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MODIFIED VACCINE VECTORS TO UNDERSTAND ADJUVANT FUNCTIONS OF LISTERIA DURING CHRONIC SCHISTOSOMIASIS

by

Stephanie K. Norwood
B.S. May 2018, Virginia Wesleyan University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

BIOLOGY

OLD DOMINION UNIVERSITY
DECEMBER 2023

Approved by:

Lisa M. Shollenberger (Director)

Wayne Hynes (Member)

Erin Purcell (Member)

ABSTRACT

MODIFIED VACCINE VECTORS TO UNDERSTAND
ADJUVANT FUNCTIONS OF LISTERIA
DURING CHRONIC SCHISTOSOMIASIS

Stephanie K. Norwood
Old Dominion University, 2023
Director: Dr. Lisa M. Shollenberger

Vaccination is one of the most effective strategies employed to prevent infectious diseases. Successful vaccination is dependent upon the induction of a specific, robust, and prolonged immune response. One of the major challenges faced by vaccine development is vaccine failure due to host-related factors that can modulate the immune system, which leads to non-responsiveness to vaccinations. The generation of new vaccine strategies is imperative to combat these effects. Live bacterial vectors are one approach used as they can elicit humoral immunity, cellular immunity, or both. *Listeria monocytogenes* is a Gram positive, intracellular pathogen that is an effective bacterial vaccine vector through strong induction of cell-mediated immunity. Specifically, wild-type Listeria expressing the HIV-Gag protein was previously shown to be a functional vector in overcoming host-related immune bias when DNA vaccines alone could not. However, there are safety concerns due to its pathogenicity and therefore new clinically relevant vectors with attenuated Listeria strains are needed. Here, a comparison of the pathogenicity (LD_{50}) is determined for several Listeria strains. Additionally, the HIV-Gag protein has been cloned into the pKSV7 shuttle vector for future use in attenuated Listeria strains. Our aim is that these non-pathogenic vectors will be clinically relevant for use in overcoming Th1/Tc1 type vaccine failure.

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I would like to dedicate this thesis to my family and close friends who provided invaluable love and support as I worked to reach my goals finishing this work. To those who know, I overcame a lot to get this point and would not be here without them. I would like to especially acknowledge my parents for their guidance, love, and encouragement pushing me to be the person I am today.

ACKNOWLEDGMENTS

I would like to extend a special thanks to my advisor Dr. Lisa Shollenberger for the opportunity to work on this project. Her mentorship and support have been an integral component throughout the course of this research. Her immense knowledge and plentiful experience have encouraged me in all the time of my academic research and daily life. My gratitude also extends to my committee members, Dr. Wayne Hynes and Dr. Erin Purcell, for their technical support and expertise during the completion of this work. I want to also thank the funding sources and departmental support that made the opportunity for this project possible.

Finally, I would like to extend special gratitude to my lab mates for a cherished time spent together in and outside the lab. Their continued support as my team and family was much appreciated.

NOMENCLATURE

°	Degrees
Δ	Delta (deletion of gene)
actA	Actin assembly-inducing Protein
APC	Antigen Presenting Cell
Bp	Base Pairs
BCG	Bacillus Calmette-Guerin
C	Celsius
CFU	Colony Forming Units
CTL	Cytotoxic T Lymphocyte
dal	D-Alanine racemase-like protein
dat	D-alanine aminotransferase
DNA	Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
IP	Intraperitoneally
inlB	Internalin B
LD_{50}	50% Lethal Dose
MCS	Multiple Cloning Site
PBS	Phosphate Buffered Saline
Tc1	Cytotoxic T cell Type 1
Th1	T-Helper cell Type 1
Th2	T-Helper cell Type 2

WT Wild-type

uL Microliter

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INTRODUCTION

Vaccines

Vaccines are an imperative global health strategy in combating infectious diseases. Vaccination is a key component of primary health care as it has been proven effective in preventing diseases that previously had high morbidity and mortality. Reducing a high burden disease has been demonstrated to be a cost-effective measure and as such immunizations have become a part of various national health programs at all stages of life [1]. Vaccines work by inducing an immune response to a particular antigen or set of antigens that provides protection against disease following exposure to a specific pathogen. To attain this response, the vaccine is composed of one or more antigens derived from either the pathogen itself or synthetically generated components of the pathogen [2]. Following immunization with a conventional protein antigen, the antigen is taken up by antigen presenting cells (APCs) that process and present specific peptides of the vaccine on major histocompatibility complex (MHC) molecules. These activated APCs, including dendritic cells, then travel to immune tissues where they can activate T cells and B cells through various signaling pathways [3,4]. These signaling processes result in lymphocyte maturation and the fine tuning of the antibody response for B cells, and induction of cytotoxic T lymphocytes (CTLs) and T - helper cells for T cells. When the APCs are activated through pattern recognition receptors (PRRs), they move to the lymph node to activate T cells through their T cell receptor (TCR). MHC class II presentation is specific to when APCs activate T cells. This also stimulates B cell maturation and clonal expansion to produce antibodies through various signaling pathways. MHC class I presentation, however, activates CD8+ T cells leading to a cell-mediated or CTL response. Eventually, memory cells are produced which allows for rapid elimination of pathogens following a secondary encounter [5]. Understanding how

vaccination induces these immune responses is important when developing the vaccines themselves.

Vaccine Development

Developing effective vaccines comes with several challenges. Vaccine efficacy is based on whether the host immune response against an antigen can successfully elicit a memory response over time for prolonged protection. An ideal vaccine candidate would provide 100% efficacy across all population types, be immunogenic but not reactogenic, and be easily stored and produced [6]. Although these characteristics are ideal for the vaccine to possess, this is difficult to achieve, and many vaccines do not contain all these features simultaneously. These factors contribute to the varying vector designs, efficacies, and host responses for the vaccines in use today. When developing vaccines, they can differ with the antigen or antigens they are targeting, administration of the vaccine (location, type), dose regimes, and vaccine components and delivery methods. Each type of vaccine strategy or platform has various advantages, disadvantages, and considerations for their use. Live vaccines are developed so that, in an immunocompetent host, they have enough replication of the vaccine antigen to induce a strong immune response but sufficient attenuation of the pathogen to avoid symptomatic disease. For this reason, some safe, live attenuated vaccines require multiple doses and induce short-lived immunity (bacterial and viral). Non-live vaccines including the whole inactivated, synthetic peptides, nucleic acid, and recombinant vaccines avoid some of the safety concerns in comparison to the live vaccine platforms. However, due to these platforms containing mainly antigenic components they require additional elements, including adjuvants, to produce a robust immune response in the host, and may not provide protection with those with certain immunodeficiencies.

DNA Vaccines

One delivery platform that is widely used for currently licensed vaccinations is DNA-based vaccines. This platform allows for the induction of humoral and cellular immune responses against various types of disease-causing pathogens. DNA vectored vaccines can induce broad-based immunity by permitting the expression of genetically engineered DNA (antigen) to induce an immunologic response within host cells [7]. One way to achieve this is through using DNA plasmids with the target antigen encoded on them. Choosing the target antigens for these vaccines is a priority when designing DNA vaccines. The selected antigens can be regulated by various promoters (including mammalian promoters) along a plasmid backbone of bacterial DNA [8].

Vaccination Challenges

Due to the disparities among vaccine platforms, vaccine failure has been observed for several vaccines in use. Many factors responsible for vaccine failures can be attributed to vaccine-related and/or host-related factors. Vaccine related factors include vaccine attenuation and development, vaccination regimes or administration [5,9]. Host related factors include host genetics, immune status, age, health or nutritional status, and immune suppression [3]. Modulation of the immune system can then lead to either skewed immune biasing or severe immune suppression. These host related factors can lead to non-responsiveness to vaccines [4,10,11]. An example of this immune biasing is chronic helminth infections, which cause systemic T-helper cell type 2 (Th2) biasing and IL-10 mediated immune suppression [11-14]. More specifically, schistosomiasis, an infection caused by parasitic worms in the genus *Schistosoma*, has displayed negative effects on vaccination and influences the responses of concurrent diseases [11,13]. Schistosomiasis is the second most devastating tropical parasitic disease after malaria with more

than 200 million cases globally [14]. After the initial infection, the presence of the parasite causes a T-helper cell type 1 (Th1) pro-inflammatory response where host signals provide cues to guide schistosome worm development through sexual reproduction. Once the adult female worms begin producing eggs, there is a reduction of the Th1 response and an induction of a polarized Th2 response. After a chronic infection has been established (around 8 weeks), host signals minimize the Th2 response biasing in favor of an IL-10-mediated immunosuppressive response. This immunosuppressive response causes non-responsiveness to vaccination. Development of vaccines against infections, such as HIV, malaria, and tuberculosis, has not been successful and comes with additional challenges [15]. In areas where these infections persist, there is also a high prevalence of coinfections with chronic helminths, including schistosomiasis. In populations where schistosome infection is endemic, reduced responses to the BCG and tetanus toxoid vaccines have been reported [16,17].

Bacteria as Vaccine Vectors

Generation of new vaccine strategies is necessary to combat these immunomodulatory effects. A solution to this problem is to use bacteria as vectors for delivering vaccine components. Using live bacteria as vaccine vectors is an effective method as they elicit effective humoral immunity, cellular immunity, or both. In terms of delivery (Fig. 1A), a variety of antigenic proteins or genes can be cloned and then transformed into live-attenuated bacterial vectors to achieve the desired expression. Antigens can be carried episomally on a plasmid, or integrated into the bacterial chromosome [6]. This is achieved through the ability to express proteins in various cellular compartments (Fig. 1B) including expressing the proteins intracellularly, on the bacterial surface, or as secreted antigens [6]. Using these vectors has several other advantages such as components that stimulate several signaling pathways of the immune

system, their ability to be genetically altered for specific targets, and are treatable with antibiotics if necessary [4,6]. These features make these vectors successful in providing protective immunity against a targeted pathogen (bacteria, virus, and/or parasite). However, as many of these vectors are pathogenic, there are several safety concerns with using live bacteria as vectors which has resulted in vaccine hesitancy to their use.

