigated.

Table 8.2 BAmICS matrix effects and performance.

Bacterial Strain	BAmICS Response* with Anti-ϕA1122 conjugated	BAmICS Response* with Anti-Y conjugated
Tryptic Soy Broth (TSB)	-	-
Brain Heart Infusion (BHI) growth media	-	-
Potassium Phosphate Buffer Saline (PBS)	-	-
0.85% Sodium Chloride (normal saline) solution	-	-
Urine	-	-
Sheep's Blood with greater than 35% hemocrit	+/-	+
Saliva	+	+
Running Buffer	-	+/-

*+ strong response, +/- slight response, - negative response.

Selectivity

To allow for maximum progeny phage production, a 10-hour infectivity study was performed with phage ϕA1122 and a bacterial concentration on the order of 10^8 cfu/mL. A control containing only bacteria as well as another control after initial adsorption of the phage to the bacteria was performed to ensure the ICS did not produce a false positive response from either the bacteria or upon initial infection. Results shown in Table 8.3 indicate that the BAmICS system was able to positively identify all *Y. pestis* strains tested. An example is shown in Figure 8.3 using phage ϕA1122 to infect *Y. pestis* CO96 isolate. To account for the increase in bacterial density over time and to assure that the higher density did not produce a false positive on the strip, bacterial controls were performed in conjunction with the infection experiments. These controls all produced negative responses to all isolates tested (results not shown).

The anti-ϕA1122 BAmICS system also gave a negative result for *Y. pseudotuberculosis* 1A when these cells were grown at 28°C, while a positive identification of this bacteria was generated when the cells were grown at 37°C. This demonstrates the negative and positive controls which are implemented in the lysis strip method with *Y. pseudotuberculosis.* The reason for the different responses at the two temperatures is due to *Y. pseudotuberculosis* having differential protein expression[8.5] on the cell surface lipopolysaccharide (LPS) layer [155-158], it is highly probable that the ϕA1122 phage is unable to access adsorption receptor sites when the cells are grown at 28°C.

The anti-ϕA1122 BAmICS system also showed negative results towards the two *E. coli* strains[8.6] when tested with all controls implemented as well as after phage infection experiments. For completeness, isolates of *Y. enterocolitica* should also be tested on the BAmICS device to assure no false positives are obtained from this *Yersinia* pathogen.

8.5 *Y. pseudotuberculosis* 1A cells express an O-antigen on the distal region of the LPS, which is temperature dependent.

8.6 The two *E. coli* strains tested were selected from the over 100 isolates tested in Chapter 2. These bacterial isolates were selected for testing on the basis of their susceptibility to lysis from all 5 yersiniophages.

Table 8.3 BAmICS selectivity testing. Results obtained after a 10 hour infectivity study with anti-ϕA1122 conjugated BAmICS and phage ϕA1122.

Bacterial Isolates	Bacterial Density Infected (cfu/mL)	Infecting Phage Concentration (pfu/mL)	MOI*	Results
Y. pestis A1122	$9.6(\pm1.3) \times 10^7$	$7.8(\pm2.0) \times 10^6$	0.08	+
Y. pestis Nepal 516	$8.4(\pm2.0) \times 10^7$	$7.8(\pm2.0) \times 10^6$	0.09	+
Y. pestis KIM 6+	$1.2(\pm0.2) \times 10^8$	$7.8(\pm2.0) \times 10^6$	0.07	+
Y. pestis CO 92 3015	$5.4(\pm2.9) \times 10^7$	$7.8(\pm2.0) \times 10^6$	0.14	+
Y. pestis CO 96 3188	$8.5(\pm1.6) \times 10^7$	$7.8(\pm2.0) \times 10^6$	0.09	+
Y. pseuduotuberculosis 1A	$1.1(\pm0.1) \times 10^8$	$7.8(\pm2.0) \times 10^6$	0.07	-
Y. pesudotuberculosis 1A	$1.1(\pm0.1) \times 10^8$	$7.8(\pm2.0) \times 10^6$	0.07	+
E. coli ECOR 4	$1.2(\pm0.2) \times 10^9$	$7.8(\pm2.0) \times 10^6$	0.007	-
E. coli ECOR 25	$1.2(\pm0.2) \times 10^9$	$7.8(\pm2.0) \times 10^6$	0.007	-

*MOI is the multiplicity of infection which is the ratio of phage to bacteria infected

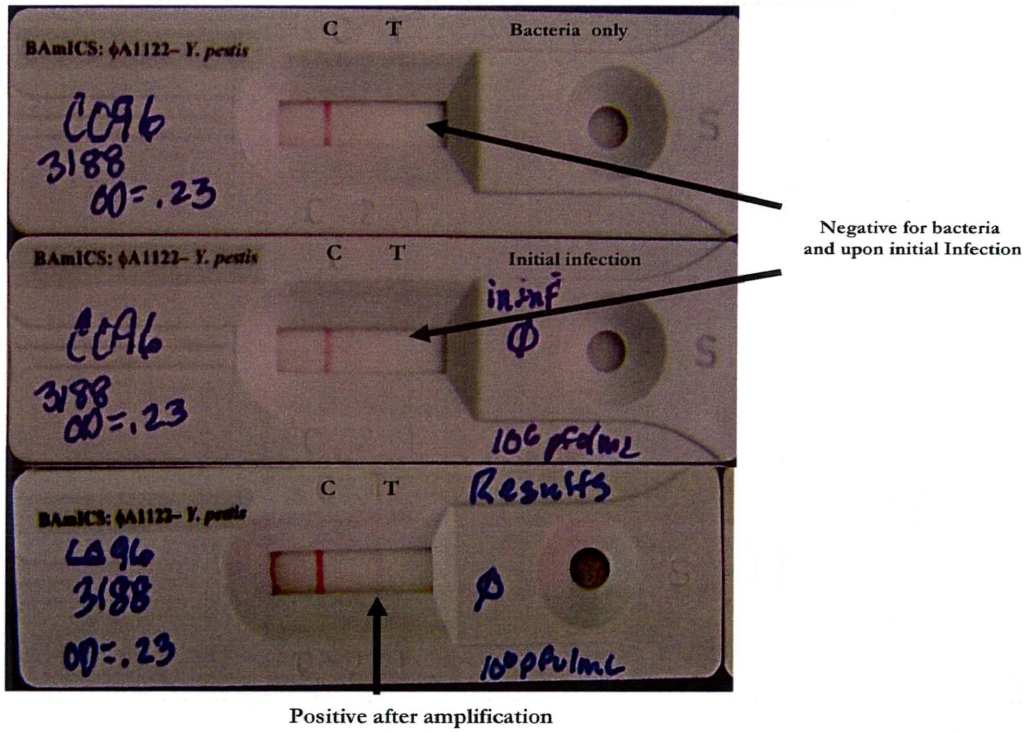

Figure 8.3 BAmICS identification of *Y. pestis* CO96-3188. Results of 10-hour infectivity study with phage ϕA1122 and *Y. pestis* CO96 isolate. C and T indicate control and test lines respectively. Top detector shows bacteria only before initial infection, the middle detector shows results after initial infection of the bacteria, the bottom detector shows results after lysis and release of progeny phage.

Speed

The speed of the BAmICS methodology was checked by performing a 3-hour timed study at bacterial concentrations on the order of 10^7 and 10^8 cfu/mL and then applying a 50μL sample followed by 25μL of running buffer to the strip. Results shown in Table 8.4 indicate that all isolates could be positively identified at a bacterial concentration of 10^8 cfu/mL 2-hours post infection, while 10^7 cfu/mL took 3-hours. Theoretical curves are included (Figure 8.4) to show that it takes time to generate this high concentration of progeny phage and to demonstrate that the time at which the signal actually becomes detectable is inherently linked to the speed of this assay. If the limit of detection could be lowered, overall assay time could potentially be decreased to approximately 40-60 minutes at these same bacterial concentrations.

Table 8.4 BAmICS rapidity testing. Results of the anti-ϕA1122 BAmICS methodology at *Y. pestis* concentrations on the order of 10^8 and 10^7 cfu/mL.

Bacterial Isolates	Bacterial Density Infected (cfu/mL)	Infecting Phage Concentration (pfu/mL)	MOI	Results 1-hour post infection	Results 2-hours post infection	Results 3-hours post infection
Y. pestis A1122	$9.6(\pm1.3) \times 10^7$	$7.8(\pm2.0) \times 10^5$	0.008	-	+	+
Y. pestis Nepal 516	$8.4(\pm2.0) \times 10^7$	$7.8(\pm2.0) \times 10^5$	0.009	NT	+	+
Y. pestis KIM 6+	$1.2(\pm0.2) \times 10^8$	$7.8(\pm2.0) \times 10^5$	0.007	NT	+	+
Y. pestis CO 92 3015	$5.4(\pm2.9) \times 10^7$	$7.8(\pm2.0) \times 10^5$	0.014	NT	+	+
Y. pestis A1122	$9.6(\pm1.3) \times 10^7$	$7.8(\pm2.0) \times 10^5$	0.08	NT	-	+
Y. pestis Nepal 516	$8.4(\pm2.0) \times 10^7$	$7.8(\pm2.0) \times 10^5$	0.09	NT	NT	+
Y. pestis KIM 6+	$1.2(\pm0.2) \times 10^8$	$7.8(\pm2.0) \times 10^5$	0.07	NT	NT	+
Y. pestis CO 92 3015	$5.4(\pm2.9) \times 10^7$	$7.8(\pm2.0) \times 10^5$	0.14	NT	-	+

+ positive result, - negative result, NT not tested

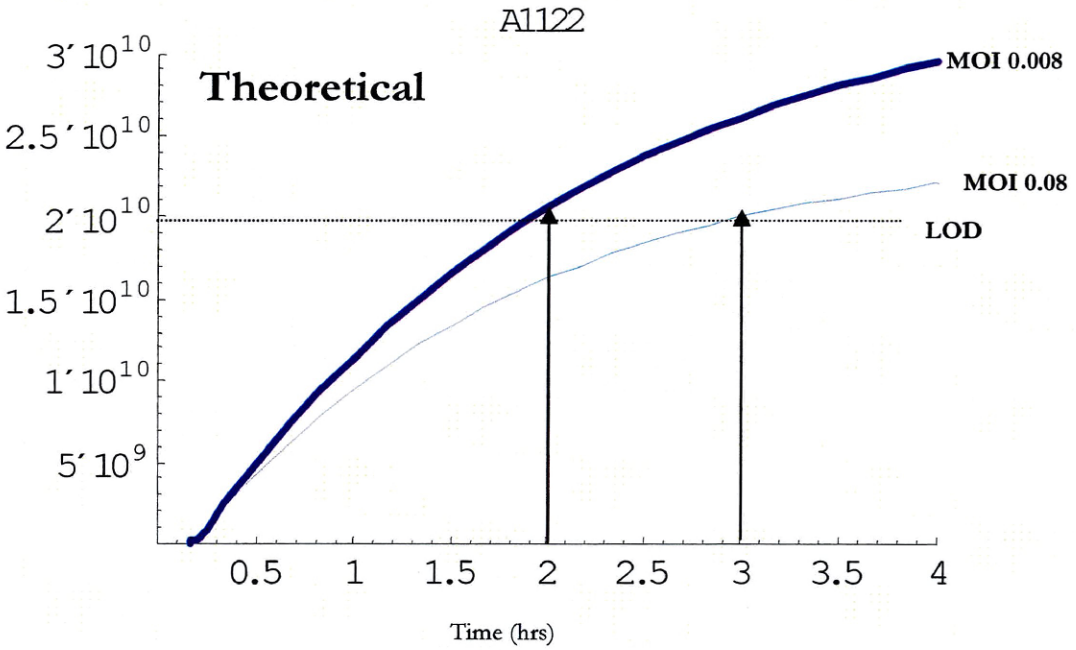

Figure 8.4 Theoretical modeling of progeny phage produced with *Y. pestis* A1122. No modeled initial infecting phage concentration could produce 10^{10} pfu/mL progeny phage to reach ICS LOD at a bacterial concentration of 10^5 cfu/mL, modeling curves are only shown for 10^8-10^6 cfu/mL for the bacteria listed in Table 8.4.

Sensitivity

Sensitivity was also tested by performing a 15.5 hour infection experiment using a concentration of 10^8-10^5 cfu/mL of *Y. pestis* A1122 bacteria. Samples were tested at 4 and 15.5 hours respectively and results are shown in Table 8.5. Results show 4-hours post-infection that bacterial concentrations of 10^7 and 10^8 cfu/mL of *Y. pestis* cells are detectable which is consistent with results obtained in the previous section. At a bacteria concentration of 10^6 cfu/mL, no signal was obtained, however testing the same samples after 15.5 hours, bacterial concentrations of 10^8-10^6 cfu/mL generated positive responses. In an effort to try and detect a bacterial concentration of 10^5 cfu/mL, the concentration of phage was decreased to 7.8(±2) x 10^3 pfu/mL, however no detectable signal was generated after 15.5 hours post infection. Modeling demonstrates that no infecting phage concentration at 10^5 cfu/mL bacteria could generate enough progeny phage to reach the detection limit on the order of 10^{10} pfu/mL. Figure 8.5 shows the theoretical modeling curves of the concentrations listed in Table 8.5. Since the detection limit is extremely high for this prototype, the sensitivity is mediocre at best and is evident by the bacterial concentrations generating a positive identification just barely exceeding the limit of detection resulting in a "light" test line response. Figure 8.6 shows actual BAmICS results from this experiment.

Table 8.5 BAmICS sensitivity testing. Sensitivity testing results of the ϕA1122-BAmICS methodology at bacterial concentrations corresponding to 10^8-10^5 cfu/mL.

Bacterial Isolates	Bacterial Density Infected (cfu/mL)	Infecting Phage Concentration (pfu/mL)	MOI	Results 4-hour post infection	Results 15.5-hours post infection
Y. pestis A1122	9.6(±1.3) x 10^7	7.8(±2.0) x 10^7	0.8	+	+
Y. pestis A1122	9.6(±1.3) x 10^6	7.8(±2.0) x10^6	0.8	+	+
Y. pestis A1122	9.6(±1.3) x 10^6	7.8(±2.0) x 10^5	0.2	-	+/-
Y. pestis A1122	9.6(±1.3) x 10^4	7.8(±2.0) x 10^3	0.08	NT	-

+ positive result, +/- slight positive, - negative result, NT not tested

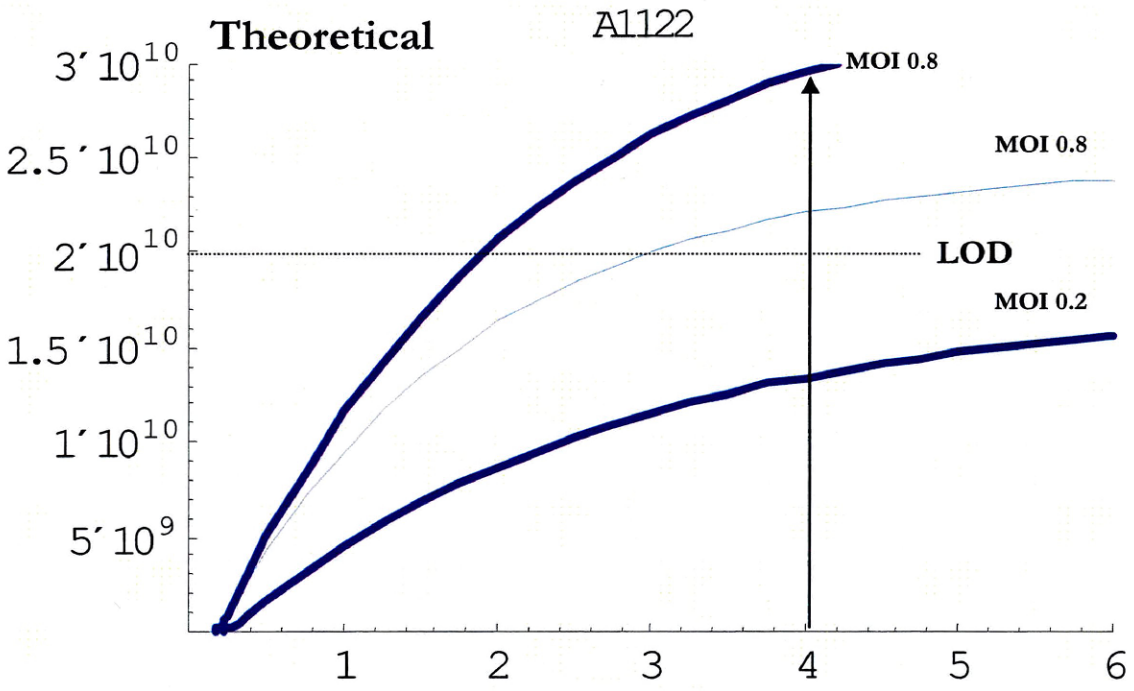

Figure 8.5 Theoretical modeling of progeny phage signal with *Y. pestis* A1122. Table 8.5 lists modeled initial infectivity concentrations.