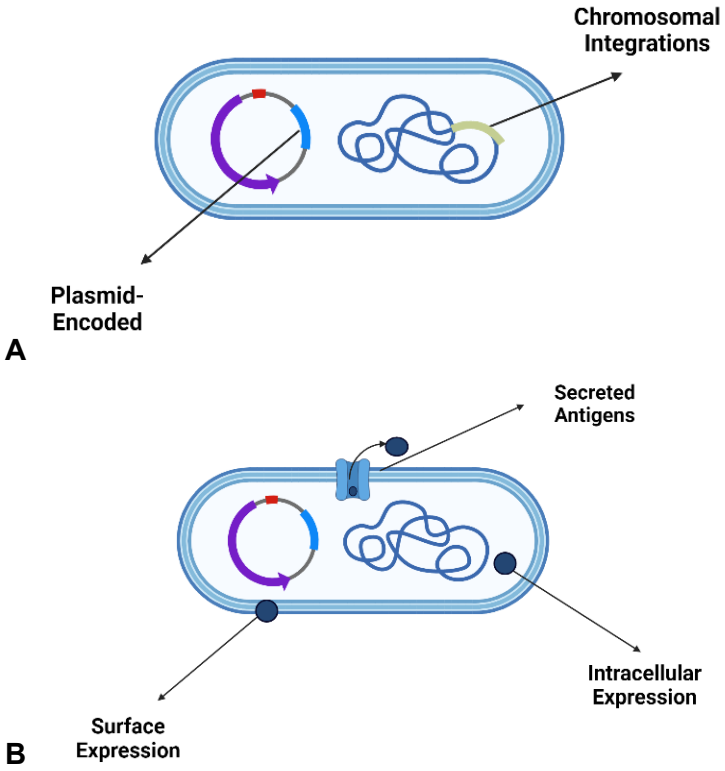

FIG 1 Using bacteria to deliver vaccine antigens. (A) Depicts how antigens can be vectored in the bacteria through plasmids or by integrating the antigens into the chromosome. When using bacteria to deliver vaccines various methods of protein expression (B) can be achieved including expressing the proteins intracellularly, on the bacterial surface, and secreting antigens using bacterial secretory systems.

Listeria as a Vaccine Vector

Due to several unique biological features, *Listeria monocytogenes* has long been studied as a model for intracellular bacterial infection and CTL-based immunity. Further, it has been an effective vaccine vector in several preclinical studies [18]. *L. monocytogenes* is a ubiquitous, Gram-positive intracellular pathogen. In humans, it is the causative agent of listeriosis, a foodborne illness contracted from consuming food contaminated with the pathogen, with the most severe effects in those who are immunocompromised and pregnant [19]. Various virulence factors (including internalins and toxins) aid *L. monocytogenes* in invading target cells and cell-to-cell spread, which allow the bacteria to survive and replicate in phagocytic cells. After engulfment, *L. monocytogenes* escapes the phagosome and enters the host cytosol where it will multiply without inducing rapid cell death. Bacterial surface proteins including internalins (InlA and InlB) interact with cell surface receptors to allow internalization of the bacterial cells. Once internalized, the bacteria utilize toxins to lyse the primary endocytic vacuole including listeriolysin O (LLO) and phospholipases. Upon replication in the cytosol, the release of actA stimulates actin polymerization by recruiting host nucleation proteins. The formation of comet tails using Actin rockets enables cell-to-cell spread of the bacteria. Lysis of the secondary vacuole by the action of LLO and phospholipases cause the release of bacterial cells into the cytosol. As a result the bacteria activate several immune pathways and is a strong inducer of cell mediated immunity [20].

Because the bacteria can activate the immune system and is self-adjuvanting, it is an attractive bacterial vaccine vector. During chronic schistosomiasis infection, previous studies have shown vaccination with *Listeria* produces a functional vaccine response, where use of a DNA vaccine alone fails [21,22]. However, due to *L. monocytogenes* being pathogenic there are

several safety concerns to using this strain as a vaccine vector even though the number of bacteria utilized is below the infectious dose for those who are immunocompetent [23,24].

To address these safety concerns, live-bacterial vectors are genetically attenuated. Live-attenuated bacterial vaccine vectors can be genetically engineered through the deletion of one or more essential genes involved in virulence regulatory systems or biosynthesis pathways. These mutant vectors are still able to mimic natural infection and contain intrinsic adjuvant properties to maintain immunogenicity while decreasing the virulence of the bacteria [18,25]. This reduces the risk to those working with the vectors as well as those receiving vaccinations. Additionally, as with wild-type vectors, various targeted proteins or genes can be cloned and transformed into the attenuated vectors to attain the required expression [10,26]. Currently, there are several of these genetically attenuated strains of *L. monocytogenes* in use for clinical trials for cancer and immunotherapies [27-30]. These features make the attenuated vectors an attractive option for vaccine and drug delivery.

SPECIFIC AIMS

Vaccination programs are one of the most effective global health strategies currently employed worldwide. The improvement of vaccines and the development of new vaccine delivery systems is necessary to defend against new and existing infectious diseases. Previous studies have shown that during chronic schistosomiasis, plasmid DNA expressing HIV-Gag is insufficient in providing protective immunity, while *L. monocytogenes* chromosomally expressing HIV-Gag elicits a robust cytotoxic T lymphocyte (CTL) response [10,21,22,30]. Therefore, there is a desperate need for the development of functional vaccines that are efficacious in schistosome-endemic areas.

The purpose of this project is to determine if fully virulent *L. monocytogenes* are necessary for the observed CTL vaccine responses. This project involves two major aims focused on the development of vaccine vector models for use during chronic schistosomiasis. The first aim will compare the pathogenic potential of various *Listeria* strains for use as vaccine vectors. The second aim is to generate attenuated Listeria-vectored HIV vaccines. This aim will include the development of the pGag DNA vector, construction of the pKSV7-Gag shuttle vector, and creating Listeria vectors episomally expressing HIV-Gag. Given that Listeria-delivered HIV antigens can generate a specific CTL vaccine response in mice chronically infected with *Schistosoma mansoni* when plasmid-delivered HIV antigens cannot, we hypothesize there are one or more molecular components of Listeria that allow it to adjuvant Tc1/Th1 responses during chronic schistosomiasis.

In future projects, the plasmid shuttle produced in this project will be used to create attenuated *L. monocytogenes* vectors expressing the HIV-Gag protein to assess functional

vaccine responses in mice. Our aim is that these attenuated vectors will become candidates for future clinical use in overcoming schistosome-induced Tc1 vaccine failure.

MATERIALS AND METHODS

Bacterial Strains

The Listeria vectors used in this project are *Listeria monocytogenes* strain 10403S. The LADD strain is a mutant strain with actin A and internalin B gene deletions (Δ*actA*Δ*inlB*). The internalin B (*inlB*) gene encodes for the surface protein used for adhesion and invasion of host cells by Listeria. The *actA* gene in Listeria is an integral membrane protein required for intracellular motility and cell-to-cell spread [31]. The LmddA strain is a mutant strain with Δ*dal*Δ*dat*Δ*actA*. The *dal* gene is the alanine racemase gene and the *dat* gene encodes for d-amino acid aminotransferase; both contribute to alanine synthesis [32].

The Lm-ZY18 and LmddA strains were received from Yvonne Patterson (UPenn) by way of Donald Harn (UGA). The LmddA strain is licensed by Advaxis for use in clinical trials. The LADD strain was received from collaborator J.D. Sauer (University of Wisconsin) and is being used for clinical trials by Aduro. The *Escherichia coli* strain used for molecular cloning was *E. coli* DH5α (ZYMO Research). All the strains used in this project are referenced in Table 1 with detailed descriptions of each strain and designations to be used for the remainder of this project.

TABLE 1 Bacterial strains

Designation	Description	Source
Lm-WT	Wild-type *L. monocytogenes* (Lm) 10403s strain	J.D. Sauer (UWisc)
Lm-ZY18	LLO-Gag chromosomally inserted into 10403S (Lm-gag)	Y Patterson (UPenn)*
LADD	10403S double-deletion Δ*actA*Δ*inlB*	J.D. Sauer (UWisc)
LmddA	10403S triple-deletion Δ*dal*Δ*dat*Δ*actA*	Y Patterson (UPenn)*
DH5α	*E. coli* strain DH5α for constructing recombinant plasmids	ZYMO Research

*Via DA Harn (UGA)

***In Vivo* Studies**

To determine the number of bacteria that can be used as an effective vaccine vector, the pathogenic potential of the bacterial strains was determined, For the Listeria vectors, this was achieved by determining the 50% lethal dose or LD_{50} of the strains in naive mice [24]. The LD_{50} is a method based on the determination of the bacterial dose required for half of the tested population to succumb to disease after a specified time duration [24]. The value is calculated by analyzing a dose-response relationship, where the dose (x-value) versus the responses (y-value) or percentage of population yields an upward-slopping sigmoidal curve. For each vector, the colony forming units (CFUs) were determined from plate counts and used to calculate the dose ranges tested to determine the LD_{50} values. The CFU dose ranges for the Listeria strains tested were chosen based on previously published LD_{50} values for Lm-WT and Lm-ZY18 strains [27,33]. In a two-week study with female BALB/c mice (6-8 weeks old), test group animals (n=4 per group) were inoculated intraperitoneally (IP) with 200 uL injections of the Listeria strains diluted in PBS. Mice were monitored for clinical symptoms of listeriosis infection and pain/distress levels over a 14-day period. All animals were included in the analysis for each strain which analyzes morbidity vs mortality rates, and the determination of the LD_{50} value.