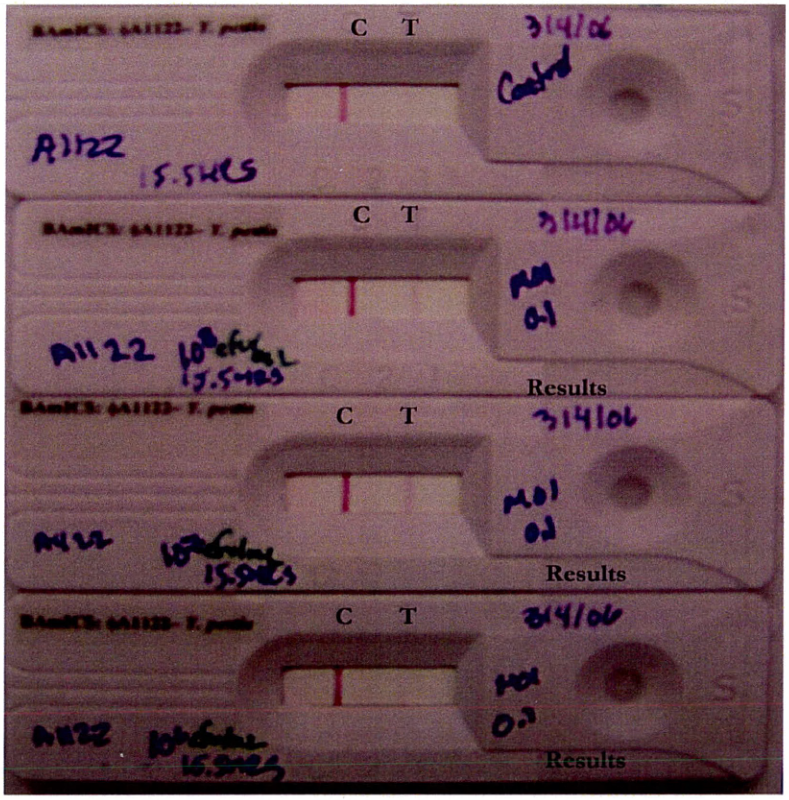

Figure 8.6 BAmICS sensitivity results. Sensitivity results of a 15.5 hour infectivity study with phage ϕA1122 and *Y. pestis* A1122. C and T indicate control and test lines respectively. Top detector shows bacteria only before initial infection, the subsequent three detectors show results of positive *Y. pestis* A1122 identification at 10^8, 10^7 and 10^6 cfu/mLbacteria respectively.

Conclusions

The anti-Y detection strips have some issues in terms of background matrices and this problem needs to be investigated further as the testing provided ambiguous results. As for the anti-ϕA1122 BAmICS system, it demonstrated a background problem with saliva, however for the current application of this detection methodology, it is unlikely that saliva will be problematic as isolates are usually grown to log phase in media broth. The anti-ϕA1122 BAmICS system also showed excellent selectivity towards the *Y. pestis* isolates as it was able to positively identify isolates from all three biovars as well as the genetically manipulated strains. BAmICS also performed as expected when phage ϕA1122 lysis was inhibited in the *Y. pseudotuberculosis* at 28°C, but positively identified the bacteria at 37°C. This is significant as this demonstrates the positive and negative controls used in the lysis strip method. In terms of rapidity, the detector was able to detect progeny phage generated from 10^8 and 10^7 bacterial cells in 2 and 3 hours respectively and this time could potentially be decreased with the production of stronger binding antibodies. In terms of sensitivity, amplification studies showed that 10^6 bacterial cells were detectable on the strip however this was inconsistently obtained with the *Y. pestis* isolates tested. Therefore it can be concluded that reliable signals can be consistently generated and positive results obtained with the strip when the viable *Y. pestis* cell concentration is 10^7 cfu/mL or greater.

This prototype demonstrates that the lysis strip method, originally developed by Cavanaugh and Quan [34] in the early 1950's has the capability to be coupled to a modern detection system like immunochromatography strips. The possibility of the BAmICS system to be purposely circumvented is extremely low, since not all cells share the same surface structures or biochemical groups on their cell walls and many of these cell surface structures have evolved to mediate specific cell surface recognition events, such as those involved with nutrient uptake, transduction of signals across the membrane, or binding to other cells. This prototype is not intended to replace other diagnostics routinely employed to identify *Y. pestis* such as the direct fluorescent anti-F1 test, enzyme immunoassays, agglutination assays or even PCR, rather it is to facilitate the detection of *Y. pestis* where these diagnostics can be

limited such as *Y. pestis* isolates from the flea vector, naturally deficient F1 strains, as well as genetically manipulated strains.

CHAPTER 9
CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

Phage amplification coupled with ICS detection has been shown to be an effective and promising method for the identification of *Yersinia pestis* strains. The modified Payne and Jansen has proven to be an excellent mathematical computer model for predicting the final concentration of progeny phage produced during an infection event as well as predicting the time in which it takes to reach a pre-determined detectable concentration provided that parameters of burst size, lysis time and the bacterial generation time are either known from the literature or are initially experimentally determined.

The production of antibodies plays a critical role in the development of bacteriophage amplification immunochromatography strips and directly affects the limit of detection and in turn the speed, sensitivity and selectivity of the assay. Because polyclonal antibodies contain of a mixture of the antibodies produced by a multitude of immune cell clones in the animal's immune system the complex mixture of antibodies can cause a high occurrence of false positive results to unrelated targets. To deal with this problem pre-adsorption of non-specific binding antibodies was performed before colloidal gold conjugation to keep the assay specific to the selected yersiniophage. As a consequence, an overall decrease in binding titer was observed to the phage target and results in the need for a very strong antigenic response to the phage during polyclonal antibody production.

9.2 Future work

Production of stronger (high affinity) polyclonal antibodies with the Freund's adjuvant would certainty be advantageous to lowering the limit of detection on the BAmICS device, however pre-adsorption of non-specific binding antibodies is still an issue. Other alternatives are available and future work to improve upon this prototype could include the production of a monoclonal antibody (MAb) for use as the antibody/reporter conjugate. Monoclonals are highly specific to a given antigenic epitope (antibody binding site) and unlike polyclonal antibodies which are produced from a multitude of immune cell clones that recognizes independent epitopes on a phage or phage protein, MAb are the result of isolating a single cell clone from the animal (typically mice) and artificially fusing them with an immortal cell line. These immortalized cell lines are referred to as hybridomas which can be grown in the laboratory by cell culture and have the capability to continue secreting antibodies into their growth medium. The high specificity of a monoclonal antibody when conjugated to colloidal is a significant advantage, as the need for pre-adsorption is minimized or may even be eliminated. While MAb are easily purified and typically have high binding titers to their target, creation is usually slow, relatively expensive and production can be variable due to mutating cell lines or cessation of antibody secretion.

Recombinant technology is also another alternative to obtain high affinity antibodies. For example, the genetic sequence of the head assembly protein of phage ϕA1122 could be inserted into a cloning (expression) vector to produce a single type of protein for antibody production (PAb or MAb). While this technique is relatively simple and cost effective a major drawback to utilizing this technique is the potential for incorrect tertiary folding of the protein upon expression, thus rendering subsequent antibodies produced with this protein antigen with weak or no binding to capability to the native phage protein. However, antibodies produced against a phage which has been genetically engineered to over express a native phage protein is a most promising idea. For example, phage ϕA1122 could be genetically engineered to over express the host assembly protein and MAb produced against

this specific protein for detection. Potentially this genetic engineering, if performed with the ϕA1122 host assembly protein, could provide a means of rapid identification without the need for progeny phage production.

Phage amplification coupled with modern detection proves to be a simple yet effective identification tool for *Yersinia pestis* strains. In addition to the use of this technique in a clinical laboratory setting, potential uses also include environmental testing and use as rapid diagnostic in bio-threat situations.

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APPENDIX A: FUNDAMENTALS OF MALDI-TOF-MS

A.1 Matrix assisted laser desorption ionization mass spectrometry

A molecule naturally possesses rotational, vibrational, and electronic energy. If it is a liquid or a gas it will also have kinetic energy. Usually if molecules in a solid have their internal energy increased (e.g. by heat or radiation) over a period of time, the molecules can equilibrate the energy individually and together as a system so that the excess energy is dissipated to the surroundings without causing any change in molecular structure. Beyond a certain threshold, however, too much energy in too short of a time, the energy cannot be dissipated fast enough, so the substance melts and then vaporizes as internal energy of vibration and rotation is turned into translational energy (kinetic energy). At the same time, electronic excitation may be sufficient so that electrons are ejected from molecules to give ions. Thus, putting a lot of energy into a molecular system in a very short time can cause melting (with increased rotational and vibrational electronic energy), vaporization (desorption, increased kinetic energy), ionization (electronic excitation energy leading to the ejection of an electron), and destruction of a material (increase in total internal energy sufficient enough to cause bond breaking). If enough energy is deposited into a sample in a very short time, it has no time to dissipate the energy to its surroundings and is effectively blasted away from the target area because of the large gain in kinetic energy and is said to be ablated. Laser desorption ionization is the process of beaming a laser light, in pulses onto a small area of sample containing analyte and matrix to desorb ions which can be examined by a mass spectrometer.

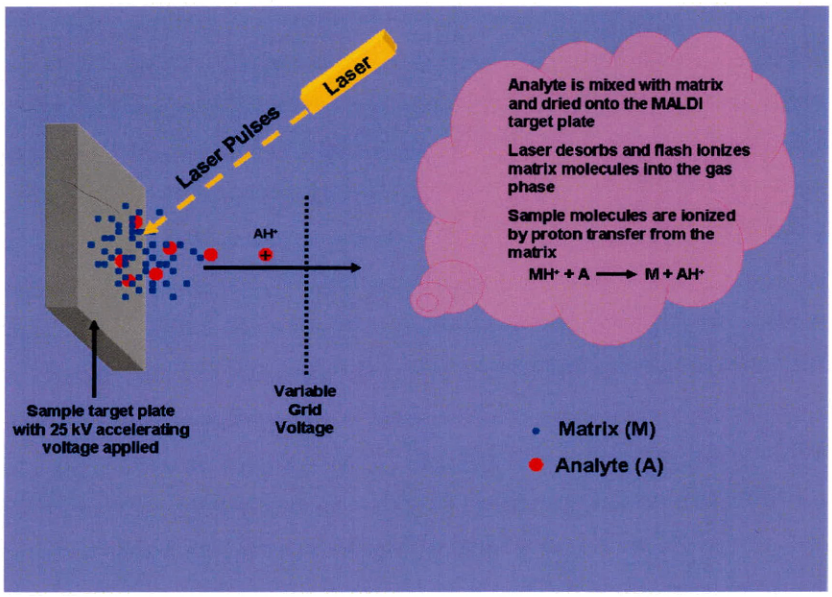

Figure A.1 Principles of MALDI [92].

For a substance to absorb the energy from laser radiation it must have an absorption spectrum (ultraviolet, visible or infrared) that matches the incident energy. It is impractical, however, to match the laser wavelength and a substance's absorption spectrum for each and every sample as it would obviously be tedious and time consuming. Instead the mixing ratio of an analyte with a matrix that closely matches the laser wavelength can be utilized to solve this problem. This is accomplished when a matrix is mixed with an analyte and allowed to dry. When irradiated, some of the energy absorbed by the matrix will be transferred to the sample causing it to subsequently desorb and ionize due to transfer of protons from the matrix to the sample (Figure A.1). As a result this technique is only dependent upon the laser energy matching an absorption band in the matrix and not the sample. This method of ionization is called matrix assisted laser desorption ionization (MALDI). Organic acids such as sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) or ferulic acid are two of several types of matrices available for this ionization method. MALDI is considered a soft ionization

technique because the ions produced are mainly protonated molecules and produce few fragment ions. Thus MALDI is an excellent ionization method for biomolecules such as peptides and proteins to yield single protonated ions in high yield because most of the abalated material is the matrix.

In theory, any type of laser can be used to effect desorption and ionization provided that it supplies enough energy at the right wavelength in a very short amount of time to the analyte/matrix mixture. In practice, however, only a few lasers are actually used. Usually, laser energies corresponding to the ultraviolet (266 nm), near-visible (337 or 355 nm), or infrared (20 mm) regions of the electromagnetic spectrum are used. In addition, pulsed lasers are much more commonly used with MALDI because the peak power delivered by the pulse is much greater than that of a continuous laser beam.

A.2 Time of flight mass spectrometry

Fundamentally the time-of-flight (TOF) ion optics is the simplest of all mass analyzers as there are no electric or magnetic fields to constrain or complicate an ion trajectory. Simply put, in linear mode the ions are extracted from the MALDI source in short pulses and directed down an evacuated straight flight tube or field free drift region to a detector (Figure A.2). After initial acceleration, the ions pass in a straight line, at constant speed, to the detector. The arrival of the ions at the detector is recorded as a trace of ion abundance against time of arrival, with the latter being converted into a mass scale to give a final mass spectrum.

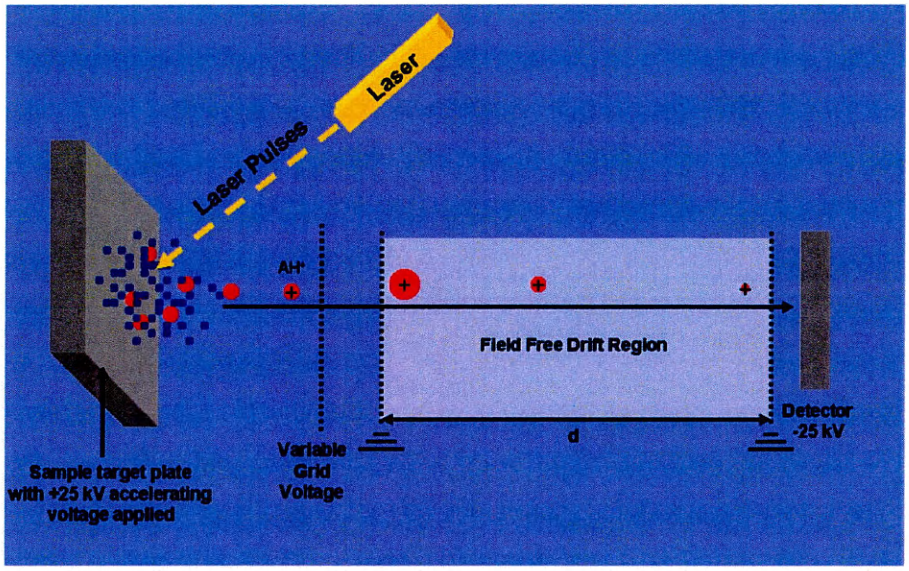

Figure A.2 TOF-MS. Field free drift region shown in linear mode. In the field free drift region ions with a greater m/z take more time to reach the detector than do ions with a smaller m/z [92].

Equations of motion are used to describe the flight of the ions from the source to the detector. For example, the time taken to travel the length of the drift region depends upon the mass of the ion and its charge (z). For singly charged ions z = 1; m/z = m, the time taken to traverse the distance (d) from the source to the detector (Equation A.1) is a function of mass. As the ion leaves the source accelerated by a potential V_s, an ion with a mass m and total charge q = ze has a kinetic energy E_k defined by Equation A.2. By solving for v in Equation A.1 and substituting variables for v^2 in Equation A.2, Equation A.3 shows that m/z can be calculated from a measurement of t. Because the distance, the potential, and the charge on an electron is constant in the instrument, the flight time of an ion is directly proportional to the square root of m/z. Fundamentally, Equation A.3 shows that the greater the mass of the ion, the slower its arrival at the detector or simply put, it takes twice as long for an ion with a m/z = 100 to reach the detector than an ion with a m/z = 25.

$$d = t/v \qquad \text{(Equation A.1)}$$

$$E_k = m^*v^2/z = z^*e^*V_s \qquad \text{(Equation A.2)}$$

$$t = (m^*d/z^*2e^*V_s)^{1/2} \qquad \text{(Equation A.3)}$$

In principle, the upper mass range of a TOF mass analyzer has no limit, which makes it ideal to be coupled to MALDI for protein analysis. For example, samples with masses greater than 300k Da have been observed by MALDI-MS. The transmission efficiency is also extremely high, resulting in very high sensitivity. For example, attomolar (10^{-18} molar) quantities of various proteins such as cytochrome C, lysozyme, and myglobin have been obtained with a TOF analyzer [159].

A.3 Resolution

While the TOF mass analyzer is sensitive, the attainable resolving power is limited for two reasons. First, the flight times are proportional to the square root of the m/z. The difference in flight times, Δt, for two ions t_m and t_{m+1} separated by unit mass is given by Equation A.4 (Figure A.4):

$$t_m - (t_{m+1}) = \Delta t = \sqrt{(m/z)} - \sqrt{(m+1/z)} * \text{constant} \qquad \text{(Equation A.4)}$$

As m increases, Δt becomes increasingly smaller. Thus, the difference in arrival times of ions arriving at the detector becomes increasingly smaller and more difficult to differentiate.

The second problem is that not all ions of any one given m/z value reach the same velocity after acceleration from the source, nor are they formed at exactly the same point in the ion source. Therefore, even for any one m/z value, ions at each m/z reach the detector over an interval of time instead of all at one time. Where separation of flight times is very short as in TOF instruments, the spread for individual ion m/z values means there will be an overlap in arrival times between ions of closely similar m/z values. One solution to this problem is modifying the instrument to include a reflectron (reflectron mode will not be discussed in this thesis); further information on reflectron mode can be obtained from reference mass spectrometry books [92]. Another solution is to include a delayed pulsed extraction developed in the 1950's by Wiley and McLaren [92]. In delayed pulsed extraction mode, the ions are initially allowed to separate according to their kinetic energy in the field free drift region. For ions of the same m/z, those with more energy move further toward the detector than the initially less energetic ions. The extraction pulse applied after a certain delay time transmits more energy to the ions that remain in the source for a longer period of time. Thus, the initially less energetic ions receive more kinetic energy from the delayed

extraction pulse and join the initially more energetic ions at the detector, effectively closing the kinetic energy gap between ions of the same m/z.

APPENDIX B: LYTIC BACTERIOPHAGE AND BACTERIAL HOSTS

B.1 Physical properties of lytic bacteriophage

Bacteriophage (phage) are viruses that infect bacteria. A phage requires specific metabolic activities of a bacterial host in order to replicate and is therefore considered to be an intracellular parasite. Phage which discriminate in their choice of host cells are said to have a narrow "host range" or limited variety of bacterial cells that it can replicate in. For example, phage which have a specific host range for a given bacterial genus or species like *Yersinia* or *Staphylococcus aureus (S. aureus),* take on the specificity of the host name such as "yersiniophage" or "aureophage".[B.1]

Phage contain only a single type of nucleic acid, such as deoxyribonucleic acid (DNA)[B.2] or ribonucleic acid (RNA), while host cells contain both DNA and RNA. The remainder of the phage is composed of proteins and/or glycoproteins and contain none of the lipids, glycolipids, simple sugars, polysaccharides, nucleotides like ATP or ADP, free amino acids, and other small molecules that occur in its bacterial host. At the biochemical level, phage, are fundamentally much simpler than their bacterial host.

In comparison to bacterial genomes, DNA phage genomes are quite small, can be single or double stranded, and appear as linear or circular shaped molecules. Phage DNA genomes vary in size over two orders of magnitude, from approximately 1.2×10^6 Da to 2×10^8 Da and are protected from the external environment by a protein and/or glycoprotein coat. Treatment of intact phage particles with nucleases would degrade any exposed nucleic acids in the surrounding solution, but does not degrade the phage particle proteins or the nucleic

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B.1 These specific names will be used interchangeably along with the generic term "phage" and "virus" throughout this thesis.
B.2 Double stranded DNA (dsDNA) will be the main focus through out this thesis.

acid contained within it. On the other hand treatments of intact phage with proteases, which degrade any exposed proteins, cause the phage proteins to degrade exposing the DNA.

Structurally, a phage is composed of a protective protein layer surrounding the nucleic acid and is termed the nucleocapsid. Due to the nanometric size of phage particles, TEM is a common method that allows visualization of phage particle structures as small as individual capsomers. The capsomers are, in turn, formed by the association of a definite number of individual proteins, often referred to as structural subunits or protomers. Early phage work performed by Crick and Watson (1956) demonstrated that the protein capsid is relatively large compared to the nucleic acid it contains. Since phages contain only a small number of genes it can code for only a limited number of proteins. In addition to the capsid protein(s), there are also nonstructural proteins that must be coded for which are necessary for phage reproduction. Due to the restraint of the small genome, the number of structural proteins is limited and as a consequence synthesizing a large number of only a few phage proteins is more practical than a small number of many proteins.

Most phage arrange their structural proteins in the form of an icosahedron, which is a three dimensional structure with 20 faces in the shape of equilateral triangles. Each of the 12 corners or vertices of the icosahedron is the intersection of five triangular faces. This configuration requires minimal energy to assemble from its subunits and therefore is the most efficient design for a capsid enclosing a spherical space.

In small phage, each of the triangular faces is constructed of three structural subunits or protomers and 60 subunits are used in the entire phage. Large phage are constructed using multiples of three structural proteins in each capsomer (multiples of 60 subunits for the entire phage), rather than synthesizing large individual proteins. In large capsid proteins the structural proteins of each face of the icosahedron join in pentameric fashion creating a pentamer, while the subunits or protomers of adjoining triangular faces form hexamers. For most phage the same protein can function in either location so distinction between pentamers and hexamers is somewhat of a moot point for infection of prokaryotes, but is of

enormous significance in animal viruses because pentons are the attachment point for long projections called spikes.

Many phage also have another basic structure in addition to the nucleocapsid, which is a tail section. The tail section can be many shapes with the most common shapes being a helical hollow tube or a short conical tail. The tail on a phage serves as an entry method of injecting its nucleic acids into the bacteria for replication. Phage tails are also found to have from one to six short thin tail fibers. For example, a T-7 phage which belongs to the Podoviridae family has a head diameter of 50-60 nm with a short tail protruding from one of its vertices and contains six short tail fibers.

B.2 Important structural features of bacterial hosts

The first interaction of phage with its host is the attachment of the phage to the bacterial cell. The cell surface and any structures associated with the surface play a crucial role in the attachment process. There are marked differences in bacterial architecture of cell organizations which produce significant differences in strategies used by the host specific phage for both entering and leaving the host cell. All bacteria are prokaryotic cells which are bound by a rigid cell wall.[B.3] Bacterial cell walls function to provide the mechanical protection from necessary for the survival of bacterial cells that are often subjected to extreme changes in their osmotic environments. The cell wall also provides the bacteria with characteristic shapes such as rods, spheres, or spirals. Bacteria are divided into two main categories, Gram positive and Gram negative, based on the architectural differences of the cell walls. These differences play a crucial role in the recognition event between phage and bacterial cells.

[B.3] All prokaryotes have cell walls with the exception of mycoplasma.

B.3 Gram-negative bacterial hosts

Gram-negative bacteria also have an outer membrane system that contains phospholipids, proteins, and a unique compound called the lipopolysaccaride (LPS) or endotoxin. The LPS plays a major role in phage/bacterial interaction. The LPS is divided into three sections: 1) lipid A; 2) core polysaccharide; and 3) O antigen. The lipid A anchors the LPS in the outer membrane though long fatty acids attached to a pair of N-acetyl-glucosamines. One of the N-acetyl-glucosamines also carry a large complex carbohydrate chain that sticks out from the membrane into the environment surrounding the cell and is the core polysaccaride. The core polysaccaride chain or keto-deoxy-octonate (KDO) is a highly branched structure and includes monosaccharides with 7 or 8 carbons. Attached to the distal end of the core polysaccharide is a specific repeating sequence of sugars termed the O antigen. The O antigen unit sequences are short and consists of 4 or 5 carbohydrates and the entire structure contains many of these units. Gram-negative cells have a peptidoglycan layer similar in construction to that of gram-positive cells, however the peptidoglycan layer is much thinner (only about one or two layers thick). In addition, the percentage of amino acids involved in cross linking the glycan chains is much less resulting in a looser overall construction. The O antigen shows enormous variability and likely plays a role in recognition events that occur at the cell surface for gram-negative bacteria. Bacteria can express either smooth LPS, which is composed of O antigen, complete core oligosaccharides, and the lipid A, or rough LPS which lack O antigen but possess lipid A and progressively shorter core oligosaccharides. In addition to the LPS, the outer membrane on gram-negative bacteria contain many integral proteins called porins, which served as transport channels for ions and other small nutrient molecules. Thus the core, O antigen sugars, and porins provide a variety of possible recognition molecules for phage/bacteria interaction.

B.4 General features of bacteriophage/host interaction

In general, the interaction between a phage and its host bacterium is the process of phage replication. Phage replication can be divided into five steps: 1) attachment; 2) penetration; 3) synthesis of nucleic acids and phage proteins; 4) maturation; and 5) release.

B.5 Attachment/adsorption

Attachment of phage, also known as adsorption, to a host cell occurs by means of specific recognition events at the cell surface. The reproduction of any phage requires that it enter a host cell and use that cell's metabolic machinery to synthesize the components of new phage. The manner in which this occurs is dependent on the architecture of the phage as well as the nature of the structures surrounding the target host cell. The host range or the different types of cells that a particular phage can infect can be broad enough to cross phylum or so narrow that it only can infect certain strains of a single species.

So what is responsible for this specificity of the phage/host interaction? It has been hypothesized that attachment of the phage to the surface of its host is the most probable explanation for phage specificity. For example, if a phage attempts to attach to an inappropriate cell, but cannot do so, the phage may be able to adsorb again on a different cell. On the other hand if the phage actually enters the cell, but is unable to reproduce because the cell is incapable of supporting its replication, the phage is effectively inactivated since it cannot produce progeny phage and it cannot leave the cell to try and adsorb to another host. Evidence exists to support the hypothesis that specificity is a cell surface phenomenon. For example, virtually all cells in an organism within a particular kingdom are capable of carrying out metabolic activities according to the Central Dogma of Molecular Biology.[B.4] If a particular phage is capable of replication in one type of cell, *E. coli* cell for example, then it would be reasonable to assume that it would infect all species of *Escherichia* , however this is typically not the case. The reason for this is that not all cells share the same

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[B.4] Transcription of DNA → RNA → protein: This dogma forms the backbone of molecular biology.

surface structures or biochemical groups on their cell walls. Many of these cell surface structures have evolved to mediate specific cell surface recognition events, such as those involved with nutrient uptake, transduction of signals across the membrane, or binding to other cells. For instance, *E. coli* bacteria resistant to infection by phage T4 can be isolated from T4 plaques. Genetic analysis of these resistant bacteria demonstrated that they had undergone a mutation which changed the structure of their cell wall LPS molecules causing the phage to be unable to adsorb to the cell surface.

Binding of phage to its host cell is probably mediated by electrostatic interaction between proteins or glycoproteins on the phage capsid and host cell surface proteins, glycoproteins, or glycolipids. Amino acids with charged side chains can bind to other charged amino acids or to the carboxyl groups on the N-acteyl-sugars commonly found at or near the end of the carbohydrate chains in glycoproteins or glycolipids. The formation of such bonds is highly dependent on the phage and the cell surface molecules that are to interact. Both molecules must have the correct three dimensional shape in order to "fit" together properly. In addition the chemical groups on each protein that will form the electrostatic bonds must have the correct ionization. Both the three dimensional shape and the ionization of proteins are influenced by the pH, ionic strength, as well as particular ions present in the medium.

The presence of particular ion(s), pH, and ionic strength have all been found to influence the attachment of phage to cell surfaces. For example, the divalent cation Ca^{2+} has been found to be necessary for T5 phage to optimally adsorb to its *E. coli* host. The Ca^{2+} may aid in attachment by providing bridges between 2 negatively charged groups and/or by influencing the three dimensional conformation of the interacting proteins. On the other hand, the pH requirements are usually fairly narrow for a particular phage, but can vary widely for different phages. pH requirements are likely a reflection of the need to have the correct ionization on different sets of interacting amino acids and/or sugars. For example, a physiological pH 7.0-7.4 is thought to create a positively charged arginine on the adenovirus which can bind to negatively charged carboxyl groups on a cell surface membrane.

The attachment of a phage to its target cell also depends on collisions between the phage and the appropriate cell surface receptor. Density of receptor molecules is a factor that can influence the efficiency of attachment, while absolute concentration of the host and the phage play a significant role in the determining the rate of attachment (dA/dt). The rate of attachment can be mathematically defined at various points in time by $(dA/dt) = kPH$, where k is the attachment rate constant, P is the free phage concentration, and H is the host cell concentration. This rate equation has important consequences as the dose-response curve is linear when one phage infects one bacterium (i.e. produces 1 plaque), while the curve becomes parabolic when more than one phage infects a cell. Note that the linear nature of the dose-response curve does not indicate that every phage initially binding to a host cell actually infects that cell.

For most phage/host systems, many phage first become reversibly attached and can be released from the cell surface if the host cell is transferred into fresh medium. After initial reversible phase of attachment, alteration in the phage and/or host cell surface may lead to irreversible binding and progressing to the introduction of the phage genome into the host cell. Efficiency of plating (EOP) is the proportion of phage that actually produce infected cell. Thus the most important factor influencing the EOP for a particular phage/host system is the efficiency with which both reversible and irreversible binding occurs. This factor is quite variable from phage to phage.

Genetic studies have provided information about the nature of phage attachment proteins and the cell surface receptors they recognize. Cell receptors for phage on a number of host bacteria have been elucidated (Table B.1). In gram-negative organisms the LPS provides many binding sites in the O antigen, core polysaccharide, and the proteins in the outer membrane. Teichoic acids in gram-positive bacteria provide the necessary specificity for the phage receptor.

Table B.1 Cell surface receptors for bacteriophage

Bacteriophage	Host Receptor
T4, T3, T7	LPS core polysaccharide
T2	OMPF porin protein
T1, T5	TonA ferrichrome transport protein
T6	Tsx nucleoside transport protein
λ	LamB maltose transport protein
X	Flagellum (Salmonella)
f1, MS2	F pilus
P-50	Teichoic acid (Bacillus)

B.6 Penetration

Penetration and uncoating introduces the phage nucleic acid into a host cell. Phage use diverse strategies for genome entry (for reviews, see references Dreiseikelmann 1994, Letellier et al. 1999, Letellier et al. 2003, Poranen et al. 2002) and mechanisms of penetration vary widely among phage, however phage architecture is one of the most crucial factors in the transfer of genomic material to the host bacteria.

For example, the genomes of T5 and T7 are transported across the membrane through a protein-rich channel partially formed by phage proteins [97, 160-162] and the transfer of the T7 genome occurs by translocation coupled to transcription by *E. coli* and T7 polymerases [163, 164].

Whereas the channel for the transfer of the λ phage genome is formed by host-derived proteins [165, 166]. Lambda phage have non-contractile tails, thus attachment and delivery of phage DNA is through the recognition of the maltose transport protein receptor on the cell surface. This receptor is located on the distal end of the tail tube and when trigged, it interrupts part of the maltose phosphotransport system in *E. coli* allowing concurrent attachment of the phage and transfer of genomic material.

Another excellent example is the T4 phage, which has a tail that contracts or injects[B.5] causing an opening or pore in the plasma membrane from the tail tube into the cytoplasm through which the genomic material can pass. The receptor binding by the tail leads to structural rearrangements, the DNA is injected with the aid of the tail, and the empty capsid is left on the cell surface.

When anyone of these processes occur, the phage as a complete entity disappears and the period between penetration and the production of new phage particles is called the eclipse period. Thickness and biochemical complexity of the bacterial cell wall make penetration a formidable task for any phage. Direct access by pili or flagella make the process easier for some phage when transferring their genomic material because of the direct attachment to the plasma membrane.

B.7 Synthesis of nucleic acids and phage proteins

Synthesis of phage proteins and nucleic acids begins the actual process of phage replication. Phage proteins and enzymes synthesized at this time are dedicated to mobilizing the bacterial host resources for phage synthesis and permits the efficient expression of the phage genome. Synthesis of phage proteins and nucleic acids can occur simultaneously, however phage proteins are normally synthesized after nucleic acid production.

For example, the T7 genome (39,937 bp) contains 56 genes that encode 59 known proteins. Genes are grouped into three classes based on their nature and time of expression.

B.5 The analogy of injection is somewhat misleading as the tail sheath does not contract, it reorganizes.

Class I genes, which enter the host and are transcribed first, moderate the transition in metabolism from host to phage. Class II genes are primarily responsibly for T7 DNA replication, and class III genes code for particle, maturation, and packaging proteins.

After adsorption the growth cycle is initiated by translocation [163, 167-169] of the T7 DNA into the host.[B.6] The entry of T7 DNA takes about ten minutes and, except for the first 850bp, is mediated by either *E.coli* or the T7 RNA polymerase. Class I genes are transcribed by *E.coli* RNA polymerase which recognizes three promoters positioned near the leading end of T7 DNA. Class I mRNAs direct the synthesis of T7 RNA polymerase (gp1), which then transcribes class II and class III genes. Replicated DNA is packaged into procapsids and, together with several other phage proteins, form progeny which begins the maturation stage.

B.8 Maturation

The process of assembling progeny phage (maturation) occurs as soon as genomic material and phage proteins become available. Phage are self assembled from nucleic acid protein components synthesized during the previous step. Classic experiments performed between 1955-1957 by Fraenkel-Conrat and colleagues demonstrated self assembly of phage by dissociating the tobacco mosaic virus into its RNA and protein components, which could subsequently recombine to re-create the infective viral particle. Similar experiments were performed on T4 phage which also demonstrated self assembly of these particles.

Since phages are only composed of proteins and nucleic acids, interactions which form the icosahedral nucleocapsid must either be protein-protein or protein-nucleic acid. Two strategies are possible for the assembly of the nucleocapsid. First, the genomic material acts as the focus for the assembly process, so that the capsomer proteins are placed around the nucleic acid to form the nucleocapsid, while the second process begins with the formation of

---

[B.6] The phage DNA is postulated to be mechanically pulled into the cell by the transcribing polymerases. The entire DNA entry process normally occupies approximately 10 min at 30°C and about 40% of the latent period.

an empty capsid that is subsequently filled by nucleic acid. The choice of strategy appears to be a function of the architecture of the capsid rather than the size or type of genomic material. Helical nucleocapsids are formed around their nucleic acids, while icosahedral nucleocapsids are first assembled into procapsids followed by subsequent filling with of the nucleic acids. Since the focus of this thesis is on T7-like species of phage having icosahedral architecture, discussion of assembly will be limited to formation of an empty capsid that is subsequently filled by DNA.

Assembly of empty procapsids begins when a sufficient concentration of structural proteins becomes available in the cytoplasm of the bacterial host. Nucleic acids then interact with a procapsid to complete the assembly. In many DNA phage form concatemers, or multigenome length molecules, which must then be cut into genome-length molecules for insertion into the procapsid. The cutting process is most often linked to the assembly of the phage and is mediated by phage components.

In general, it is speculated that the order of assembly for simple DNA phage appears to occur in association with the inner surface of the cytoplasmic membrane where the growing procapsid is held in place by a hydrophobic phage protein. A series of intermediate-sized structures can be isolated from infected cells, providing evidence that the assembly proceeds by sequential additions to a growing procapsid.

DNA phage such as the T7-like species, are composed of an iscosahedral capsid, short conical tail and a connecting region at one of the vertices called a portal vertex connector (also known as a DNA translocating vertex). This head-to-tail connector plays an important role in the first steps of head assembly and is the central piece of the DNA packaging machinery. This structure functions as the initiation complex for assembly of the head unit and as the opening for the movement of DNA into the head during maturation and DNA out of the head during penetration. The portal vertex is located at the junction of the head and tail and the portal proteins secure the head and tail together. Mounted on the inside of the connector is the "core" [96], which is a cylindrical protein complex. Scaffolding proteins are required in the construction of the procapsid as it helps to create the proper

overall shape and in building the inner shell, however scaffolding proteins (assembly proteins) are not found as part of the mature phage.