Construction of pGag Vector

To determine the effectiveness of the bacterial-vectored vaccines, a control DNA vaccine was required to compare their abilities of inducing CTL responses. Table 2 outlines the major genetic features of the HIV-Gag insert and the plasmid backbones used to generate the DNA control vector for this project. The HIV-Gag gene (GenBank accession number U12055.1) was selected as the target antigen for the vaccine model developed in the project. Previous antibiotic susceptibility testing on the Listeria strains used in this project determined that ampicillin

resistance was needed for the control DNA vector. The pCI mammalian expression vector (Promega) which promotes constitutive expression of cloned DNA inserts in mammalian cells, was chosen as the backbone for the DNA control vector. The pCI vector (GenBank accession number U47119) contains the human cytomegalovirus (CMV) enhancer/promoter region, an ampicillin resistance gene, and a multiple cloning site (MCS) for insertion of the gene of interest. To generate the vector, the HIV-Gag gene, flanked by EcoRI and Xbal restriction sites, was synthesized and ligated into the pCI backbone at the Multiple Cloning Site (MCS) using the EcoRI and XbaI sites (Azenta). This resulting constructed vector, pGag (Fig. 2), is a plasmid encoding the HIV-Gag gene, ideal for vaccine delivery.

TABLE 2 Vectors and inserts for cloning

Name	Format	Features	GenBank #	Source
Gag	Gene	HIV-1 lllB strain CTL epitope (AMQMLKETI)	U12055.1	Synthesized
pCI	Plasmid	CMV promoter Ampicillin resistance	U47119	Promega
pKSV7	Plasmid	CMV promoter ColE origin Ampicillin resistance Chloramphenicol resistance Thermosensitive region	n/a	Yvonne Patterson (UPenn) via Donald Harn (UGA)

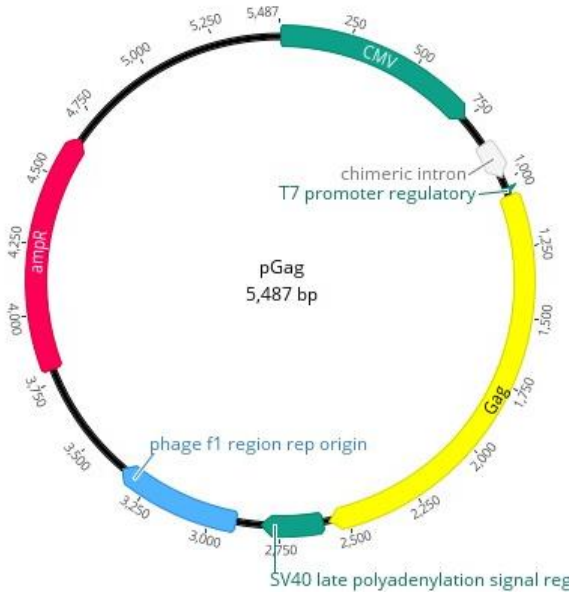

FIG 2 Plasmid map of pGag plasmid including the plasmid pCI backbone and HIV Gag insert. The map highlights the major features including the CMV promoter, ampicillin resistance gene, and f1 origin.

Cloning of Gag Gene

To generate bacterial-vectored vaccines expressing HIV-Gag, the generation of a shuttle vector with HIV-Gag was required. A shuttle vector is a constructed plasmid with the ability to propagate in two different host species. DNA inserted into a shuttle vector can be tested or manipulated in two different cells. Plasmid pKSV7 (Fig. 3), a shuttle vector capable of replication in *E. coli* and temperature-sensitive replication in Gram-positive bacteria such as *L. monocytogenes* [34]. This donor vector was used to create a shuttle vector for cloning Gag in the Listeria strains, as this vector allows for homologous recombination in Listeria. The shuttle vector for this project was received from Yvonne Patterson (UPenn) by way of Donald Harn (UGA). Plasmid pKSV7 was double- digested with EcoRI and Xbal (New England BioLabs) restriction sites to linearize the plasmid backbone. The linearized pKSV7 backbone was purified by 1%

agarose gel electrophoresis and the plasmid DNA was isolated through column purification (Qiagen QIAquick® Gel Extraction Kit). To isolate the HIV-Gag for cloning pGag was also double-digested using EcoRI and Xbal restriction sites. The HIV-Gag was separated from the pGag backbone using 0.8% gel electrophoresis and column purified as above (Qiagen). To generate the shuttle expressing the Gag gene, HIV-Gag was ligated into the linearized pKSV7 shuttle with utilizing the sticky end sites. Ligation reactions (25 uL) including instant-sticky end ligase/master mix (NEB), pKSV7 plasmid DNA and HIV-Gag insert DNA were incubated overnight at 16ºC in PCR tubes. For these reactions, 1:1, 1:3, and 1:5 molar ratios of vector to insert were used, along with reactions without ligase and/or insert DNA. Ligation reactions were transformed into chemically competent *E. coli* DH5α (ZYMO), using a 42ºC heat shock for 1 minute, followed by a one-hour recovery period at 37ºC with SOC media. Post-recovery, 200 uL of *E. coli* transformants were plated on selective media with 100 μg/mL of ampicillin and incubated for 24 hours at 37ºC. Colonies from the selective plates were used for colony PCR (described below) to confirm the ligations of plasmid pKSV7 with the Gag insert. Colonies with confirmed ligations were used to isolate the newly generated pKSV7-Gag shuttle plasmid from bacterial DNA using the QIAprep Spin Miniprep Kit (Qiagen). Prepared pKSV7-Gag plasmid DNA was then used for future cloning steps with Listeria strains.

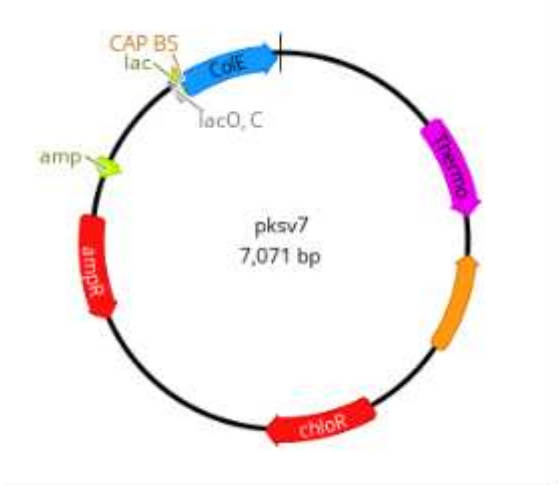

FIG 3 Plasmid map of pKSV7 shuttle vector. This map highlights the main features of the plasmid including the pKSV7 backbone with ampicillin and chloramphenicol resistance genes, ColE origin for replication in *E.coli,* and thermosensitive regulatory region. The pKSV7 vector allows for homologous recombination in Listeria.

Construction of Listeria Vectors Expressing Gag

To generate the Listeria strains episomally expressing HIV-Gag, *gag* was isolated from the pGag DNA vector and then cloned into the pKSV7 plasmid to generate the pKSV7-Gag. Using the pKSV7-Gag shuttle vector, *gag* was then transformed into the mutant Listeria strains (Lm and LADD), as shown in the cloning workflow in Figure 4. The previously generated pKSV7-Gag shuttle vector was transformed into electro-competent Listeria strains through electroporation. For the Listeria transformations, 50 uL of competent Listeria cells were mixed with 1 µg of pKSV7-Gag plasmid DNA and incubated on ice for five minutes. In a 1 mm cuvette, the solution was electro-pulsed at 1 kV, followed immediately by the addition of 1 mL recovery media (BHI-sucrose). The cells were incubated statically at 30ºC for a 2-hour recovery period. Transformants were plated (100 uL) on selective media (BHI with 10 µg/mL of chloramphenicol) incubated at 30°C for 2 days. Single colonies were picked and streaked onto new selective media (BHI with 10

µg/mL chloramphenicol and 50 µg/mL streptomycin) plates and incubated overnight at 30°C. The addition of streptomycin for this step ensures growth for Listeria strains but no other bacteria. Single colonies (20 colonies per plate) were picked for analysis to confirm the presence of the shuttle plasmid in the listeria strains. Colonies were streaked onto RAPID' *L.mono* medium selective chromogenic media (BIO-RAD) to ensure that the colonies were *L. monocytogenes* species. Future analysis will also use colonies selected and used for colony PCR. Orientation specific primers spanning the 5' ligation site will be used to amplify along the entire Gag insert and spanning the pKSV7 backbone and Gag gene insert.

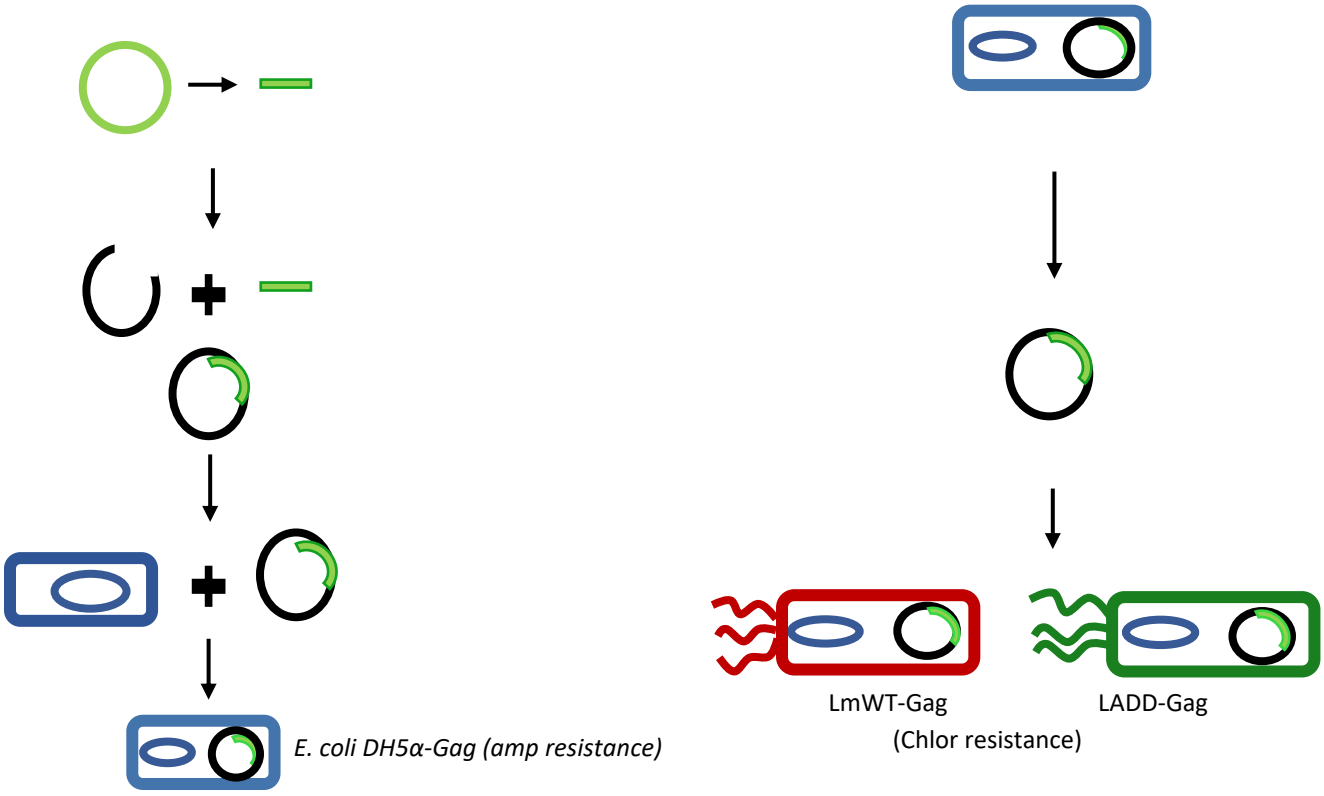

FIG 4 Cloning workflow. This workflow provides an overview of the cloning steps used to generate the Listeria strains expressing Gag.

Polymerase Chain Reaction (PCR)

To confirm the construction of the pGag DNA vector, the HIV-Gag gene flanked by EcoRI and Xbal restriction sites was amplified in a nested PCR in 25 uL reactions. Reactions included Q5 High-fidelity 2X master mix (New England BioLabs), 0.5 mM of each F/R primer (table 3), 2 uL of pGag template DNA or deionized water (diH2O) for negative controls. The conditions for thermal cycling were as follows: 95°C for 10 min, 5 cycles of 95°C for 30 s, 42.4°C for 40 s, and 72°C for 2 min, 30 cycles of 95°C for 30 s, 63.6°C for 40 s, and 72°C for 2 min and final extension cycle at 72°C for 5 min. The initial 5 cycles first amplified the targeted *gag* DNA and the additional 30 cycles at the higher temperature continued to amplify the entire targeted sequence that included the flanked restriction sites.

TABLE 3 Target regions for amplification

Target region	Primer names	Primer sequences (5'-3')	Target size (bp)
Gag-RE	Gag-EcoRI-F	GGAGAATTCGCAATGGGTGCGAGA	1515
	Gag-Xbal-R	GGAGGAAAGATCTGGATTATTGTGACGAGGG	
Gag	Gag-F	ATTAAGCGGGGGAAAATTAGATCGATGGGA	1500
	Gag-R	CCTTGTCTATCGGCTCCTGCTTCTGA	
pKSV7	pKSV7-F	GTAGTAGCGACAGCTATTAACTTTCGGTTGCAAA	3980
	pKSV7-R	CTAATCCTGTTACCAGTGGCTGCTGC	

Colony PCR

To confirm the presence of the Gag insert in the pKSV7 shuttle vector, colony PCR was performed on isolated transformed colonies. Ligation reactions transformed into chemically competent *E. coli* DH5α were plated at 200 uL onto selective media (100 µg/mL ampicillin). After the 24-hour incubation at 37ºC, the transformants were numbered and 20-40 colonies were tested

per plate. Plate colonies tested also included negative controls (transformants without plasmids) and positive controls (transformants with plasmid pGag). Individual colonies were picked with sterile 10 μL pipette tips and swirled into PCR tubes containing 25 μL of the master mix including primers to amplify the desired target regions. The reaction master mix included Taq 2X master mix (New England BioLabs) and 0.2 mM of each the F/R primer. The primers amplified the HIV-Gag gene insert using forward and reverse primers along Gag insert sequence. Primer sequences are listed in Table 3. The conditions for thermal cycling were as follows: 95°C for 10 min, 35 cycles of 95°C for 30 s, 57.5°C for 45 s, and 68°C for 1 min 30 s and final extension cycle at 68°C for 5 min. PCR products were visualized using 0.8% Ethidium bromide (EtBr) agarose gel for bands longer than 1000 bp.

RESULTS

LD_{50} Determination of Listeria Strains

The pathogenic potential of four Listeria strains were compared using the LD_{50} values. The LD_{50} value for the tested Listeria strains was expressed as CFU and calculated using the AAT Bioquest *Quest Graph*™ LD_{50} *Calculator*. For each Listeria strain, the graphs in Fig. 5 depict the percent total average weight loss per group (left) and the percent survival of the population (right) for the mice over the two-week period for various CFU doses. For the Lm-WT strain (Fig. 5A), a sharp decrease in weight is seen for the 1E7 and 1E8 doses, with no recovery from the infection. Mice given the 1E6 dose experienced an initial weight loss over the first 3 days, but 80% of the mice from the group completely recovered from the infection. Additionally, 100% of the population succumbed to infection for doses 1E7 CFU and above, but 100% survival was observed for 1E5 CFU and below, indicating the LD_{50} value to be between these two doses. For the Lm-Gag strain (Fig. 5B), the CFU dose given was from previously published LD_{50} data for this strain. This dose was tested in our lab to verify this value. However, even after the mice from this group displayed symptoms of infection and weight loss over the first 5 days, 100% of mice in this group recovered and survived. For the LADD strain (Fig. 5C), a sharp decrease in weight is seen for the 1E11 dose, with no recovery from the infection. Mice given the 1E4, 1E6, 1E8, and 1E10 doses experienced an initial weight loss over the first 2 days, but 100% of the mice from these groups completely recovered from the infection. For the LADD 1E11 CFU dose, 100% of the population succumbed to infection but 100% survival was observed for 1E110 CFU and below, indicating the LD_{50} value to be between these two doses. For the triple deletion mutant strain LmddA (Fig. 5D), an LD_{50} was indeterminate due to the expected lack of pathogenicity. The mice from this group were given the highest amount of CFU per 200 uL for the LmddA yet displayed little to no

symptom of infection. A comparison of the calculated LD_{50} values and previously published values for each of the strains (Table 4) is also shown as attenuation is increased. Using the data from Figure 5, the LD_{50} value for each of the strains was determined. By plotting dose-response curves, which yield an upward-slopping sigmoidal curve, the dose at the point where 50% mortality is observed represents the LD_{50} value (Fig. 6). The LD_{50} value for the wild-type strain was determined to be 7.3E5 and 5E10 for the LADD strain.