Assembly of the procapsid to the mature capsid proceeds through several steps. The first construct of the double shell, referred to as procapsid I, consists of 415 coat proteins on the outside and scaffolding proteins on the inside which is nucleated around the cylindrical protein complex. The procapsid I structure which usually has a spherical rather than icosahedral shape is typically smaller in dimension than the mature capsid. When the scaffolding proteins are digested or recycled, the procapsid becomes an empty shell and is considered a procapsid II. In preparation or concurrent packaging of DNA, conformational changes in the coat proteins caused by proteolytic cleavages cause the head to expand to its final dimensions and assume the icosahedral shape found in a mature head.

Phages also have complex architectures, which can include a helical or conical tail and its associated tail fibers. While the steps outlined above are occurring to build the mature capsid, separate protein assembly lines are occurring for construction of the phage tail and tail fibers. Final assembly of the head to the tail occurs after the capsid has been packaged with DNA.

B.9 Release

The last step in the interaction of a phage with its host is the liberation of newly synthesized progeny phage. Release is a function of both phage and bacterial host. Almost all phage are naked (non-enveloped) and cause their bacterial host to lyse or break apart spilling the progeny phage into the extracellular environment. Lysis can be accomplished by at least two fundamentally different mechanisms [170].[B.7] In particular, double-stranded DNA (dsDNA) phage use the holin-endolysin strategy to achieve an efficient host lysis [171, 172]. A two component lysis system is needed because lysis involves two different activities since a bacterial cell is surrounded by a cytoplasmic membrane and a complex cell wall.

[B.7] dsDNA phage relevant in this thesis use only the holing-endolysin mechanism for release of progeny, however autolysin is also another fundamental mechanism of lysis, but will not be discussed.

To produce host cell lysis and release progeny phage, first the cytoplasmic membrane is destabilized by the action of phage proteins called holins creating lesions in the membrane. Holins are small membrane proteins which are extremely diverse (comprised of at least 34 unrelated protein families) and are essential for creating holes in the membrane in order for endolysin to gain access to the peptidoglycan layer [171].[B.8] The timing of the holin function is not fully understood, however once the cytoplasmic membrane has been disrupted by holin proteins, a phage enzyme called endolysin is used to degrade the peptidoglycan layer of the bacterial cell wall. These endolysins either attack the glycosidic linkages between the amino sugars of the peptidoglycan layer (muramidases, glucosaminidases) or the *N*-acetylmuramoyl-L-alanine amide linkage between the glycan strand and the cross-linking peptide (amidases).

B.10 Bacteriophage amplification

The interaction between a phage and its host bacterium described above is the process of phage replication or "bacteriophage amplification". When bacteriophage amplification is coupled with analytical detection, MALDI-MS or ICS, it provides a new methodology for rapid identification of bacterial pathogens. Since phage infection is both specific and rapid and there is a natural increase in phage proteins during this process, identifying the increase in phage proteins after amplification by MALDI-MS or ICS provides an indirect, sensitive and rapid method of detection for a targeted bacterium.

[B.8] Most phage-encoded endolysins of both Gram-negative and Gram-positive bacteria lack a secretory signal peptide needed for *sec*-dependent transport across the cytoplasmic membrane. In a few Gram-positive phage however, there is a functional *sec* system and holins are not needed because the endolysins can utilize the *sec* system to gain access to the peptidoglycan layer.

APPENDIX C: PROTOCOLS

C.1 EFM staining protocol of phage particles

Objective:

EFM staining of phage particles

Experimental:

Materials:

- 0.02 micron pore size, 47 or 25 mm diameter aluminum oxide Anodisc filters (Whatman)
- 0.22 micron pore size, 47 mm diameter cellulose acetate membrane filter system
- Glass slides and microscope cover slips.
- Plastic Petri dish, pipettes and tips
- Sample preserved with 1% 0.02 micron filtered formalin
- SYBR Green I solution (Molecular Probes, Inc.)

SYBR Green I Solution Preparation:

- Make a stock solution by diluting 1:10 of the original concentration with 0.02 micron filtered distilled de-ionized water. Store frozen.
- From the above stock solution, make a 2.5% working solution (2.5×10^{-3} dilution of stock) just before use. For 100 μL drop, 2.5 μL of working stock to 97.5 μL of filtered distilled deionized water.
- For 10 samples: prepare 1 mL, 25 μL of working stock to 975 μL of filtered distilled de-ionized water (same as above just larger quantity).

- For double the concentration, 5%, 5 μL of working stock to 97.5 μL of filtered distilled de-ionized water.

Antifade Mounting Solution Preparation:

- Best results if antifade solution is made daily.
- 50% Glycerol.
- 50% PBS.
    - PBS Solution Preparation
        - 120 mM NaCl
        - 10 mM NaH_2PO_4 pH 7.5
- 0.1% p-phenylenediamine.

Method:

- Place 0.02 micron Anodisc filter over a pre-wetted 0.22 micron cellulose acetate membrane filter.
- Apply vacuum after placing the Anodisc filter on the cellulose acetate membrane filter.
- The Anodisc filter should moisten and stick to the cellulose acetate membrane filter. The Anodisc filter should be flat and smooth with no air bubbles between the two filters.

To be performed in the dark.

- Prepare a 100 μL drop of SYBR Green I, see working stock above and place on the bottom of the Petri dish.
- If there is a visible orange precipitate or viruses do not look bright on slides, double the concentration of the SYBR Green I, see working stock above for preparation.

Sample Preparation for Slide:

- Viral samples should be filtered through the Anodisc filter (20 KPa or 150 Torr).
- Once filtered, leave vacuum on and remove Anodisc filter.
- Any remaining water on back of Anodisc filter should be removed with a kimwipe (wipe carefully the back of the filter for ~ 2min), being careful not to touch the top of the filter. It is important that the filter be completely dry.
- The Anodisc filter should look uniformly dry and opaque when held up to the light.

To be performed in the dark.

- Place the Anodisc filter sample side up on the staining reagent in the Petri dish for 15 minutes (note the shorter amount of time on stain, the faster the fluorescence fades).
- After staining, pickup filter with forceps, leaving most of the stain in the dish.
- Remove any remaining stain from bottom of filter with a kimwipe as before and allow to dry for a few minutes.
- Again the Anodisc filter should look uniformly dry and opaque when held up to the light.
- Complete drying before and after staining is important for bright fluorescence and to prevent fading.

Mounting Anodisc Filter to Glass Slide:

- Place the completely dried Anodisc filter onto a glass slide.
- Put approximately 30 μL of the antifade mounting solution on a 25 mm cover slip and invert it over the filter.
- Push the cover slip to be sure the mounting solution fills the square space under the cover slip.

Epifluorescence Microscopy (EFM):

- View slide with blue excitation.
- If viruses are in more than one focal plane or appear to move, remake the slide.

If the viruses fade rapidly, the filter was not properly dried or antifade solution was not applied.

References:

Birge, EA Bacterial and Bacteriophage Genetics, 4th ed., Springer-Verlag: 2000, New York, 175-181.

Noble, RT and JA Fuhrman. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat. Microb. Ecol. 14: 113-118.

C.2 EFM differential bacterial staining protocol

Objective:

To differentially stain and determine viability of *Y. pestis* bacterial cells by Viagram Red+ Bacterial Stain.

Background:

Fluorescent staining provides a method by which Gram negative and Gram positive bacterial species can be differentiated while discriminating viable and non-viable bacterial cells based on the integrity of the plasma membrane.

Materials:

- Viagram Red+ Bacterial Gram Stain and Viability Kit (V-7023):
    - Contents of kit:
        - Component A: 40 µL DAPI solution, blue live-cell stain
        - Component B: 40 µL STYOX Green Solution in anhydrous DMSO, green dead-cell stain
        - Component C: 1 mg Texas Red-X conjugate of wheat germ agglutinin (WGA), lyophilized, red gram-positive stain
        - Component D: 1 mL reconstitution buffer of 0.1 M sodium bicarbonate, pH 8.3 for use with component C.
- BSA-saline solution:
- 0.25% bovine serum albumin (BSA)
- 0.15 M NaCl filter sterilized (0.876 g NaCl in 10 mL sterile DIH_2O)
- (0.025 g BSA in 10 mL of the 0.15 M NaCl solution)
- 100k Da spin filter
- Microcentrifuge and balance spin filter or microcentrifuge tube

Method:

1. Prepare 2 mg/mL stock solution of Texas Red-X conjugate of WGA by adding 500 µL of buffer (component D). For 20 preparation use 50 µL. Store the remaining solution in aliquots at -20 °C.
2. Prepare working solution of the viability indicators by adding 3 µL of DAPI (component A) and 3 µL of STYOX Green (component B) to 54 µL of sterile and 0.2 µm filtered water for a final volume of 60 µL.
3. Microcentrifuge 50 µL of the *Y. pestis* bacterial suspension for 3-4 minutes at 4000 x g in a 100k Da spin filter.
4. Wash cells in 50 µL of BSA-saline solution by pipetting up and down several times.
5. Microcentrifuge suspension for 3-4 minutes at 4000 x g. and re-suspend in 50 µL BSA-saline solution.
6. Add 2.5 µL of the WGA conjugate and mix by pipetting up and down several times and incubate at room temperature for 15 minutes.
7. Microcentrifuge suspension for 3-4 minutes at 4000 x g to remove the WGA and re-suspend in 50 µL BSA-saline solution.
8. Add 2.5 µL of the DAPI/STYOX working solution and incubate at room temperature for 10 minutes.
9. Transfer 10 µL of the solution to a clean glass slide, apply cover slip and observe in the epifluorescent microscope.

	Gram-Negative	**Gram-Positive**
Live Cells	Blue	Blue interior red surface
Dead Cells	Green	Green interior red surface

Reference:

Molecular Probes, Incs. www.probes.com

C.3 Gram staining

Objective:

Gram stain of *Y. pestis* bacterial cells.

Materials:

- Bunsen Burner, matches
- Tongs and Kim Wipes
- Crystal Violet, Iodine, 95% ETOH, Saffarine Red
- DIH_2O
- Glass Slide, Cover Slip, Microscope
- Waste Container
- Bacteria from streak plate or liquid dilutions and Wire loop

Experimental:

1. Smear bacteria onto slide and heat fix to glass slide.
2. Add 1-2 drops crystal violet on top of the bacterial spot for 1 minute.
3. Wash with DIH_2O for 5 seconds.
4. Add 1-2 drops iodine on top of the bacterial spot for 1 minute.
5. Wash with DIH_2O for 5 seconds.
6. Add ETOH drop wise until the crystal violet appears to have washed out of the bacteria. This is subjective so you may not be able to get all of the crystal violet out.
7. Add 1-2 drops saffarin counter on top of the bacterial spot stain for 1 minute.
8. Wash with DIH_2O for 5 seconds.
9. Allow to dry, visualize with microscope.

Reference:

Any introductory microbiology text book.

C.4 Correlating viable cell counts to optical density measurements

C.4 Correlating viable cell counts to optical density measurements

Objective:

To correlate *Y. pestis* viable cell counts to optical density measurements.

Background:

Optical density is a measure of the amount of light absorbed by a suspension of bacterial cells and is measured by a spectrophotometer (i.e. Spec-20). The measured absorbance has been shown to be proportional to the molar concentration of the absorbing species and the thickness of the sample the light passes through. This is known as Beer's Law, and is expressed: A = (molar absorption coefficient) * thickness * (concentration). Optical density is most useful when repeated estimates of growth are necessary. Absorbance values are used to plot the growth of bacteria and to also gauge the concentration in suspension cultures. It is standard to use matched glass tubes or cuvettes, thus taking optical density measurements at wavelengths ranging from 600 - 620 nm is optimal.

Materials:

- Tyrptose blood agar or plate count agar can be used
    - Preparation per 1L is given for plate count agar:
        - Casein peptone 10g
        - Tryptone 5g
        - Yeast Extract 5g
        - Glucose 10g
        - Agar 15g
        - DI Water 1L
- *Y. pestis* streak plate
- TSB broth and culture tube
- Spec - 20 set to a λ = 620 nm

- 9 mL tube tube for Spec-20 measurement
- Incubator set to 37 or 28°C
- Shaker table set to 150 cycles/min

Procedure:

Autoclave the plate count agar mixture for 25 min at 121°C. After a period of cooling and using sterile technique, pour the agar into Petri dishes and allow time for polymerization. Pick a single colony of *Y. pestis* from the streak plate and infect 5 mL of BHI broth in a sterile culture tube and incubate with shaking (150 cycles/min) at 28°C or 37°C. Grow the *Y. pestis* culture to optical densities of 0.2, 0.5 and 1 and plate serial dilutions at each optical density.

Over a wide range of concentrations, light passing through a suspension containing particles, such as microbes, will be scattered, the amount of light scattered is proportional to the concentration of particles. It is usual to use matched glass tubes or cuvettes, thus taking optical density measurements at a wavelength of 620 nm is optimal.

Make 1 mL serial dilutions with media broth of each *Y. pestis* as stated in Table C.1.

Table C.1 Serial dilutions of bacteria

Sample #	Bacteria (uL)	Media (uL)	Dilution Factor	Plate
S	1000	0	-	DNP
1	100 of stock	900	10x	DNP
2	100 of sample 1	900	100x	Plate
3	100 of sample 2	900	1000x	Plate
4	100 of sample 3	900	10^4x	Plate
5	100 of sample 3	900	10^5x	Plate
6	100 of sample 3	900	10^6x	Plate
7	100 of sample 3	900	10^7x	Plate

- The dilutions should be mixed thoroughly
- Transfer the 100μL sample dilutions to separate Petri dishes.
- Use a sterile bend glass rod or the like to spread sample evenly across agar plates
- Allow time for drying
- Invert and incubate for 24-72 hours.
- Select the plates containing between 100 - 300 colonies and count the number of colonies present.

- From this count the viable population of the original sample can be calculated.
    - Count all colonies
    - Record the data. Calculate CFU/mL.

$$CFU/\ mL = Count \times 10^{(DF + 1)}$$

C.5 Bacteriophage stock preparation

Objective:

Preparation of stock phage (by days) for ϕA1122 with *Y. pestis* A1122

Materials:

- Fresh *Y. pestis* A1122 streak plate
- Bacteriophage ϕA1122 with titer at least 10^8 pfu/mL
- TSB or HIB
- Incubator and shaker
- Wire loop
- 50 mL culture tubes
- Heart Infusion broth
- 1L baffle flask
- Absorbance meter
- Pipettes and tips
- 0.22 and 0.45 micron filters
- Centrifuge

Procedure:

Phage stock preparation steps (by days)

Day 1

- Inoculate and streak out a fresh culture of strain A1122 on a plate to ensure purity of the culture.
- Incubate the plate overnight at 37°C or 28°C.

Day 2

- Use a wire loop to pick up growth from the pure A1122 culture plate to inoculate 5ml of TSB or HIB in a 50ml sterile conical tube.
- Incubate the tube overnight at appropriate growth temperature for 12-18 hours in a shaking incubator.
- This is the starting culture tube.

Day 3

- Make sure that the optical density in the starting culture is about 2-3 units at OD_{620}. When turbidity is satisfactory, transfer 5 ml of the starting culture to 100 ml TSB or HIB in a 1000ml flask.
- Incubate this larger culture at appropriate growth temperature with constant shaking (~150 rpm/min) in a shaking incubator.
- After 9-12 hours of incubation inoculate this A1122 culture with 0.1 ml of bacteriophage stock (10^8-10^9 pfu/ml) and continue to incubate in shaker.
- Monitor the OD_{620} of the culture until you see at least a 10-fold drop in the OD or if you see the OD bottom out and then begin to rise again (bacterial culture may not completely clear).
- This flask of material is the preparative stock. Chill the culture on ice ~30 minutes then centrifuge the preparative stock at 10,000 x *g* for 30 minutes to remove cells/cell debris.
- Use a pipet to draw off the supernatant as the pellet is easily disrupted. Sterilize the cleared supernatant through a 0.45 μm filter, then a 0.22 μm filter.
- Store the filtrate at 4°C.
- Remove a 5-ml aliquot to a sterile tube.
- This tube will serve as the source of titration and for future seed stock.

C.6 Propagation of phage by liquid lysate method

Objective:

Propagation of phage by liquid lysate method.

Background:

Propagation of bacteriophage (phage) provides a stock for bacteriophage amplification experiments. Many times phage (depending on source) are lyophilized for long term storage and require proliferation of a stock. The plate lysate (see protocol D.6) and liquid lysate are two routine methods to achieve a stock solution of phage. While both methods are functional, the main difference between the two methods is the obtainable titer. On average the plate lysate gives 1-2 orders of magnitude higher titer than does the liquid lysate procedure.

Materials:

- 25 mL culture of *Y. pestis* in TSB broth
- 100 µL phage
- 100 µL pipettes and tips sterile.
- Vacuum
- Sterile 0.22 µm bottle top filter.
- 2 50 mL sterile centrifuge tube to use as bottle receiver for filtering.

Method:

- Grow *Y. pestis* to an optical density (OD_{620}) of ~0.4 (mid log phase) at 28° C or 37°C with shaking (150 rpm/min).
- At OD ~ 0.4 add 100 µL phage and continue to shake at appropriate temperature overnight or until culture clears.

- Centrifuge down bacteria (9,000 x g at 4 °C for 15 mins) in sterile 30 mL glass pyrex tubes or the like.
- Collect supernatant and vacuum filter through 0.22μm filter, filtering with 0.45 or 0.8μm maybe required prior to 0.22μm filter.
- This will be your stock phage. Be sure to titer to determine stock concentration.