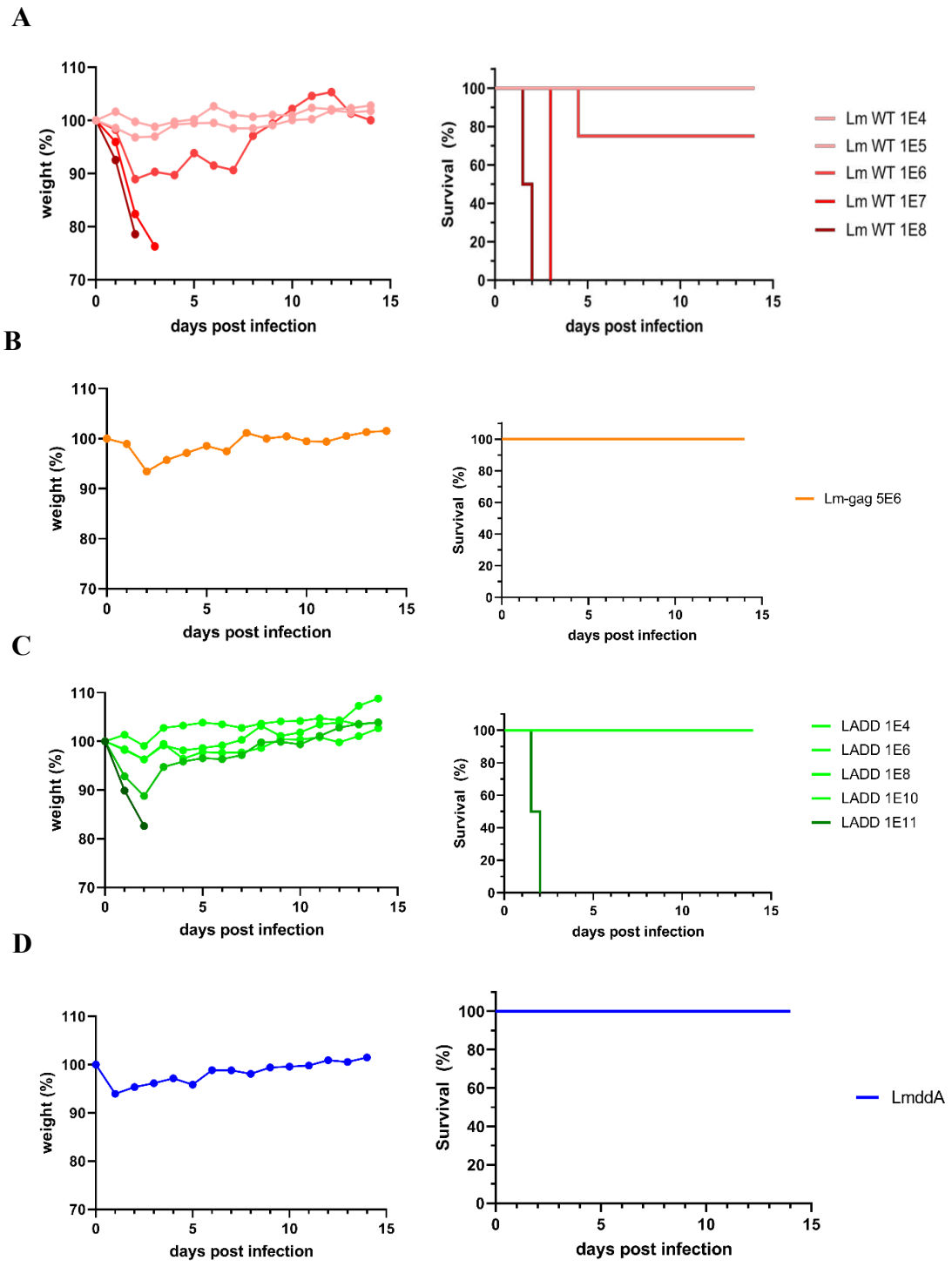

FIG 5 Morbidity and mortality for LD_{50} testing *in vivo*. For each Listeria strain there are the percent weight lost (left) and the percent survival of the group population results from the duration of 14-day LD_{50} testing in naïve mice. (A) Responses to Lm-WT strain for five tested CFU doses.

FIG 5 (Continued) (B) Responses to Lm-Gag strain for one tested CFU dose. (C) Responses to LADD strain for five tested CFU doses. (D) Responses to LmddA for one tested CFU dose.

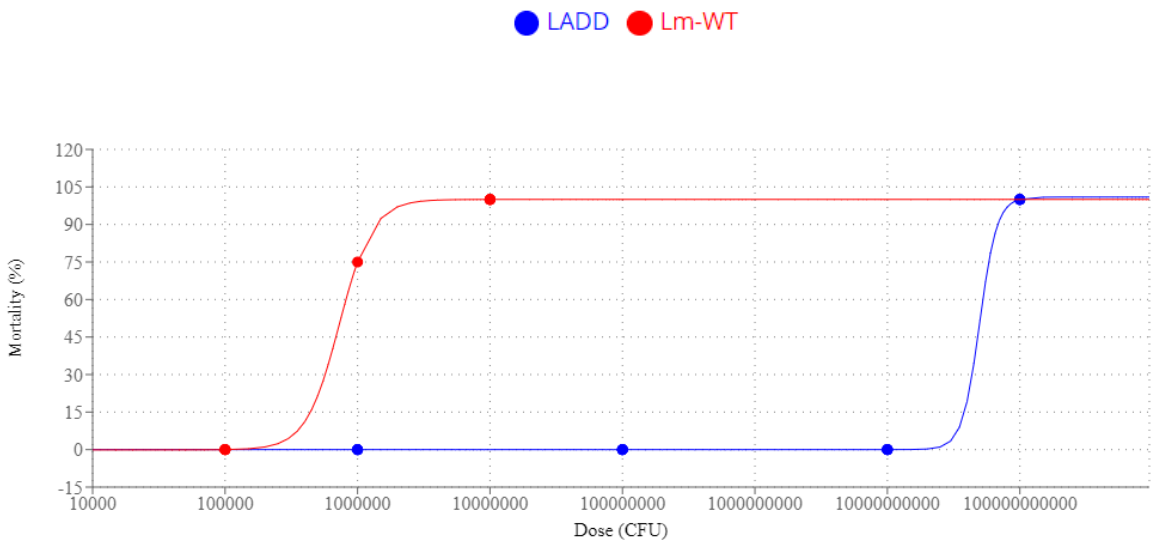

FIG 6 LD_{50} determination for Listeria strains. Dose-response curves, where the dose (x-value) versus the mortality % (y-value) yields an upward-slopping sigmoidal curve. Using this curve, the dose at the point where 50% mortality is observed represents the LD_{50} value. The LD_{50} value for the wild-type strain (Red) is determined to be 7.3E5 and for the LADD strain (blue) is 5E10.

Table 4 Comparison of LD_{50} doses. LD_{50} values (CFU/ 200 uL injection causing 50% of population to succumb to infection) were calculated for three Listeria strains; 7.5E5 CFU for Lm-WT, 5E6 CFU for Lm-Gag, and 5E10 for LADD.

Bacterial Strain	**Published LD_{50} values**	**Calculated LD_{50} values**
Lm-WT 10403s	1E4	7.3E5
Lm-Gag	5E6	N/A
LADD	1E10	5E10
LmddA	ND	ND

ND = LD_{50} values were not determined for the LmddA strain due to lack of pathogenicity

Confirmation of pGag

Here, the HIV-Gag gene was synthesized and then ligated into the pCI backbone by Azenta. The resulting construct is the DNA vector, pGag, a plasmid encoding the HIV-Gag gene (Fig. 2). To confirm the Gag gene had been inserted into the pCI backbone and in the correct direction, the pGag vector was digested with BglII which cuts along the pCI backbone and inside the Gag insert (Fig 7, 8). Additionally, to confirm the Gag gene was inserted in the correct directionality, PCR was performed targeting amplification of the Gag gene flanked by the EcoRI and XbaI restriction sites. Figure 9 shows a singular band at around 1.5 kb indicating positive amplification of the Gag gene. The size of the bands created by these digests or amplified regions during PCR were visualized through gel-electrophoresis, using the 1kb Plus DNA Ladder (NEB).

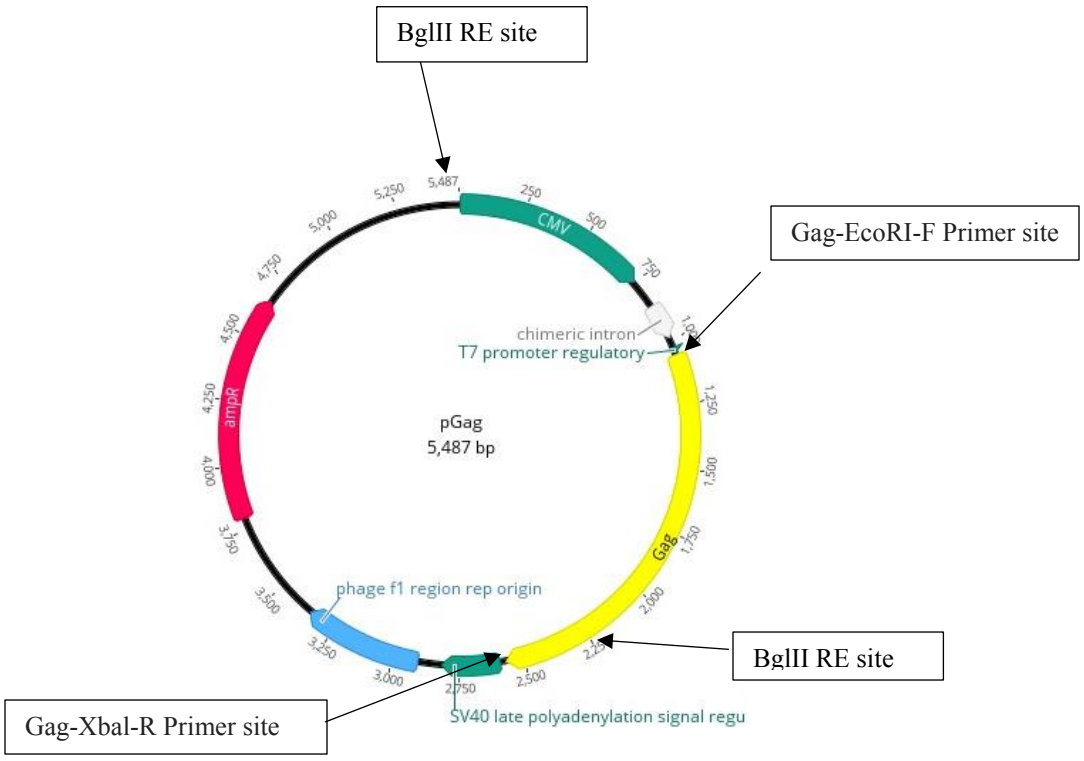

FIG 7 pGag DNA vector RE and amplification sites. Plasmid map shows the location of the BgIII cut sites and the primers used to amplify Gag region in the plasmid.