C.7 Propagation of phage by plate lysate method

Objective: Phage propagation by plate lysate method

Materials:

To propagate phage using the plate lysate method require the following material: 3 bottom agar plates, 5 mL culture of bacteria, two culture tubes filled with 3 mL of ¼ strength soft strength agar (3.75 g/L) warmed to 50°C, spectrophotometer, 100 µL of re-hydrated stock phage, incubator set to 37°C, water bath set to 50°C, centrifuge and centrifuge tubes, vacuum, sterile 0.22 µm bottle top filter with 50 mL culture tube, 1000 µL and 100 µL pipettes and tips sterile.

Procedure:

- Pour bottom plates using sterile growth conditions.
- Culture bacteria to an optical density of ~0.5. Add 100 µL of phage and 300 µL to the ¼ strength soft agar tube and spread over the bottom agar plate.
- Follow the same procedure above excluding 100 µL of phage for a control. Incubate plates right side up at the appropriate temperature overnight.
- Pour enough PBS solution to cover plate, collect under sterile conditions into sterile centrifuge tubes. Pellet bacterial debris by centrifugation (9,000 x g at 4°C for 15 mins).
- Collect supernatant and vacuum filter through a 0.22 micron cellulose acetate filter. May need to larger filter first, 0.45 or 0.80 micron will work
- Store stock either in refrigerator or separate into 2 mL aliquots and store in cryovials at -70°C.
- Spot titer to determine concentration.

C.8 Determination of plaque-forming unit, spot titer method

Objective:

To determine the concentration of propagated stock phage ϕA1122.

Background:

The spot titer is a method that provides a measure of the phage concentration in a sample. This method is a modified version of the titer method. Instead of mixing the phage and bacteria in the soft (top) agar and overlaying onto an entire Petri dish, the spot titer allows for several phage dilutions to be performed on the same plate, saving time and materials.

Materials:

- 5 mL overnight culture of *Y. pestis*
- Stock phage ϕA1122
- PBS (pH=7.4) or normal saline solution for phage dilutions
- 2 Petri dishes with bottom TSB agar
- 2 sterile tubes containing 3 mL ½ top (soft) TSB agar warmed to 45 °C
- Bunsen burner
- Incubator set at 37°C or 28°C
- Pipettes and tips

Procedure:

- Make serial dilutions of phage as shown in Table C.2.

Table C.2 Serial dilutions of phage

Sample #	Phage (uL)	Media (uL)	Dilution Factor	Plate	Plate
S	100	0	-	DNP	DNP
1	100 of S	900	10x	Y	Y
2	100 of sample 1	900	100x	Y	Y
3	100 of sample 2	900	1000x	Y	Y
4	100 of sample 3	900	10^4x	Y	Y
5	100 of sample 4	900	10^5x	Y	Y
6	100 of sample 5	900	10^6x	Y	Y
7	100 of sample 6	900	10^7x	Y	Y
8	100 of sample 7	900	10^8x	Y	Y

- Rocking the micro-centrifuge tubes gently back and forth should mix the dilutions. DO NOT vortex, vortexing will decrease the number of infectious phage.

Allow the bottom TSB agar plates (2) to warm to room temperature. Divide each plate into 4 sections and label 1-8 (Figure C.1). Add 300 µL of *Y. pestis* overnight culture to the 3mL of top TSB agar and overlay. Allow top agar to harden. After hardening spot 5µL of each phage dilution to the corresponding section of the plate and allow to completely dry on the bench top invert and incubate at 37°C or 28°C. Check plates after 4-6 hours for signs of lysis.

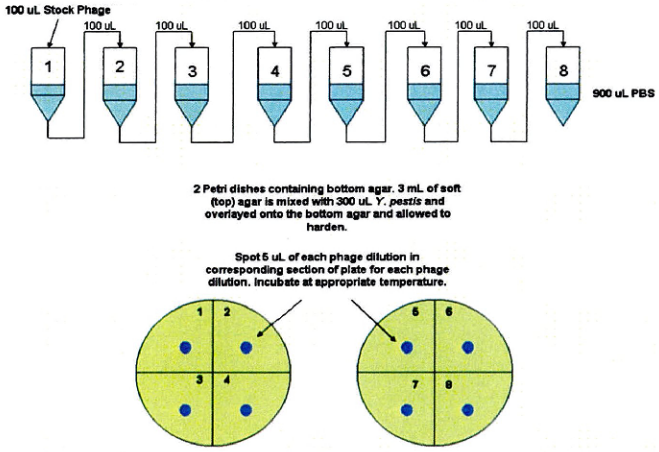

Figure C.1 Spot titer scheme

Reference:

Sambrook, et. al Molecular Cloning: A laboratory manual; 1989, Cold Spring Harbor Laboratory Press.

C.9 Phage lysis susceptibility testing

Objective:

To determine the susceptibility of given bacterial strains to phage lysis.

Background:

In order to produce selective assay for *Y. pestis* using the phage amplification technique, the spectrum of bacteria that a given phage can infect must be investigated. While it is impossible to test for all bacteria, a broad range representative panel at the Family level can be investigated. Families which demonstrate lysis by a particular phage can be subsequently investigated at the genus and species level. The objective of the host range study is to select a phage which infects all types of *Y. pestis* species while minimizing phage cross reactivity with other microorganisms.

Cross reactivity and host range studies were determined by bacterial susceptibility to lysis from each phage. Briefly, bacterial isolates were cultured overnight in TSB at 37°C and or 28°C. Three hundred micro liters of each culture were added to separate 3mL tubes of soft TSA agar and overlaid onto a Petri dishs containing bottom agar to create the bacterial lawns. A 5μL spot of each phage was dropped onto each bacterial lawn, incubated overnight, and checked for lysis the following morning.

Materials:

- Phage samples
- TSA plates, divide and label into sections corresponding to the number of phages you want to test on each strain
- 3 mL soft agar tubes warmed to 45°C
- Bacterial strains

Procedure:

- Plating of bacterial lawns
    - To 3mL of soft agar add 300μL of bacteria and allow to harden
- Spot 5μL of each phage into respective labeled section and allow to dry
- Incubate overnight at appropriate incubation temperature and check for clearings in the bacterial lawn.
- Clearings represent a positive results that the bacterial strain is susceptible to lysis by that particle phage.

C.10 One step growth experiment

Objective:

Determine the burst size of phage ϕA1122, R and Y per *Y. pestis* bacterium using a one-step growth curve at 30°C (Figure C.2).

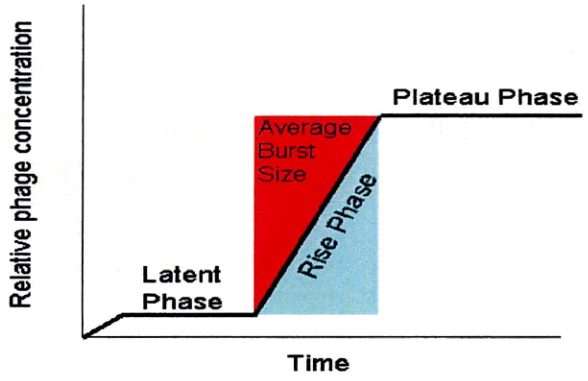

Figure C.2 One-step growth experiment to determine burst size

Background:

The average number of phage released during lysis can be determined by adding a small amount of phage to a known quantity of bacteria and then lysing the cells at 5 minute intervals with chloroform. The chloroform lysed cells, in turn are mixed with bacteria, plated out and incubated. Plotting a graph of relative phage concentration (as determined by the number of plaques on each plate) vs. time it is possible to determine the average burst size

(Figure C.2). The average burst size per bacterium is then determined by the subtracting the plateau phase – the latent phase.

Materials:

- 13 Petri plates with bottom BHI agar
- 13 5 mL sterile culture tubes with 3 mL of top (soft) BHI agar, store at 45 °C in water bath
- Small bottle of chloroform (20 μL ~ 4 drops)
- Pipettes 100 μL, 1000 μL and 5000 μL and tips
- Rack to hold 5 mL sterile tubes
- Water bath set to 45 °C and incubator set at 30 °C
- Overnight culture of A1122 *Y. pestis*, diluted with BHI to known density (OD 1 (λ=620nm)
- Stock of ϕA1122, R and Y phages
- 1 14 mL sterile tubes labeled "ADS"
- 1 14 mL sterile tubes labeled "ADS 2" with 9.9 mL of sterile BHI broth
- 26 14 mL sterile culture tubes with 9.9 mL of sterile BHI

Procedure:

The addition of 2 mL of *Y. pestis* and 0.1 mL of phage to the "ADS" tube indicates when adsorption begins. Time should be recorded as "time zero". Tubes should be initially setup as shown in Figure C.3 and then the "ADS" and "ADS 2" tubes are to be incubated at 30 °C. Figure C.4 shows the overall scheme of the experiment for plating.

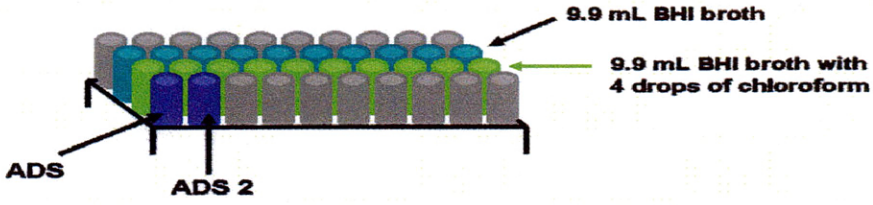

Figure C.3 One-step growth experiment tube scheme.

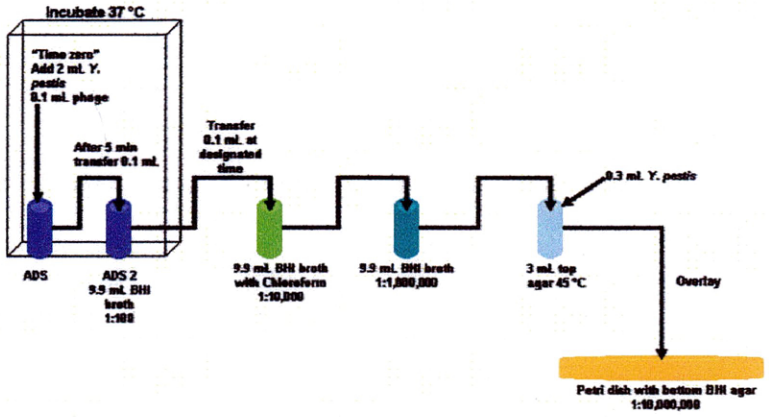

Figure C.4 One-step growth experiment transfer procedure for each at a given time interval.

After the "ADS" tube has been incubated for an initial period of 5 minutes, 0.1 mL of the mixture is transferred to the "ADS 2" tube and incubated at 30 °C for an additional 10 minute period. Next 0.1 mL of the mixture in "ADS 2" is transferred to the tube containing the chloroform. The tube is rocked back and forth so that any unlysed cells can be lysed by the chloroform. After the lysing step in the chloroform, 0.1 mL is transferred to a tube containing BHI broth, mixed and subsequently 0.1 mL of this mixture is transferred to a

tube containingn 3 mL of top agar. To the top agar 0.3 mL of Y. pestis is also added and then overlayed onto the Petri dish. Each dish should be given ample time to polymerize, inverted and incubated at 30 °C overnight. This procedure should be performed at 5 minute intervals after the initial plating.

Reference:

Benson, Microbiological Applications Lab Manual, Eighth edition V. Bacterial Viruses 30. Burst Size Determination: A One-Step Growth Curve. McGraw-Hill Companies, 2001, 120-124.

C.11 PEG precipitation of phage (semi-purification)

Objective: Precipitate phages

In general, the precipitation of proteins by PEG is explained on the basis of the volume exclusion effects according to which proteins are sterically excluded from the regions of solvent that are occupied by PEG linear chains that fold when in solution [173]. Virus particles and proteins are thus concentrated and finally precipitated when the solubility is exceeded. Thermodynamically, the steric exclusion leads to an increase in the chemical potential of the protein until it exceeds that of the "pure solid state," leading to precipitation of the virus particle. This happens mainly because of a large unfavorable free energy of interaction between the PEG and the viral particle leading to preferential hydration of particle. The preferential hydration helps maintain the native structure of virus in the presence of PEG [174, 175].

The mechanism by which a virus and/or protein is precipitated from solution by a polymer such as polyethylene glycol (PEG) is displacement from solution. Juke's (1971) showed through thermodynamic reasoning that the solubility (S) of a protein in a polymeric solution follows the "salting out" equation of:

$$\log S = K - \beta^{\omega} \qquad \text{Eqn 1}$$

where K is a constant, β is the rate of displacement with change in concentration of the polymer, and ω is the polymer concentration. Juckes also observed that the factor log β is a linear function of the Stokes radius[B.1] of displaced substances and the maximum rate of displacement from solution is dc/dPEG. Thus the log β factor is small for low molecular

B.1 Diffusion coefficients can be correlated to molecular weights of particles or molecules for a specific solvent/polymer system. Using the Stokes-Einstein equation a diffusion coefficient for a particle or molecule can be correlated to its Stokes radius.

weight substances and large for high molecular weight substances, predicting that in a mixture of components with different molecular weights, the largest molecular weight will be the first to be displaced.

The work of Ogston (1958) further demonstrated that the available volume (a) for a colloidal particle (protein or virus) in the presence of the linear polymer equals:

$$a = \exp [-\pi 1 (r_s - r_r)^2] \qquad \text{Eqn 2}$$

Where 1 is the total length of the polymer per unit volume, while r_s and r_r are the hydrodynamic radii of the solute and polymer molecules respectively. This explains why the displacement action is most effective using PEG polymers that have molecular weights greater than 6,000 Da and is a result of the ability of the polymer to fold or coil in solution allowing substances to diffuse into these coils. Typical PEG concentrations range from 7-10% for precipitation purposes. Concentration of viral particles or proteins naturally follows this method because the precipitated particles/molecules are centrifuged into a pellet with subsequent re-suspension into a smaller volume of buffer solution. The exclusion by the folded PEG molecules is similar in many ways to size exclusion chromatography (SEC). Similarities worth mentioning are listed in Table C.3.

Table C.3 Similarities using PEG and SEC

Exclusion by PEG	SEC
If the particles are large (i.e. viral particles) they do not enter the coils ofthePEGpolymerbutwillremain in the intermolecular "watery" spaces	If the molecules are too large for the pores they will not diffuse into the gel particle.
If the substance has a very low molecular weight the substance will diffuse in and out of the PEG coils so that the distribution coefficient C_i/C_e will approach unity and will not be excluded to any useful degree by the polymer	Distribution coefficient will also approach unity.
If the substance is of a molecular size which enables it to diffuse into the coils of the polymer to a limited degree, its distribution or partition coefficient between the solvent and the solvent incorporated in the coils of the polymer will depend upon the degree of folding of the polymer, which in turn will depend upon the concentration and chain length of the polymer.	The concentration of the gel determines the partition coefficient between the gel particles and the medium which in turn determines the position in elution order.

Polyethylene glycol can also be used to purify viruses, rendering the particles free from extraneous proteins in solution [173, 176]. This was demonstrated by recovering more than 95% of influenza virus particles from extraneous proteins using 1-4% concentrations of PEG 6000. The resulting purified viruses were shown to have superior antigenic properties for antibody production raised in guinea pigs [177]. However, the drawbacks to using solely PEG for purification purposes is the low concentration of viral particles obtain from solution and the potential for proteins or aggregates thereof to co-precipitate along with the viral particles which are similar in molecular weight.

Method:

This is a modified procedure taken from Sambrook et al., 1989. For 500 mL of 0.22 micron filtered viral stock, add 50 g of PEG 8000 (10% w/v) and 30 g of NaCl to the viral

stock and dissolve with a magnetic stir bar. Salt is added to promote the dissociation of virus particles from any remaining nucleic acids in solution. Place the mixture on ice for no less than 1 hour (preferably overnight) to precipitate the virus particles. Pellet precipitated phage by centrifugation (11,000 x g for 15 min at 4°C). Glass centrifuge tubes are also recommended as viral particles tend to stick to the plastic tubes. Depending upon the original volume used to precipitate, the pellet may look like a thin film on the side of the centrifuge tube. Pour off supernatant and re-suspend pellet in 8 mL of phosphate buffer saline (PBS, pH = 7.4) solution for every 500 mL of viral stock precipitated. Add to this centrifuge tube an equal amount of chloroform, vortex for 30 seconds, and centrifuge at 4,000 x g for 15 minutes at 4°C. Carefully collect the aqueous phase (top layer) containing viral particles. Repeat differential centrifugation process 1-2 more times to remove remaining PEG. Titer purified phage stock to determine concentration.

C.12 CsCl purification of phage

Objective:

CsCl equilibrium gradient purification of phage for polyclonal antibody production

Purification of T7 Phage and T7 DNA

T7 is purified from clarified lysates by precipitation with polyethylene glycol (PEG 8000) followed by banding in a CsCl step gradient. The technique requires an ultracentrifuge equipped with a swinging bucket rotor. Procedures are described below for purifying phage from different volumes of lysate. In each case, phage are extracted from the PEG pellet in 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and the concentrated phage solution is under layed in four steps of different density CsCl solutions in a clear ultracentrifuge tube (e.g., Beckman SW50.1 for small scale cultures, Beckman SW41 for larger scale preparations).

Although the SW50.1 rotor is convenient for small scale purification, the larger Beckman SW41 rotor and Beckman 344059 centrifuge tubes (14 × 89 mm) allow gradients to be loaded with 1–5 ml PEG-extracted phage. This volume range is suitable for banding of phage from 50 ml to 500 ml initial culture volume per tube.