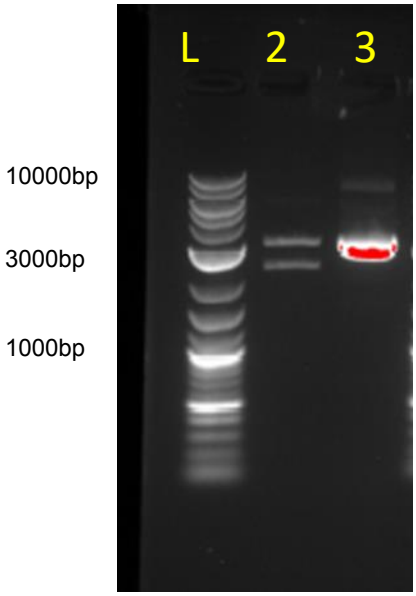

FIG 8 pGag confirmatory digest. Digested pGag plasmid with BglII, which cuts the backbone and Gag to confirm presence of Gag insert. Confirmation gel shows the ladder for band sizes (lane L) and digested plasmid with 2 bands (lane 2) to show the 2 fragments made by the cut. Lane 3 shows one band for the uncut plasmid.

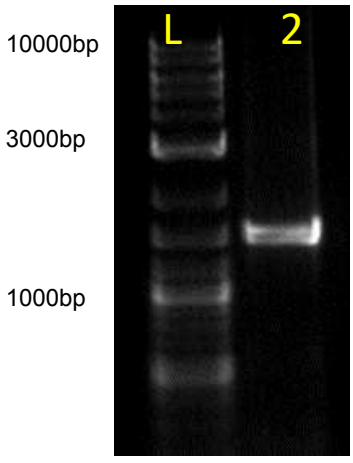

FIG 9 PCR confirmation of pGag construction. Confirms the insertion and correct orientation of the Gag insert. Lane 2 depicts PCR amplification of HIV-Gag gene insert in pGag DNA vector through singular band at 1500 bp with band size confirmed by the ladder for band sizes (lane L).

TABLE 5 Plasmid constructs

Plasmid	Backbone	Gene insertion	Restriction sites
pGag	pCI	Gag	EcoRI, Xbal
pKSV7-Gag	pKSV7	Gag	EcoRI, Xbal

Cloning of pKSV7-Gag Shuttle Vector

The HIV-Gag gene was cloned into the pKSV7 shuttle vector using sticky-end cloning. The pKSV7 plasmid was digested to allow for ligation of the Gag insert into the shuttle. Confirmation of the linearized pKSV7 plasmid was visualized following agarose gel electrophoresis (Fig. 10). Here, the uncut plasmid yields several large bands greater than 7 kb (lane 2) while the linearized plasmid shows one band at around 7 kb (Lane 3). The purified HIV-Gag gene was cloned into the shuttle to yield the plasmid construct pKSV7-Gag (Fig. 11, Table 5). After transformation with *E. coli*, positive clones were selected from growth on LB-Amp plates for pKSV7-Gag. Single colonies were then selected to isolate the Gag plasmid constructs. Colony PCR was performed and confirmed the insertion of the Gag into the shuttle backbone with primers (Table 3). Figure 12 shows the amplification of the Gag gene within positive clones containing pKSV7-Gag using forward and reverse primers to amplify the Gag sequence with bright bands present around 1500 bp. Once positive clones were selected, the colonies were miniprepped to isolate the plasmid constructs.

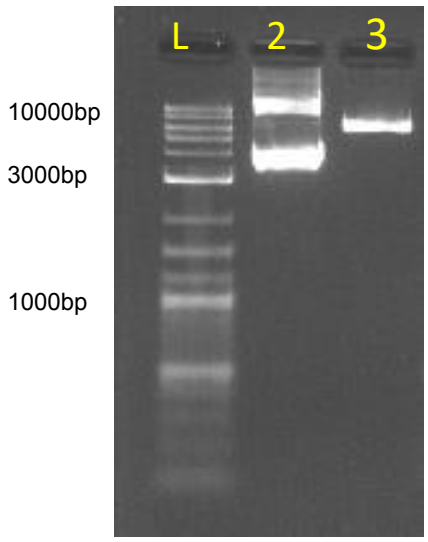

FIG 10 Linearization of pKSV7 vector. Gel image depicting the digest of plasmid pKSV7 with EcoRI and Xbal, with the ladder in lane L for band sizes. Lane 2 is the uncut plasmid and Lane 3 shows the linearized pKSV7 vector at 7071 bp. Confirmation of linearization needed for successional cloning steps.

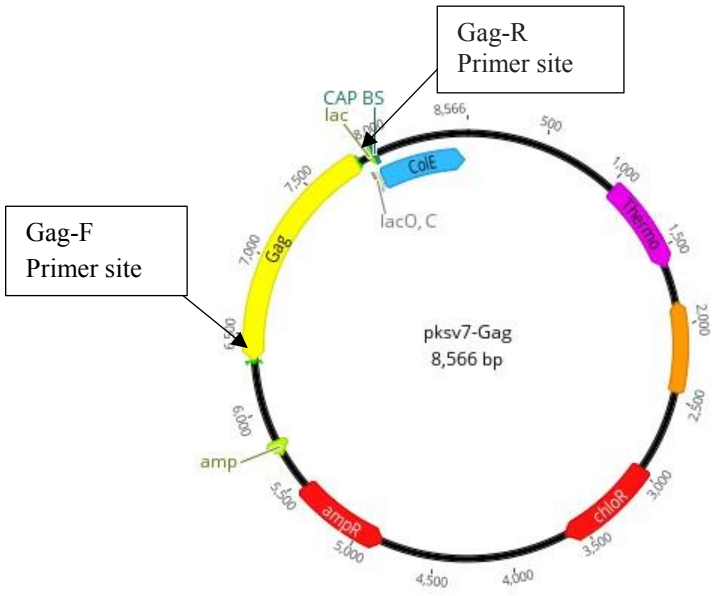

FIG 11 pKSV7-Gag plasmid map. Plasmid map shows the location and orientation after insertion of the HIV-Gag gene into the linearized pKSV7 backbone to generate the pKSV7-Gag shuttle vector. Amplified regions for PCR are also depicted.

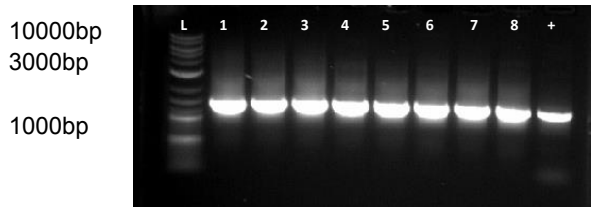

FIG 12 Amplification of Gag in Positive pKSV7-Gag clones. Bright bands at 1500 bp indicate amplification of Gag gene present in the constructed pKSV7-Gag plasmids. Lane 1 (L) is the ladder for band sizes, lanes 2-8 are colonies isolated to test for pKSV7-Gag, and lane 9 is a positive control for amplifying Gag using pGag.

Listeria Strains Expressing Gag

The plasmid construct pKSV7-Gag was introduced into the wild-type and LADD strains through electroporation. Passaging at the lower 30ºC temperature allowed for the Listeria to obtain the plasmid with active replication of the plasmid where the Gag gene is expressed episomally. The pKSV7-Gag plasmid contains genes for both ampicillin and chloramphenicol resistance. Once in Listeria, the plasmid allows the Listeria strains to also be chloramphenicol resistant. For both wild type and LADD strains, the growth on selective media plates with chloramphenicol indicated the presence of the pKSV7-Gag plasmid in the Listeria strains (Fig. 13). Additionally, as seen in Fig.13, the colonies re-streaked on the chromogenic agar also had growth. The colonies on these plates also contained a blue coloration, which is indicative of the colonies being *L. monocytogenes* species. These Listeria strains episomally express Gag through the pKSV7-Gag plasmid, and this expression of Gag will be analyzed in future studies through Western blot analysis.

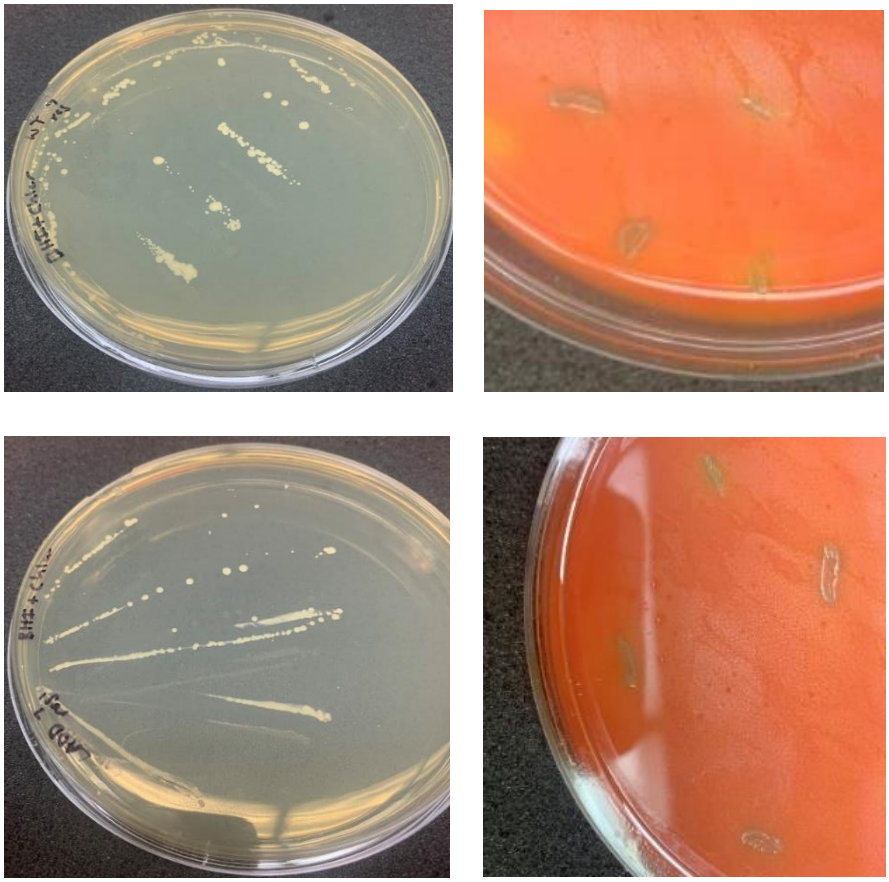

FIG 13 Growth of Listeria strains expressing Gag. The BHI plates (left) show growth on selective media for the wild-type Listeria (top) and LADD strain (bottom) transformed with pKSV7-Gag. The chromogenic agar (right) also shows that the colonies are *Listeria monocytogenes* species.

DISCUSSION

The improvement of vaccine delivery systems is necessary as vaccination is a key public health strategy for preventing infectious disease. The ideal vaccine candidate would elicit a strong immune response, not be reactogenic, be 100% efficacious, and easily produced. To date, this is difficult to achieve. These traits can be improved through alternative delivery strategies, including using bacteria to deliver vaccine antigens. Listeria's self-adjuvanting properties make it an ideal candidate for vaccine delivery. However, due to safety concerns since it is a pathogen, attenuated strains need to be evaluated for their ability to induce similar CTL responses to the wild-type strain. Here, the pathogenic potential of three attenuated Listeria strains were evaluated. From this data as the attenuation of the Listeria strain increases, the bacterial dose required for 50% of the population to fall to disease also increases. As such, using attenuated versus WT Listeria vaccine vectors increases safety. Using attenuated vectors is safer for laboratory development and vaccine administration, as the reduction in virulence genes also decreases the risk of harmful interactions with the Listeria. This data was also used to calculate the 0.2 LD_{50}, the dose that will be used for future feasibility testing of the vectors in naïve mice for functional CTL responses.

The purpose of this project was to generate tools for developing new vaccine models using the HIV-Gag gene. Here, the HIV III B Gag gene was selected as the model antigen for the DNA control vector, the shuttle vectors, and to be used with the Listeria strains. However, the vaccines developed in this project are not to provide protective immunity against HIV infection. HIV-Gag is useful as a model antigen to test functional CTL vaccine responses in mice as it contains a CTL epitope (AMQMLKETI) that can be targeted directly in immunoassays (ELISA/ELISpots). The DNA vector pGag is a plasmid expressing the Gag gene that will be used as a differential control for this vaccine model and to show that during helminth infection, the DNA vectored vaccines fail

to induce CTL immunity. The pCI backbone for this vector was selected based on the features required for proper expression of the HIV-Gag gene in bacterial and mammalian cells, as well as selection of clones. The pGag vector's ability to induce functional CTL responses, including during chronic schistosomiasis infection, will be directly compared to the attenuated Listeria strains expressing Gag. Plasmid construct pKSV7-Gag will be used in future projects as the shuttle vector to create the attenuated Listeria strains that express Gag. Construction of this shuttle vector with Gag was necessary as its origin of replication allows for plasmid replication inside the bacteria and contains a cassette that allows for integration of desired insert DNA into the Listeria chromosome.

CONCLUSIONS & FUTURE DIRECTIONS

Despite a large proportion of clinically licensed vaccines being DNA vectored vaccines, there are various immune conditions, including helminth infections, which can cause these types of vaccines to be ineffective. As a result, new delivery methods need to be explored to combat this issue. The vectors constructed in this project will be used in future projects that will aim to identify the adjuvant properties of Listeria that make it a suitable vaccine vector. The newly constructed pKSV7-Gag shuttle vector will be used to generate Listeria with Gag strains, both plasmid-expressed and chromosomal integrates. The thermosensitive region allows the use of temperature change to create different expression types. At the lower 30°C temperature, the Listeria will express the Gag through the shuttle plasmid itself. However, a temperature increase to 40-42°C, allows for homologous recombination for insertion of the Gag gene into the Listeria chromosome. Strains can then be tested as vaccine vectors for functional CTL responses during chronic schistosomiasis infection. The comparison of the episomal versus chromosomal expression of the Gag gene will aid in determining the component of Listeria responsible for adjuvanting the CTL response.

REFERENCES

1 Delany, I., Rappuoli, R. & Seib, K. L. Vaccines, reverse vaccinology, and bacterial pathogenesis. *Cold Spring Harb Perspect Med* **3**, a012476 (2013). https://doi.org:10.1101/cshperspect.a012476

2 Del Tordello, E., Rappuoli, R. & Delany, I. in *Human Vaccines* (eds Kayvon Modjarrad & Wayne C. Koff) 65-86 (Academic Press, 2017).

3 Kaufmann, S. H. The contribution of immunology to the rational design of novel antibacterial vaccines. *Nat Rev Microbiol* **5**, 491-504 (2007). https://doi.org:10.1038/nrmicro1688

4 Mora, M., Donati, C., Medini, D., Covacci, A. & Rappuoli, R. Microbial genomes and vaccine design: refinements to the classical reverse vaccinology approach. *Curr Opin Microbiol* **9**, 532-536 (2006). https://doi.org:10.1016/j.mib.2006.07.003

5 Singh, K. & Mehta, S. The clinical development process for a novel preventive vaccine: An overview. *J Postgrad Med* **62**, 4-11 (2016). https://doi.org:10.4103/0022-3859.173187

6 Lin, I. Y., Van, T. T. & Smooker, P. M. Live-Attenuated Bacterial Vectors: Tools for Vaccine and Therapeutic Agent Delivery. *Vaccines (Basel)* **3**, 940-972 (2015). https://doi.org:10.3390/vaccines3040940

7 Ulmer, J. B. *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745-1749 (1993).

8 Ferraro, B. *et al.* Clinical applications of DNA vaccines: current progress. *Clin Infect Dis* **53**, 296-302 (2011). https://doi.org:10.1093/cid/cir334

9 Pollard, A. J. & Bijker, E. M. A guide to vaccinology: from basic principles to new developments. *Nat Rev Immunol* **21**, 83-100 (2021). https://doi.org:10.1038/s41577-020-00479-7

10 Mata, M., Yao, Z. J., Zubair, A., Syres, K. & Paterson, Y. Evaluation of a recombinant *Listeria monocytogenes* expressing an HIV protein that protects mice against viral challenge. *Vaccine* **19**, 1435-1445 (2001). https://doi.org:10.1016/s0264-410x(00)00379-0

11 Molehin, A. J. Current Understanding of Immunity Against Schistosomiasis: Impact on Vaccine and Drug Development. *Res Rep Trop Med* **11**, 119-128 (2020). https://doi.org:10.2147/rrtm.S274518

12 Da'Dara, A. A. *et al.* Helminth infection suppresses T-cell immune response to HIV-DNA-based vaccine in mice. *Vaccine* **24**, 5211-5219 (2006).

13 Pearce, E. J. & MacDonald, A. S. The immunobiology of schistosomiasis. *Nat Rev Immunol* **2**, 499-511 (2002).

14 Colley, D. G., Bustinduy, A. L., Secor, W. E. & King, C. H. Human schistosomiasis. *Lancet* **383**, 2253-2264 (2014). https://doi.org:10.1016/s0140-6736(13)61949-2

15 Kaufmann, S. H. & McMichael, A. J. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat Med* **11**, S33-44 (2005). https://doi.org:10.1038/nm1221

16 Malhotra, I. *et al.* Helminth- and Bacillus Calmette-Guerin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *Journal of immunology (Baltimore, Md. : 1950)* **162**, 6843-6848 (1999).

17 Sabin, E. A., Araujo, M. I., Carvalho, E. M. & Pearce, E. J. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with *Schistosoma mansoni*. *J Infect Dis* **173**, 269-272 (1996).

18 Witte, C. E. *et al.* in *Advances in Immunology* Vol. Volume 113 (eds R. Unanue Emil & A. Carrero Javier) 135-156 (Academic Press, 2012).

19 Radoshevich, L. & Cossart, P. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nat Rev Microbiol* **16**, 32-46 (2018). https://doi.org:10.1038/nrmicro.2017.126

20 Chen, G. Y., Pensinger, D. A. & Sauer, J. D. *Listeria monocytogenes* cytosolic metabolism promotes replication, survival, and evasion of innate immunity. *Cell Microbiol* **19** (2017). https://doi.org:10.1111/cmi.12762

21 Shollenberger, L. M., Bui, C., Paterson, Y., Allen, K. & Harn, D. Successful vaccination of immune suppressed recipients using *Listeria* vector HIV-1 vaccines in helminth infected mice. *Vaccine* **31**, 2050-2056 (2013). https://doi.org:10.1016/j.vaccine.2013.02.037

22 Shollenberger, L. M., Bui, C. T., Paterson, Y., Nyhoff, L. & Harn, D. A. HIV-1 vaccine-specific responses induced by *Listeria* vector vaccines are maintained in mice subsequently infected with a model helminth parasite, *Schistosoma mansoni*. *Vaccine* **31**, 5651-5658 (2013). https://doi.org:10.1016/j.vaccine.2013.09.067

23 Bécavin, C. *et al.* Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. *mBio* **5**, e00969-00914 (2014). https://doi.org:10.1128/mBio.00969-14

24 Casadevall, A. The Pathogenic Potential of a Microbe. *mSphere* **2** (2017). https://doi.org:10.1128/mSphere.00015-17

25 Thompson, R. J., Bouwer, H. G., Portnoy, D. A. & Frankel, F. R. Pathogenicity and immunogenicity of a *Listeria monocytogenes* strain that requires D-alanine for growth. *Infect Immun* **66**, 3552-3561 (1998).