The four CsCl layers are made by mixing a stock solution of 62.5% CsCl (25 g CsCl + 15 ml deionized water) with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) in the following ratios (volume in centrifuge tube):

CsCl:TE	SW50.1	SW28	SW41
	1 ml	3 ml	2 ml
1:1	1 ml	3 ml	2 ml
2:1	1 ml	3 ml	2 ml
1:0	0.5 ml	1.5 ml	1 ml

SW28	CsCl:TE	CsCl	TE	Total	Phage	Tube Total
3 ml	1:2	3 ml	6 ml	9 ml		
3 ml	1:1	3 ml	3 ml	6 ml		
3 ml	2:1	6 ml	3 ml	9 ml		
3 ml	1:0	3 ml	0	3 ml		
35 ml tubes for SW28				27 ml	5.5 ml	32.5 ml

Successively denser solutions are underlayered in the tube using a long loading needle. Because of the large differences in density between the layers, this can be done easily with little mixing. Up to 1.5 ml (SW50.1 tubes) and 5 ml (SW41 tubes) of concentrated phage solution is layered on top of the CsCl steps. The tubes are centrifuged at room temperature for 35 min at 40,000 rpm (SW50.1) or for 60 min at 35,000 rpm (in an SW41 rotor).

After centrifugation, the tubes typically contain a thick layer of debris and empty phage heads atop the 1:2 layer, a sharp, turbid band of phage particles above the 2:1 layer, and in many cases a lower turbid band atop the 1:0 layer. The lower band is a carbohydrate fraction that contains no phage particles and is present in variable amounts from one preparation to the next. The turbid bands are seen most easily when the tube is illuminated from above and

the room light is dim. The band of purified phage particles can be collected by dripping through a puncture in the bottom of the tube, or by careful removal from above with a Pasteur pipet (after initial removal of the top layers).

Phage particles at this stage are pure enough for most purposes. They may be further purified by adding enough 62.6% CsCl to make the solution denser than the 2:1 CsCl:TE mixture, then floating up to an interface with an upper layer of 1 M NaCl again centrifuging at the same speeds and times cited above.

Purified T7 particles may be stored in the CsCl solution in the refrigerator, where they are reasonably stable. They typically have a titer around 4×10^{11} pfu/ml per A_{260} unit (i.e. a solution with $A_{260} = 1$ has 4×10^{11} pfu/ml). For convenience, stocks in use are usually diluted to an A_{260} of 6 in 0.2 M NaCl, 2 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, where they also are quite stable. For higher dilutions, autoclaved gelatin (100 μg/ml) will help to protect against inactivation. However, purified stocks, whether diluted or not, gradually lose titer over periods of many months. Purified T7 particles may also be stored at -80°C in solutions containing 8% glycerol, but indications are that a slow loss of titer occurs under these conditions as well.

Reference:

Sambrook, et. al Molecular Cloning: A laboratory manual; 1989, Cold Spring Harbor Laboratory Press.

C.13 CsCl equilibrium gradient purification of phage

To obtain purified phage from polyethylene glycol precipitated phage is typically performed by banding in a CsCl step gradient [93]. The technique requires an ultracentrifuge equipped with a swinging bucket rotor or rotors that can obtain centrifugal forces greater than 100,000. The gradient densities utilized in the steps are dependent upon the type and density of phage to be purified.

In general, phage are extracted from the PEG pellet in one of three types of buffers: 1)1 M NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA; 2) phosphate buffer saline (PBS) solution (pH 7.4); or 3) 0.85% sodium chloride (normal saline) solution. The concentrated phage solution is layered atop four steps of different density CsCl solutions in clear polyallomar ultracentrifuge tubes. A Beckman SW28 (35 mL capacity) or SW41 (17 mL capacity) are typical swinging bucket rotors used for large scale phage purifications. Phage volume per tube can range from 1-7 mL depending upon rotor.

This procedure outlines the CsCl gradient for 35 mL capacity tubes. Phage added per tube should be 5-7 mL to obtain adequate banding. Table below outlines four CsCl layers which are prepared by mixing a stock solution of 62.5% CsCl (25 g CsCl + 15 ml deionized water) with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) using the following ratios listed in Table C.4.

Table C.4 CsCl density gradient layer ratio

CsCl:TE	Volume Ratio for 35 mL tube	Total Volume SW28 Tube
1:2	3 mL : 6 mL	9 mL
1:1	3 mL : 3 mL	6 mL
2:1	6 mL: 3 mL	9 mL
1:0	3 mL	3 mL

Successively denser solutions are underlayered in the tube using a syringe with a long needle. Because of the large differences in density between the layers, underlaying each layer can be prepared easily with little mixing and interfaces marked on the outside of the tube. Loading is performed in the following sequence: 1) load 5-7 mL of concentrated phage solution; 2) underlay the 1:2 ratio of CsCl:TE; 3) underlay 1:1 ratio; 4) underlay the 2:1 layer; and 5) underlay the heaviest ratio, 1:0, last. Centrifuge at room temperature overnight at 25,000 rpm (SW28). After centrifugation, the tubes typically contain a thick layer of debris and empty phage heads atop the 1:2 layer, a sharp, turbid band of phage particles above the 2:1 layer, and in many cases a lower turbid band atop the 1:0 layer. The lower band is a carbohydrate fraction that contains no phage particles and is present in variable amounts from one preparation to the next. The turbid bands are seen most easily when the tube is illuminated from above and the room light is dim. The band of purified phage particles can be collected by puncturing with a syringe needle just below the band. Typically recovery amounts range from 5-7 mL of the starting phage added to the tube. Phage titers average around 4×10^{11} pfu/ml per A_{260} unit (i.e. a solution with $A_{260} = 1$ has 4×10^{11} pfu/ml).

Purified phage particles may be stored in the CsCl solution at 4°C, where they are reasonably stable. However, purified stocks kept in CsCl gradually lose titer over the course many months. Particles may also be stored at -80°C in solutions containing 8% glycerol.

C.14 Dialysis of CsCl purified phage

Objective:

Dialysis and filter sterilization of CsCl purified phage to bring purified samples to physiological pH for injection of antigen into mice.

Materials:

- 0.22μm filter sterilized phage stocks
- Sterile distilled de-ionized water
- 0.85% saline solution or PBS
- CelluSep 6,000 (or the like) dialysis membranes
- Dialysis clips
- Sterile syringe and 0.2μm low protein binding filter
- pH paper or pH meter
- 600mL beaker

Procedure:

- Soak dialysis membrane in sterile distilled de-ionized water for 15-30 minutes.
- Close one end of the dialysis tubing with the dialysis clip
- Load phage sample into other end and clip with the other dialysis clip
- Stand vertically in 600mL beaker
- Dialyze against 0.85% sodium chloride solution (or PBS) for 18-24 hours changing the solution frequently to remove the CsCl.
- Using a syringe filter dialyze phage through a 0.2 μm filter
- This is considered to e 1x purified phage stock
- Store at 4°C

- Depending upon the stability of the phage, may need to add stabilizing protein, such as BSA to the purified stock.

Reference:

Sambrook, et. al Molecular Cloning: A laboratory manual; 1989, Cold Spring Harbor Laboratory Press.

C.15 TEM staining protocol for phage

Objective:

Transmission electron microscopy (TEM) of phages from gradient purified preparations

Materials:

- 1% uranyl acetate
- EM grids: a formvar and carbon film (be sure grids are glow discharged before use)
- High concentration of phages at least 10^{10} pfu/mL (can make dilutions if necessary)

Negative Staining Protocol:

- Make up a 1% uranyl acetate (1% w/v) solution using distilled water. It can be a little slow to dissolve. This is your negative stain solution.
- Put about 1μL of phage sample on the carbon side of the grid, let it sit for a minute, blot off most of the liquid by touching the grid to filter paper at an angle (don't put the grid face down on the filter paper)
- Add a series of drops of negative stain to the grids and shake off the drops after a few seconds then blot the last drop of stain (blot the same way with the filter paper as you did with the phage, but not face down on it)
- Let it dry and its ready for viewing (the grid is dry when the last bit of stain is dry). You want to see a very think layer dry gradually from the edges. If the stain beads up instead, the glow discharge did not work properly.
- The glow discharge is supposed to make the carbon surface hydrophilic
- On the scope, look around the grid. You many find variable thickness of stain.
- Usually a very thin layer of stain provides the best images.
- Analyze on a with a Philips CM10 transmission electron microscope (FEI, Inc. Hillsboro, OR) operated at 80kV or the like.

- Phage were digitally photographed at a magnification of x92,000

C.16 Animal protocol: cheek puncture technique

Procedure:

Mouse Cheek Puncture Technique

Practical: Taught by Marc Dolan. CDC/NCID/DVBID Ft. Collins CO

Objective:

Cheek puncture provides a method for blood collection from mice for initial bleed (negative control) and to monitor titer through out polyclonal antibody production from mice.

Materials:

- Gloves
- Lab Coat
- Mice
- Size 11 scalpel
- 70% EtOH
- Isofurane (~3mL for every 5-6 mice), anesthesia
- Mason jar with cotton cloth wired in lid
- Large forceps (Large tweezers)
- 1 mL Blood collection tubes with lids
- Cotton absorbent pad
- Picric acid solution with cotton swab to mark mice control group

Procedure:

- Add 3mL of isofurane to cotton contained in lid of mason jar, place lid back over jar while not using.

- Using forceps, place mouse in mason jar and cover with lid. Watch mouse until breathing slows significantly.
- Remove mouse from jar, and place mouse on left side on absorbent pad.
- Place blood collection, near head
- Grab mouse between ears and pull cheeks taught with fingers.
- Take scalpel with angled pointed away from you and stab at 90 degree angle just above zygomatic in sinus cavity, just back and to the left of the eye. If too low will hit hard bone (Zygomatic arch), see Figure C.5.

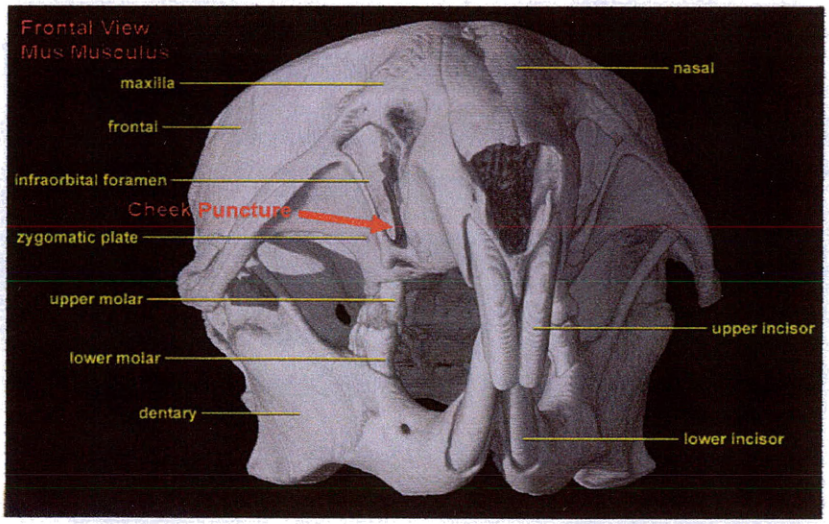

Figure C.5 Cheek puncture.

- Blood droplets should form readily.
- Collect droplet in collection tube.
- Be sure to release pressure on head between droplet formation, otherwise could suffocate mouse.
- Can collect as much as 500μL per mouse using this technique
- Be sure to stop bleeding by turning mouse cheek onto cotton cloth and apply pressure for 15-30 seconds or until stops bleeding.
- Put distinguishing marks onto each mouse (i.e. yellow head or yellow head and yellow tail)
- Place back into cage.
- Cap blood collection tube and place in 4°C fridge overnight before spinning out to collect serum.
- Spray 70% EtOH on clean area of absorbent cloth and clean scalpel in between cheek punctures.
- May need new scalpel ~ every 6 mice, idea is to maintain sharp scalpel.

C.17 Animal protocol: subcutaneous administration of antigen

Procedure:

Subcutaneous administration of antigen

Practical: Taught by Leon Carter. CDC/NCID/DVBID Ft. Collins CO

Objective:

Subcutaneous administration of antigen provides a method for delivery of antigen (immunization) of mice for production of polyclonal antibody production from mice.

Materials:

- Gloves
- Lab Coat
- Mice
- 1 mL 5/8" 26 gauge needles (TB needles)
- Antigen (phage: 1 x 10^{10} pfu/mL)

Procedure:

- Fill each syringe with 0.8mL of appropriate antigen
- Remove mice and place atop of cage, should stay put as long as do not scare them or make sudden motions.
- Hold mice appropriately (tail and head) exposing stomach and so that it is in capable of biting or moving during immunization.
- Insert needle just beneath the skin in the dorsal area of the mouse. You will know if giving injection subcutaneously as the area should puff out a bit as you inject the antigen. Add 0.3mL per each of two dorsal sites.

- Before pulling out needle, be sure to shake needle slighty as this prevents leakage of antigen after injection.
- Place mouse back into cage.

C.18 Animal protocol: terminal mouse bleed by cardiac puncture

Procedure:

Mouse Cheek Puncture Technique

Practical: Taught by Leon Carter and Scott Bearden CDC/NCID/DVBID Ft. Collins CO

Objective:

Terminal bleed provides a method for blood collection from mice for final bleed for collection of polyclonal antibodies from production in mice.

Materials:

- Gloves
- Lab Coat
- Mice
- Tb syringes
- 70% EtOH
- Isofurane (~3mL for every 5-6 mice), anesthesia
- Mason jar with cotton cloth wired in lid
- Large forceps (Large tweezers)
- 1 mL blood collection sera tubes with lids
- Cotton absorbent pad

Procedure:

- Add 3mL of isofurane to cotton contained in lid of mason jar, place lid back over jar while not using.
- Using forceps, place mouse in mason jar and cover with lid. Watch mouse until breathing slows significantly.
- Remove mouse from jar

- Hold the mouse by the scruff of skin above the shoulders so that its head is up and its rear legs are down.
- Using 70% ETOH wet chest area to outline the sternum
- Using a 1 ml Tb syringe, insert needle just below the sternum towards the animal's chin, 5-10 mm deep, holding the syringe 25-30 degrees away from the chest
- If blood doesn't appear immediately, withdraw 0.5 cc of air to create a vacuum in the syringe. Withdraw the needle
- Without removing it from under the skin and try a slightly different angle or direction.
- When blood appears in the syringe, hold it still and gently pull back on the plunger to obtain the maximum amount of blood available.
- Pulling back on the plunger too much will cause the heart to collapse.
- If blood stops flowing, rotate the needle or pull it out slightly.
- Perform cervical dislocation by placing forcep horizontally with slight pressure across the back of the neck and pull the tail to ensure the animal is dead.
- After performing a terminal blood collection, *always* be certain that the animal is dead before placing the carcass in the freezer. Remember that the anima will stop breathing before the heart stops.

C.19 Direct ELISA

Objective:

To determine the polyclonal antibody dilution factor (binding response) on mouse produced anti-ϕA1122 sera and anti-Y sera by Enzyme linked immunosorbent assay (ELISA). This is total Ig content to ensure antibodies are being produced during the course of mouse immunization.

Note this ELISA is not to be used for quantitation of Mouse IgG. After purification with protein G for isolation of mouse IgG, quantitation can be performed with the Pierce protein quantitation kit. A second ELISA maybe performed using secondary antibody specifically for IgG.

Materials:

11 mL of purified phage with concentration of $10^{10} - 10^{12}$ pfu/mL

Alpha Diagnostics International (ADI) ELISA kit for Mouse Antibodies (Cat. No 80150) which includes:

- 0.15 mL Goat Anti-Mouse Ig HRP-conjugate stock (40321) this is secondary antibody
- 25 mL Coating buffer 10x (80050)
- 25 mL Blocking buffer 10x (milk based, 80062)
- 25 mL Antibody/Conjugate 10x (80070)
- 25 mL Wash buffer concentrate 100x (80083)
- Ready-to-use TMB Substrate Soln (80091)
- 10mL Stop Soln for TMB, 10x (80100)

  Note: Reagents included in kit contain the preservative thimerosal, disposal should be put into the biowaste or heavy-metal waste. Storage of all solutions are stable at 2-8°C until expiration date.

- 96 well high protein binding micro-plate
- Multichannel (8 or 12) and single pipettes and tips (1-200µL)
- Adhesive cover films for covering plate
- Pre-bleed sera
- Anti-phage sera

Procedure preparation:

SOLUTION PREPARATION:

Wash Buffer Concentrate:

- Dilute 1:100 (1mL: 99mL) with DIH_2O
- Note: occasionally crystals form at 4°C but these will re-dissolve at room temperature

Coating Buffer Concentrate:

- Dilute 1:10 (25mL: 225mL) with DIH_2O
- This is a neutral pH buffer (likely PBS)

Blocking Buffer Concentrate:

- Dilute 1:10 (25mL:225mL) with DIH_2O, so 1x blocking buffer working solution.
- The diluent will be used at 200µL per well (96 wells x 200 µL = 19.2 mL for entire plate) or make entire bottle

Antibody/HRP-Conjugate Diluent:

- Dilute 1:10 (25 mL: 225 mL) with DIH_2O, gives 1x Antibody/HRP-Conjugate
- A red dye has been added for better visibility.

- If BSA or goat/calf serum interferes with your assay, try another buffer (call ADI for alternative buffer systems)

Ready-to-Use TMB Substrate Solution:

- Do not contaminate the bottle, withdraw the necessary amount into another container.
- Dispense a total of 10 mL into separate container (100µL/well x 96 well = 9.6 mL for the entire plate)

Stop Solution:

- Dilute 1:10 (1mL:9mL) with DIH_2O, gives 1x stop solution
- (100µL/well x 96 well = 9.6 mL for the entire plate)
- This is a diluted H_2SO_4 solution

Secondary Antibody HRP Conjugate Working Solution:

- Secondary antibody: Anti-Mouse HRP conjugate stock: Reconstitute in 2 mL of sterile water.
- Add the reconstituted Anti-mouse conjugate to 48 mL of 1x antibody/HRP conjugate diluent, this give a 1:25 (1 mL in 25mL) which I will call the concentrated antibody/HRP conjugate working solution.
- Store in cyrovials (or microfuge tubes) in 1 mL increments at 4°C
- Concentrated antibody/HRP conjugate working solution should be used within 1 year of reconstitution

Secondary antibody/HRP conjugate working solution:

- Start with 1:2500 dilution (1:100 of concentrated antibody/HRP conjugate working solution)

- Add 300 μL of concentrated antibody/HRP conjugate working solution to 30 mL of 1x antibody/HRP conjugate diluent , I will call this the antibody/HRP conjugate working solution
- If too concentrated then try:
- Dilute 1:5000 dilution (1:200 of concentrated antibody/HRP conjugate working solution)
- Add 200 μL of concentrated antibody/HRP conjugate working solution to 40 mL of 1x antibody/HRP conjugate diluent
- Unused of antibody/HRP conjugate working solution should be discarded.
- Make 10-fold serial dilutions of pre-bleed sera and antibody (anti-sera) in the 1x Antibody/HRP-Conjugate using the following dilution schemes in Table C.5. This is enough for 2 duplication as only 100μL is used per well.

Table C.5 Direct ELISA: antibody/HRP-conjugate 10-fold dilution scheme.

Serum Sample Designation	Mouse Anti-Phage Serum Sample	Antibody/HRP-Conjugate	Total Volume	Sample Dilution Factor
1	25μL undiluted anti-phage sera	225μL	250μL	10x
2	25μL of 1	225μL	250μL	100x
3	25μL of 2	225μL	250μL	10^3x
4	25μL of 3	225μL	250μL	10^4x
5	25μL of 4	225μL	250μL	10^5x
6	25μL of 5	225μL	250μL	10^6x

Procedure per 96 well plate:

1. Coating Plate:
    a. Add 100μL/well of purified phage solution (already in neutral buffering solution 0.85% saline) to high binding 96 well plate and incubate at room temperature for 3-6 hours (or overnight at 4°C).
    b. Remove excess coating phage solution by aspiration and tapping plate on paper towel
2. Blocking Plate:
    a. Add 200μL/well of 1x blocking buffer solution to each well and incubate for 2-6 hours at room temperature (can allow to go overnight at room temperature).
    b. Remove excess blocking buffer solution by aspiration tapping plate on paper towel
    c. Do not wash plate as it will wash out the stabilizing agent present in the blocking buffer.
    d. Allow plate to air dry at room temperature for 30-60 minutes
    e. If needed can store plates at in sealed bag at 4°C until use (Plates blocked as stated above will remain stable for several months depending upon nature of the phage).
3. Antibody Assay
    a. Aspirate and tap to remove excess blocking agent and dispense prebleed and anti-Y and anti-ϕA1122 dilutions in the 1x Antibody/HRP-Conjugate 100μL per well
    b. Cover plates with adhesive films
    c. Incubate at room temperature for 30 minutes : 1st incubation with antisera
    Time Start:_____ Time Stop:_____

d. After incubation, wash plates 3 times with 200µL/well, tap plate over paper towel after each wash. (0.6 mL x 96 = ~58 mL of total wash solution) Note that improper washing leads to high background
e. Add 100µL/well the 1x Antibody/HRP-Conjugate
f. Cover plate with adhesive film and incubate at room temperature for 30 minutes
    i. 2nd incubation with conjugate Time Start:_____ Time Stop:_____
g. After incubation, wash plates 4 times with 200µL/well, tap plate over paper towel after each wash to remove all trace solution. (0.8 mL x 96 = ~77 mL of total wash solution).
h. Add 100µL/well TMB substrate soln. Incubate plates for 15 minutes at room temperature (incubation can be continued for up to 60 minutes to increase color)
    i. 3rd incubation with TMB conjugate: Time Start:_____ Time Stop:_____
i. Be sure to gently shake the plate 1 or 2 times to mix color evenly through the well.
j. A blue color should develop in antibody positive wells.
k. Add 100 µL/well of 1x stop solution to each well. The blue color will turn to a yellow color. Be sure to gently shake the plate for a few seconds to mix color evenly through the well.
l. Read plates and score (at 450nm) within 15-30 minutes
m. The yellow color will fade over time
n. Use microplate reader, read results at 450 nm.

Reference:

Alpha Diagnostics, Inc. www.alpha.com

C.20 Affinity protein G purification of mouse sera

Objective:

To purify IgG polyclonal anti-phage mouse sera using affinity chromatography (protein G)

Materials and Method:

- Syringe Pump
- Microfuge tubes and microfuge brick
- 200 μL pipette and tips

MAb Kit Contents: (Amersham Bioscience 17-1128-01, now GE Healthcare)

- 1 HiTrap Protein G HP 1 ml
- 1, 1/16" male/luer female
- 1 Stop plug female 1
- 1 Syringe, 5 ml
- 1 Binding Buffer, 50 ml, 10 x concentrate containing 20% ethanol as a preservative
- 1 Elution Buffer, 15 ml, 10 x concentrate 1
- 1 Neutralizing Buffer, 25 ml, containing 20% ethanol as a preservative

MAbTrap Kit contains enough material to perform up to 20 purifications when operated with a syringe.

1. Column Preparation:

- Allow the column and buffers to warm to room temperature. The column is delivered with a top-cap on the inlet and a snapoff end on the outlet. The medium is supplied in 20 % ethanol.

2. Buffers

The buffers have been prepared using salts and water that has been filtered through a 0.45 μm filter.

Buffer preparation, for one purification, using a syringe

- Dilute the 10 x buffer concentrates as follows:

  a) 2.5 ml binding buffer concentrate + 22.5 ml high quality water to a total volume of 25 ml.

  b) 0.5 ml elution buffer concentrate + 4.5 ml high quality water to a total volume of 5 ml.

However, if you need to make more buffering solution use:

- Binding buffer: 20 mM sodium phosphate, pH 7.0
- Elution buffer: 0.1 M glycine-HCl, pH 2.7
- Neutralizing buffer: 1 M Tris-HCl, pH 9.0

3. Tube preparation

Prepare collection tubes by adding 60–200 μl of neutralizing buffer per ml of fraction to be collected. This allows for immediate renaturing of the purified IgG to preserve the activity of labile IgGs.

4. Sample preparation:

- The sample should be adjusted to the composition of the binding buffer. This can be done by diluting the sample with binding buffer.

- The sample should be centrifuged or if there are particles present or the serum appearance is cloudy. Dilute the serum sample 1:1 with prepared working binding buffer.

Purification Procedure:

1. Prepare collection tubes by adding 60-200 µl neutralizing buffer per ml of fraction to be collected.

2. Remove the stopper and connect the column to the syringe (with the provided adaptor), or pump tubing. Remove the snap-off end at the column outlet.

3. Fill the syringe or pump tubing with 5 mL of DI water to wash out the ethanol preservative at ~1 drop/sec (~2 ml/min). Add "drop to drop" to avoid introducing air into the column.

4. Wash the column with 5 column volumes of binding buffer at 1 ml/min.

5. Apply the sample 0.500 mL of mouse sera , using a syringe fitted to the luer adaptor, add by pumping it onto the column with syringe pump.

6. Wash with 5-10 column volumes of binding buffer or until no material appears in the effluent.

7. Elute with 2-5 column volumes of elution buffer.

Storage

- Before storage wash the column 5-10 column volumes with of 20% ethanol to prevent microbial growth. Store the column in 20% ethanol at +4 to 8 °C.

Reference:

Amersham Biosciences, Inc. Protein Separation and Affinity Chromatography Handbook.

C.21 BCA quantification of total protein

Objective:

Quantifying total protein content of mouse IgG affinity purified fractions by BCA Protein Microassay

Background:

The BCA protein assay is a detergent- compatible formulation based on bicnichoninic acid (BCA) for the colormetric detection and quantitation of total protein. The method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu+1) using the BCA reagent. The purple colored reduction reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex, is water soluble, and exhibits a strong absorbance at 562nm and is linear with increasing protein concentration over a range of 0.20 mg/mL – 2 mg/mL. This method is not a true end point method, as the final color continues to develop over time. Following incubation, however, the rate of color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds, and the presence of four amino acids: cysteine, cystine, tryptophan, and tyrosine are reported to be responsible for color formation with BCA. Studies with di-, tri-, and tetrapeptides suggest that the extent of color formation is caused by more than the mere sum of individual color producing functional group. Protein concentration generally are determined and reported with reference standards of a common protein such as bovine serum albumin (BSA) or bovine gamma globulin (BGG). Serial dilutions of known concentrations are prepared from the protein standard and assayed alongside the unknown IgG concentrations.

Materials:

- 96 well sterile standard well plate
- Microplate reader (550 nm)
- Microfuge tubes
- 200 µL repeater pipette, single pipette, and tips
- BCA Kit (Pierce 23227):
- Albumin Standard Ampules: 2mg/mL (Pierce 23209)
- Bovine Gamma Globulin: 2 mg/mL (Pierce 23212), note this std not in kit
- BCA Reagent A 500 mL: contains sodium carbonate, sodium bicarbonate, bicnichoninic acid and sodium tartrate in 0.1 M sodium hydroxide.
- BCA Reagent B 25 mL

Method:

1. Prepare the BCA working reagent by combining 9.8 mL of BCA reagent A with 0.2 mL of reagent B. This will be enough to working reagent for 1 plate.
2. Prepare standards in separate microfuge tubes as outlined in Table C.6.

Table C.6 BCA Assay: BSA preparation of standards

Vial	Volume of Diluent (µg/mL)	Volume and Source of Standard (µg/mL)	Final Concentration of Standard (µg/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B	750
E	325	325 of vial C	500
F	325	325 of vial E	250
G	325	325 of vial F	125
H	400	100 of vial G	25
I	400	0	0

3. Pipette 200 µL of working reagent into microplate wells.

4. Pipette 25 µL (10 µL) of each standard or unknown sample into microplate wells. This gives a sample to working reagent ratio of 1:8 (1:20 if use 10 µL, however working range of assay limited to 125 – 2000 µg/mL).

5. Cover and mix thoroughly on a plate shaker if available for 30 seconds.

6. Cover plate and incubate at 37°C for 30 minutes.

7. Cool plate to room temperature.

8. Measure absorbance with a plate reader at or near 562 nm, wavelengths between 540 – 590 nm are successful as well.

Reference:

Pierce Biotechnology, Inc.

Smith, et. al 1985. Measurments of protein using bicinchoninic acid, 150, 76-85.

C.22 MALDI-MS detection of proteins

Objective:

MALDI-MS detection of IgG protein from affinity purified mouse sera.

Material and Method:

- MALDI-MS
- MALDI target plate, hydrophobic 96 well (100 well plate or the like will also work)
- Ferulic acid
- Formic acid
- Acetonitrile
- De-ionized water
- Purified IgG mouse sample
- 0.2-2.5 μL pipette and tips
- 1000 μL pipette and tips

Matrix: Ferulic acid (15 mg/mL) in a 17:33:50 mixture of 88% formic acid, acetonitrile and de-ionized water solution.

Sample Application to Target Plate: Samples were applied to a hydrophobic target plate using the dried droplet method in a sandwich fashion as follows: 0.5 μL of matrix (allow to dry): 0.5 μL of sample: 0.5 μL of matrix (co-crystallized with sample).

Instrument Settings: Mass spectra were obtained with a 337 nm N_2 laser in linear mode using a matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) PerSeptive Biosystems Voyager-DE STR+ (Applied Biosystems, Inc., Framingham, MA, USA).

The following parameters were used to collect spectra: accelerating voltage 25kV, grid voltage 80%, delayed extraction time of 500-800 ns and low mass ion gate set to 5k Da. Mass spectra were acquired as an average of 150 laser shots taken from 3 replicate sample spots (50 shots per spectrum). Raw data from Data Explorer (Applied Biosystems, Inc. Framingham, MA, USA) was noise reduced and if needed exported into SigmaPlot 7.0 (Point Richmond, CA USA) for spectral comparison.

C.23 SDS-PAGE

Objective:

To compare the protein staining profile of affinity purified mouse ϕA1122 IgG polyclonal antibodies fractions to that of the prebleed sera, non-binding fraction, and the whole sera collected at from the day 21 bleed. Using SDS-PAGE and colloidal blue staining. This same procedure can also be used to look at the proteins bands from purified and semi-purified phage preparations

Background:

The purpose of this method is to separate proteins according to their size (i.e. molecular weight), and no other physical feature. In order to understand how this type of separation works, a theoretical understanding of how both SDS and PAGE work are essential.

In order to create a banding profile of proteins contained in a virus or antibody it is necessary to linearize the proteins so they no longer have any secondary, tertiary or quaternary structure. Consider two proteins that are each 500 amino acids long but one is shaped like a closed umbrella while the other one looks like an open umbrella. If you tried to run down the street with both of these molecules under your arms, one would be more likely to slow you down, even though they weigh exactly the same. This analogy helps point out that not only the mass but also the shape of an object will determine how well it can move through and environment. In order to linearize all proteins to the same shape SDS is used. SDS is a detergent (soap) that can dissolve hydrophobic molecules and impart a negative charge on the linearized molecule by attaching a sulfate to it.

Therefore, if a cell or virus is incubated with SDS, the membrane or particle will be dissolved and the proteins will be solubilized by the detergent and all the proteins will be covered with many negative charges. The end result has two important features: 1) all proteins contain only primary structure and 2) all proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.

Materials:

- Phage
- PBS
- 4x LDS NuPage sample buffer
- 10x NuPage reducing agent
- Microfuge tubes
- Heat block set at 70 C
- X Cell Surelock Mini Cell vertical gel apparatus
- Nu Page 4-12% Bis Tris Gel
- 20x NuPage MES Running Bugger
- NuPage Antioxidant
- Bio-Rad 200/2.0 Power Supply
- Multi-mark MW Protein Buffer Standard
- 10 uL pipettor, 200 uL pipettor
- Scoopula
- Staining Tray (Top of pipette box)
- Methanol, glacial acetic acid
- Rocking platform
- Colloidal Blue Stainer A (Invitrogen #2004-005A), Stainer B (Invitrogen #2004-005B)
- Digital Camera or scanner

Method:

1. Preparation of fraction samples:

- Put 13 uL of into separate microfuge tubes:

- purified IgG fractions
- non-binding sera fractions

2. Preparation of whole sera samples

Put 13 uL of sera into separate microfuge tubes to give the following ratios listed in Table C.7

Table C.7 SDS-PAGE sample dilutions

Ratio	Sterile DIH_2O (uL)	Sera (uL)
1:0	0	13
1:1	13	13

3. Preparation of samples for denaturing and gel

- Combine concentrated phage and bacterial samples with 4x loading sample buffer and 10x reducing agent as follows:

- 13.0 uL of concentrated phage or bacteria
- 5.0 uL of 4x sample buffer
- 2.0 uL of 10x reducing agent (this will be added just before denaturing)
- Denature these samples by heating to 70 °C for 10 minutes

Start_____ Stop_____

- Microfuge samples for 2 minutes at 12,000 rpm to collect liquid on sides of tube and pellet insoluble material.
- Leave samples at room temperature until loading.
- Standard Preparation
- Molecular weight protein standard is ready to use.
- Running Buffer Preparation
- Using a 50 mL conical tube, measure 50 mL og 20x MES/SDS running buffer and transfer to a 1L graduated cylinder
- Add deionized water to 1000mL and mix by inversion.
- Transfer 200 mL of 1x running buffer to a 250 mL graduated cylinder.
- Add 200 mL of antioxidant to the 200 mL running buffer, mix by inversion. (This is for upper gel reservoir).

6. Gel Preparation

- Remove gel from wrapper and rinse with de-ionized water.
- Remove the lower slot tape
- Place gel into the XCell II apparatus with wells toward the electrode core (words should be facing outward so you can read them)
- Use a second gel reversed as a dummy gel for the other side of the electrode core, rewrap and save this gel for later use.
- Lock the gels using the compression wedge. Press firmly and evenly to prevent leakage.
- Pour about 180 mL of upper reservoir buffer into the core and check for leakage to the lower reservoir.
- Rinse the gel wells with upper buffer using a 200 uL pipettor.

7. Loading

- Each lane will be loaded with 10 uL.
- If you are using 15 wells, only load dye in the last lane on right and left this will allow the gel to run uniformly. These lanes should not be loaded with sample.
- Load 1 lane with MW standards (NuPage MES).
- Write down what lane you load what sample in.

8. Running Conditions

- 35 minute run as follows:
- Start: 200 V
- End: 200 V
- Note that the end of the run is signified by the blue dye front entering slots by the end point.

9. Staining with Colloidal Blue

Prepare 2 solutions:

Fixing Solution:

- 40 mL DI water
- 50 mL MeOH
- 10 mL Glacial Acetic Acid

Staining Solution

- 55 mL DI water
- 20 mL MeOH
- 20 mL stainer A solution
- *5.0 mL stainer B solution (*do not added until later)

- Remove gel from cassette using a scoopula to the gel staining tray. Pop sides of plastic using scoopula and use plastic wrap to transfer to plastic tray.
- Cut the comb teeth off of the top of the gel and the large ridge at the bottom of the gel.
- Fix the gel in 100 mL of fixing solution and place on rocking platform at a setting of approximately 3 for 10 minutes.
- Pre-stain the gel with the staining solution that does not contain stain solution B and place on rocking platform (setting on rocker = 3) for 10 minutes.
- Add the 5 mL of stainer B solution and continue to rock for 3 hours at the same rocking speed as above. Be sure when you add the B solution that you add around the edges and not directly onto the top of the gel.
- Destain the gel with deionized water, rinse a couple of time with deionized water and then allow to sit overnight in fresh deionized water on the rocking platform overnight.

Results:

- Interpret bands according to molecular weight markers. Note that the smallest molecular weight proteins will migrate further through the gel than heavier proteins.
- Reported sensitivity of this stain is 10 ng for BSA.

- For instance say the lowest concentration producing a visible band at say 150 kDa (g/mol) occurs at a dilution factor of 8. Then one may roughly assume that this is approximately 10 ng.

- 10 ng x 8 = 80 ng of IgG in the sample

- 80 ng/6.5 uL = 12.3 ug/mL of IgG in the affinity purified antibody sample.

- If you wanted to find out how many IgG protein per mL, do a back calculation

$$\frac{6 \times 10^{23} \text{ proteins}}{150{,}000 \text{ g/mol}} = 4.0 \times 10^{18} \text{ IgG proteins/g}$$

$$= 4.0 \times 10^{15} \text{ IgG proteins/mg}$$

$$= 4.0 \times 10^{12} \text{ IgG proteins/ug}$$

$$4.0 \times 10^{12} \text{ IgG proteins/ug} \times 12.3 \text{ ug/mL} = 4.9 \times 10^{13} \text{ IgG proteins/mL}$$

Reference:

Invitrogen, Inc.

C.24 Bacteriophage amplification : analysis by MALDI-MS

Objective:

Example of a bacteriophage amplification experiments of *Y. pestis* using ϕA1122 and MALDI-MS detection.

Experimental:

- Stock of ϕA1122 titer: 2×10^{11} pfu/mL
- Y. pestis $OD_{620} = 0.2 = 8.0(\pm 2) \times 10^{7}$ cfu/mL
- Microfuge tubes
- Incubator set to 28°C
- 100 and 1000 μL pipettes and tips
- Shaker table
- NaCl
- PEG 8000
- 0.2 micron filters and syringe
- PBS or normal saline
- MALDI-MS, target plate (I like hydrophobic 2x96 well plate, but any will do)
- "magic juice" matrix: 15mg/mL Ferulic acid in 17:33:50 mix of formic acid: acetonitrile: distilled water

Procedure:

Add following ratios of bacteria to phage listed in Table C.8 and incubate overnight and for time study respectively:

This gives maximum phage production at given MOI (modeling will help here)

Start Time: Stop: Overnight

Table C.8 MOI for phage amplification experiments

Bacteria	Phage	MOI
8.0×10^7 cfu/mL	2×10^7 pfu/mL	0.25
8.0×10^6 cfu/mL	2×10^6 pfu/mL	0.25
8.0×10^5 cfu/mL	2×10^5 pfu/mL	0.25
8.0×10^4 cfu/mL	2×10^4 pfu/mL	0.25

Bacteria	Phage	MOI
8.0×10^7 cfu/mL	2×10^6 pfu/mL	0.025
8.0×10^6 cfu/mL	2×10^5 pfu/mL	0.025
8.0×10^5 cfu/mL	2×10^4 pfu/mL	0.025
8.0×10^4 cfu/mL	2×10^3 pfu/mL	0.025

Can keep lowering MOI same as two tables above if need to....

For timed study at given MOI of 0.0025 (Modeling will help here)

Bacteria	Phage	Time (hrs) sample at
8.0×10^8 cfu/mL	2×10^6 pfu/mL	Initial Infection time "0"
8.0×10^8 cfu/mL	2×10^6 pfu/mL	1
8.0×10^8 cfu/mL	2×10^6 pfu/mL	2
8.0×10^8 cfu/mL	2×10^6 pfu/mL	3
8.0×10^8 cfu/mL	2×10^6 pfu/mL	4

Same for each subsequent bacterial concentration

Bacteria	Phage	Time (hrs) sample at
5.0 x 10^7 cfu/mL	2 x 10^5 pfu/mL	Initial
5.0 x 10^7 cfu/mL	2 x 10^5 pfu/mL	1
5.0 x 10^7 cfu/mL	2 x 10^5 pfu/mL	2
5.0 x 10^7 cfu/mL	2 x 10^5 pfu/mL	3
5.0 x 10^7 cfu/mL	2 x 10^5 pfu/mL	4

- Spin bacterial debris and transfer supernatant to new tube (put through 0.2 micron filter if possible)
- Add NaCl and PEG, put on ice for 1 hour
- Spin at 11,000 x g to pellet, discard supernatant
- Resuspend pellet equal volume of PBS
- Add equal amount of chloroform and vortex 30 sec
- Spin at 4,000 x g to differentially separate phases, collect aqueous phase (may need to perform this step twice).
- Analyze by MALDI-MS.

C.25 Preparation of antibody/colloidal gold conjugates

Objective:

Preparation antibody/colloidal gold conjugates

Background:

Conjugation of antibodies to gold particles depends upon three separate but dependent phenomena: (a) ionic attraction between the negatively charged gold and the positively charged protein; (b) hydrophobic attraction between the antibody and the gold surface; (c) dative binding between the gold conducting electrons and sulfur atoms which may occur within amino acids of the protein. In order to form a strong absorption between gold and antibody a preliminary titration must be performed to determine the optimum conditions for conjugation.

In addition to the titration for judging the minimum amount of protein it is also necessary to determine the correct pH for the conjugation. This is best performed at, or near to the isoelectric point of the protein. It is found by performing the preliminary titration at different pH values (for example pH 7-9 in 0.2mL increments).

Materials:

Procedures for the preparation of gold colloids of various particle sizes are published elsewhere. The example given here assumes that the gold colloid is prepared from a 0.01% gold chloride solution. This is not necessarily the same procedure that Millenia Diagonstic, Inc. used in the preparation of the phage antibodies to the colloidal gold conjugates in Chapter 7.

The antibody should be affinity purified and of the highest quality. On the day of preparation make up a 0.1μg/μl solution of antibody in 2mM borax and dialyze for at least 4 hours in 1 liter of borax at pH9. Centrifuge the antibody at 100,000g for 1h at 4°C just before use. Keep at 4 °C.

All glassware must be very clean. Glass and plastic containers and stirrers should be cleaned in aqua regia, thoroughly washed in deionized water. All reagents must be of high quality analytical grade and should be filtered immediately before use. Water should be double distilled or high quality de-ionized water.

Materials:

Dialyzer

0.1% gold chloride solution

Antibodies

3mL plastic tubes

2mM borax solution

10% NaCl solution

pH meter

Centrifuge (high speed or ultra)

Procedure:

Preliminary titration of colloidal gold conjugates

1. Adjust the gold colloid to pH9. Pipette 1ml of colloid into each of a series of 3ml clean plastic tubes.
2. Adjust the antibody (0.1μg/ul) to pH9.2 with 100nM K_2CO_3 or 100mM HC1.
3. Add the antibody to each tube in a series from 0-150μL (i.e. 0-15ug in steps of 0, 1, 2, 3 to 15μg).
4. Make up each tube to 1.15ml with 2mM borax.
5. Shake each tube and leave for approximately 5 minutes to conjugate.
6. To each tube add 100ul of 10% NaCl and agitate for 1 minute.
7. The tube containing the minimum amount of protein required to stabilize the gold is indicated by the one in which the color of the gold does not change from red to blue upon the addition of NaCl.

Reference:

Beesley J (1989) "Colloidal Gold. A new perspective for cytochemical marking". Royal Microscopical Society Handbook No 17.Oxford Science Publications. Oxford University Press.

C.26 Modeling phage amplification in liquid culture

Objective:

To provide modeling syntax only for Mathematica for modeling the phage amplification in liquid cultures

Background:

A working knowledge of Mathematicia. It is recommended if your going to attempt some modeling to take a math class or Mathematicia short course to become familiar with the syntax. It is too cumbersome to explain in any great detail in this thesis, however I have provided all the syntax you would need for modeling the phage derived signal as a function of time. The rest is up to you!

Materials:

- Mathematicia 5.1 or similar version, Wolfram Research, Inc.
- Computer
- Predetermined experimental parameters for burst size and lysis time from one-step growth experiments and doubling time of bacteria

Input parameters (1st kernel)

Where (a) is the doubling time, b is the transmission coefficient, m is the decay rate, k is the lysis time and L is the burst size. This is performed in the first kernel.

```
a=.67;
b=.0000001;
m=.0000000000001;
k=1.5;
L=1000;
```

2nd kernel is the differential equation input

```
NDSolve[{x'[t]==x[t](a-b v[t]),y'[t]==y[t] a+b v[t] x[t]-k y[t],v'[t]==k L y[t]-b v[t] x[t]-m
v[t],x[0]==1000000,y[0]==0,v[0]==300000},{x,y,v},{t,0,60}]
```

3rd kernel is an evaluation of the phage concentration as a function of time, which has been interpolated between 0 to 60 hours

```
vv190=Evaluate[v[t]/.%448]
```

4th kernel is the graphical output of the evaluation from 0 to 19 hours

```
Plot[vv190, {t,0,19},PlotStyle→{{Thickness[.012],RGBColor[0,1,0]}}]
```

C.27 Phage ϕA1122 host assembly protein sequencing information and amino acid composition

Sequencing information is provided from reference [44] and the Swissprot database.

Name:

Head assembly protein

Nucleotide position:

24924–25340

Regulatory element:

ϕ13

Gene:

13

Gene Translation initiation region:

TACGGGATGGTTTTCTTATGATG

Shine-Dalgarno sequence is double underlined and initiation codon is underlined.

Number of amino acids:

138

Primary accession number:
Q858K2

Molecular mass of protein: 15,795 Da

Amino acid protein sequence:

```
          10                  20                  30                  40
MMTIRPTKST DFEVFTPAHH DILEAKAAGI EPSFPDASEC

          50                  60
VTLSLYGFPL AIGGNCGGQC

          70                  80                  90                 100
WFVTSDQVWR LSGKAKREFR KLIMEYRDKM LEKYDTLWNY

         110                 120
VWVGNTSHIR FLKTIGAVFH

         130
EEYTRDGQFQ LFTITKGG
```

GLOSSARY

A1122 a laboratory avirulent strain of *Y. pestis*, missing 70 kb plasmid

Adjuvant a substance injected with antigens (usually mixed with them but sometimes given before or after the antigens) which non-specifically enhances or modifies the immune response to the antigens

Adsorption Adhesion of a phage to the cell surface of a bacterial cell

Analytical sensitivity. The probability that a test will detect an analyte, or amutation or an alteration when it is present in a specimen. Usually expressed as a percentage.

Antibody titer measures the amount of antibodies in the blood

Antigen Foreign substance, such as phage that causes the immune system to make a specific immune response

Attachment Specific binding of a phage to its host cell's surface.

Bacteriophage A virus that infects bacteria. Also called a phage

Burst size Number of phage released per bacterium

Binding titer The titer of a binding is the dilution factor used to bind a specific ligand (antibody and antigen)

Capsid Protein coat that surrounds the phage nucleic acid.

caf1 Gene complex encoding F1 antigen

Capsomer. The morphological unit of the capsid that is composed of one or several structural subunits or protomers.

Cell wall. A rigid structure external to the plasma membrane in a bacterial cell.

Cell culture. Growth of dispersed cells in vitro.

Cell density optical density of a suspension of cells is directly related to cell mass or cell number, after construction and calibration of a standard curve

Colony forming units (cfu's) is related to the viable number of bacteria in the sample

Conjugate The joining together of the antibody and a reporter molecule, such as a gold particle.

Concatemers. A nucleic acid molecule in which the sequence of a genome is repeated numerous times.

Core polysaccharide. A chain of sugars attached to lipid A in a lipopolysaccharide.

Cross reactivity. A test produces positive and negative results for something other that what it was designed for.

DAPI. 4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters.

Doubling time Time it takes for cells to divide go through 1 division, also known as the generation time and is equal to the time in minutes or hours divided by the number of doublings or generations

Early genes. Phage genes expressed at the beginning of an infective process, usually before and during replication of the genome.

Efficiency of plating. (EOP) the proportion of phage that actually produce infected cells.

Endolysin. An enzyme with one or more muralytic activities against the glycosidic, amide, or peptide bonds of the peptidoglycan layer in Gram-negative and Gram-positive bacteria.

Facultative anaerobe. An organism, usually a bacterium, that make ATP by aerobic respiration if oxygen is present but that switches to fermentation under anaerobic conditions.

Gram-negative bacteria. Bacteria with a thin layer of peptidoglycan and an external membrane system.

Gram-positive bacteria. Bacteria with a thick layer of peptidoglycan and no external membrane system.

Hexamer. One of six protein subunits that meet on adjoining triangular faces of icosahedral phage.

Holin. Small membrane proteins which is required to induce bacterial lysis by dsDNA phage.

Host range. The type of different bacterial cells that a given phage can infect.

Humoral immune response an immune response that is mediated by B cells which make the antibodies

F1-antigen A temperature regulated glycoprotein or capsular protein (antigen) expressed on the cell surface of most *Y. pestis* strains at temperatures greater than 33°C

Immunization technique used to cause an immune response that results in resistance to a specific disease, especially an infectious disease

Insertional sequences. Any of several discrete DNA sequences that repeat at various sites on a bacterial chromosome, on certain plasmids, and on bacteriophages which can move from one site to another on the chromosome, to another plasmid in the same bacterium, or to a bacteriophage.

In vitro In an artificial environment, such as a test tube

In vivo Pertaining to a biochemical process or reaction taking place in a living cell or organism

Icosahedron A solid figure having 20 triangular faces.

Isoelectric point pH at which an amphoteric molecule (such as a proteins) has a net charge equal to zero

Late genes. Phage expressed toward the end of the infective process, usually after replication of the genome.

Limit of detection. The minimum concentration f a substance being analyzed and has a 99% probability of being identified.

Lyse. (lysis) causes dissolution or destruction.

Lysis from within Form of lysis which is properly connected with intracellular progeny phage proliferation

Lysis from without A mechanism of phage lysis which causes a phenomenon to occur when infecting bacteria at high phage concentration, resulting in little to no progeny phage to be produced

Lysogenic phage. A phage that can become integrated into it's host chromosome.

Lytic cycle The steps in phage production that usually lead to cell lysis

Lytic phage. Phage that reproduces itself and then disrupts its host cell.

Maturation. Process of assembly of phage during the phage replication process.

Multiplicity of infection The ratio of phage to bacterium upon initial infection

Muralytic activity. Enzymes that degrade the bacterial cell wall.

Monoclonal antibody. Antibody of a single specificity produced by the progeny of a single original antibody-producing cell.

Non-fastidious. Uncomplicated or nonspecific nutritional requirements.

Non-specific binding As opposed to binding to the receptor of interest, non-specific binding is to the other sites

Nucleases. Specific digestive enzyme which breaks the bonds between nucleotides.

Nucleocapsid. Nucleic acid and its surrounding capsid proteins.

O-antigen. Constituent of the outer membrane of a Gram-negative bacteria, consisting of a chain of repeating sequences of sugars.

One-step growth experiment. Classic experiment demonstrating that phage replication involves assembly rather than division.

pCad plasmid that carriers the Lcr gene complex, 70 kb also known as pCD1

pCD1 plasmid that carriers the Lcr gene complex, 70 kb also known as pCad

Peptidogylcan. Constituent of bacterial cell walls, consiting of chains of N-acetyl-glucosamine and N-acetyl-muramic acid crosslinked by short chains of amino acids.

pFra plasmid encoding F1 gene complex, 110 kb also known as pMT1 and pTox

Phage lysis susceptibility the ability of a phage to infect, replicate and burst a cell creating plaques on a bacterial lawn.

Plasmid Autonomously replicating, extrachromosomal circular DNA molecules, distinct from the normal bacterial genome and nonessential for cell survival under nonselective conditions. Some plasmids are capable of integrating into the host genome.

Plaque assay. Method of titering phage based on counting the cleared areas (plaques) on a continuous cell sheet or "lawn" produced by phage destruction of their host cells.

Plaque forming units (pfu's) is related to the viable number of phage in the sample

pMT1 plasmid encoding F1 gene complex, 110 kb also known as pFra and pTox

Polyclonal antibody Antibodies produced from several different antibody-producing cell.

Porosity. Amount of air in the three dimensional structure of a membrane.

Procapsid or prohead. An empty icosahedral capsid during the assembly process.

Proteases. Specific digestive enzyme which degrade proteins.

Protomer. One of the individual proteins that form a phage capsid; also called structural subunit.

Prototype An original type or form serving as a basis or standard for later stages

pTox plasmid encoding F1 gene complex, 110 kb also known as pMT1 and pFra

Scaffolding proteins. Proteins that assist in assembly of an icosahedral procapsid but do not become part of the mature capsid.

Sensitivity. An operating characteristic of a diagnostic test that measure the ability of a test to detect a disease when it is truly present.

Specific binding Binding to the receptor of interest is called specific binding.

Structural subunit. Individual proteins of the capsomer also called a protomer.

Teichoic Acids. Teichoic acids are polyol phosphate polymers bearing a strong negative charge. They are covalently linked to the peptidoglycan in some gram-positive bacteria, are strongly antigenic and are generally absent in gram-negative bacteria.

Titer The concentration of a substance in a solution, or the strength of such a substance detected by titration

Transcription. Is the process through which a DNA sequence is enzymatically copied by an RNA polymerase to produce a complementary RNA. In the case of protein-encoding DNA, transcription is the beginning of the process that ultimately leads to the translation of the genetic code (via the mRNA intermediate) into a functional peptide or protein. Transcription has some proofreading mechanisms, but they are fewer and less effective than the controls for DNA; therefore, transcription has a lower copying fidelity than DNA replication.

Transcription proceeds in the 5' → 3' direction, and is divided into 3 stages: *initiation*, *elongation* and *termination*.

Virulence factor A specific factor possessed by an organism rendering the organism pathogenic

Zoonotic. A human infection caused by an organism which occurs naturally in other animals and has crossed into humans.