26 Peng, X., Treml, J. & Paterson, Y. Adjuvant properties of listeriolysin O protein in a DNA vaccination strategy. *Cancer Immunol Immunother* **56**, 797-806 (2007). https://doi.org:10.1007/s00262-006-0240-9

27 Brockstedt, D. G. *et al. Listeria*-based cancer vaccines that segregate immunogenicity from toxicity. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 13832-13837 (2004). https://doi.org:10.1073/pnas.0406035101

28 Gunn, G. R. *et al.* Two *Listeria monocytogenes* vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *Journal of immunology (Baltimore, Md. : 1950)* **167**, 6471-6479 (2001).

29 Chen, Z. *et al.* Episomal expression of truncated listeriolysin O in LmddA-LLO-E7 vaccine enhances antitumor efficacy by preferentially inducing expansions of CD4+FoxP3- and CD8+ T cells. *Cancer Immunol Res* **2**, 911-922 (2014). https://doi.org:10.1158/2326-6066.Cir-13-0197

30 Deng, W. *et al.* Recombinant *Listeria* promotes tumor rejection by CD8(+) T cell-dependent remodeling of the tumor microenvironment. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 8179-8184 (2018). https://doi.org:10.1073/pnas.1801910115

31 Wood, L. M. & Paterson, Y. Attenuated *Listeria monocytogenes*: a powerful and versatile vector for the future of tumor immunotherapy. *Front Cell Infect Microbiol* **4**, 51 (2014). https://doi.org:10.3389/fcimb.2014.00051

32 Verch, T., Pan, Z. K. & Paterson, Y. *Listeria monocytogenes*-based antibiotic resistance gene-free antigen delivery system applicable to other bacterial vectors and DNA vaccines. *Infect Immun* **72**, 6418-6425 (2004). https://doi.org:72/11/6418 [pii]
10.1128/IAI.72.11.6418-6425.2004

33 Ghosh, P., Zhou, Y., Richardson, Q. & Higgins, D. E. Characterization of the pathogenesis and immune response to *Listeria monocytogenes* strains isolated from a

sustained national outbreak. *Sci Rep* **9**, 19587 (2019). https://doi.org:10.1038/s41598-019-56028-3

34 Li, G. & Kathariou, S. An improved cloning vector for construction of gene replacements in *Listeria monocytogenes*. *Appl Environ Microbiol* **69**, 3020-3023 (2003). https://doi.org:10.1128/aem.69.5.3020-3023.2003

APPENDIX

Introduction

The work outlined in this appendix includes preliminary experiments that were required to complete the project discussed in this thesis and the plans I designed for the continuation of this project with other students (chromosomal integrations and LmddA strain with episomal expression of Gag). For this project, all protocols for growing bacterial strains were evaluated and standardized for use in our lab, with stocks made for each strain. Bacterial stocks were made for use for cloning, as well as vaccine stocks for use in the LD_{50} testing in mice. Additionally, antibiotic susceptibility was tested for all strains to identify antibiotic selection for cloning steps and for laboratory safety. For cloning steps, all shuttle backbones and other plasmid stocks were prepared prior to cloning using the QIAprep Spin Miniprep Kit (Qiagen). Protocols were also standardized for making competent cells (Listeria strains) and the parameters for transformation of these cells with plasmid DNA.

Antibiotic Susceptibility for Vector Strains

In order to be able to work with the bacterial strains for use as vectors and design the pGag DNA vector, the antibiotic susceptibility needed to be tested for all bacterial strains. Understanding the antibiotic susceptibility is additionally important to know which antibiotics can be used as treatments in case of an infection with one of the pathogens in the laboratory. For the Listeria 10403S strains, each strain was grown for 24 hours at 37ºC on BHI plates. Individual colonies were then picked to inoculate 3 mL of BHI broth containing antibiotics. Broth cultures were analyzed for their optical density after 24 hours and 48 hours of growth at 37ºC, using an

uninoculated broth as the control. The results for visible growth and measured OD are shown in table 6.

Table 6 Antibiotic Susceptibility Testing for WT Lm.

		24 hours		48 hours	
Antibiotic	**Concentration**	**Growth**	**OD**	**Growth**	**OD**
None	N/A	Yes	0.544	Yes	0.821
Ampicillin	100 μg/mL	None	0.0	None	0.0
Chloramphenicol	170 μg/mL	Not visible	0.039	Not visible	0.051
Erythromycin	20 μg/mL	Yes	0.309	Yes	0.429
Kanamycin	50 μg/mL	Not visible	0.040	Not visible	0.031
Streptomycin	50 μg/mL	Yes	0.580	Yes	0.612

LmddA Expressing Gag

To continue to investigate if fully virulent *L. monocytogenes* are necessary for the observed CTL vaccine responses, additional attenuated vectors are needed to be compared to the wild-type strain. Here, outlines the plans developed to generate LmddA-vectored vaccines expressing HIV-Gag, a triple-deletion *L. monocytogenes* strain. To develop this, the generation of a shuttle vector using the pAdv134 plasmid will be required. This plasmid for this project was received from Yvonne Patterson (UPenn) by way of Donald Harn (UGA). Plasmid pAdv134 is a bacterial shuttle system without any plasmid-controlled antibiotic resistance genes. The plasmid instead contains the Listeria *dal* gene which uses alanine supplementation for growth. Plasmid pAdv134 will be double-digested with Xhol and Xmal (New England BioLabs) restriction sites to linearize the plasmid backbone. The linearized pAdv134 backbone will be purified by 1% agarose gel electrophoresis and the plasmid DNA isolated through column purification (Qiagen QIAquick® Gel Extraction Kit). To isolate the HIV-Gag for cloning, pGag will also be double-digested using

XhoI and XmaI restriction sites. The HIV-Gag will be separated from the pGag backbone using 0.8% gel electrophoresis and column purified as above (Qiagen). To generate the shuttle expressing the Gag gene, HIV-Gag will be ligated into the linearized pAdv134 shuttle utilizing the sticky end sites. Ligation reactions (25 uL) including Instant-sticky end ligase/master mix (NEB), pAdv134 plasmid DNA and HIV-Gag insert DNA will incubate overnight at 16ºC in PCR tubes. Ligation reactions will be electroporated into electro-competent *E. coli* Mb2159 cells (D-alanine deficient for use with LmddA, UGA), followed by a one-hour recovery period at 37ºC with SOC media. Post-recovery, 200 uL of *E. coli* transformants will be plated on selective LB media with and without 100 μg/mL of D-alanine and incubated for 24 hours at 37ºC. For pAdv134, once the plasmid is present, alanine supplementation is no longer required for growth. Colonies from the selective plates will be used for colony PCR (described above) to confirm the ligations of plasmid pAdv134 with the Gag insert. Prepared pAdv134-Gag plasmid DNA will then be used for future cloning steps with the LmddA strain. Figure 14 shows the plasmid map and major features for pAdv134-Gag shuttle plasmid.

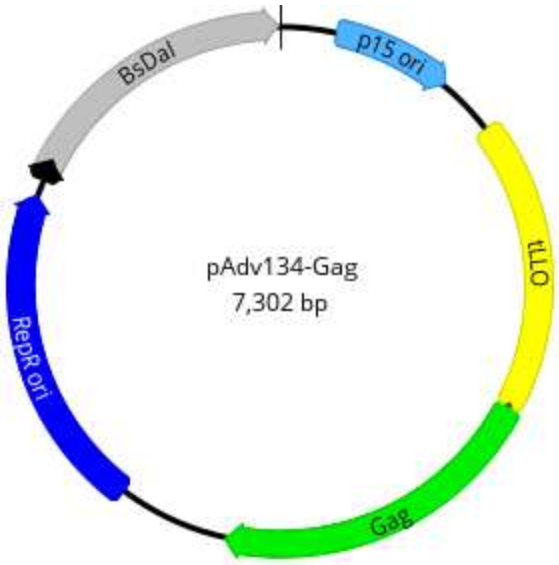

FIG 14 Plasmid Map for pAdv134-Gag. Plasmid map shows the location and orientation after insertion of the HIV-Gag gene into the linearized pAdv134 backbone to generate the pAdv134-Gag shuttle vector. This map also highlights the key features of the plasmid including the pAdv134 backbone with *dal* gene, p15 origin for replication in *E.coli,* RepR origin for replication Listeria*,* tLLO (truncated Listeriolysin), and the Gag insert.

Generation of Chromosomal Integrants

Once the shuttle plasmids with pAdv134 containing Gag have been developed, they will be transformed into the Listeria strains (Lm, LADD, LmddA) to generate the chromosomal integrants. For the Wild-type and LADD strains, Gag will be first cloned into the shuttle vector pKSV7. For the LmddA strain, Gag will be first cloned into the shuttle vector pAdv134 to generate pAdv134-Gag (described above). For pAdv134, once the plasmid is present, alanine supplementation is no longer required for growth. The prepared shuttle vectors with Gag inserts will then be transformed into their respective *Listeria* strains to allow for homologous recombination. Plasmid constructs will be electroporated into electrocompetent Lm, LADD and LmddA strains (1 mM cuvettes at 1kV) and then incubated in BHI-sucrose recovery media at 30°C for 2 hours. Transformants will then be plated onto selective (BHI-chlor 10µg/mL) plates and

incubated at 30°C for 2 days. Single colonies will be picked and streaked onto new (BHI-chlor 10μg/mL + streptomycin 50 μg/mL) plates and incubated overnight at 30°C. Colonies will then be used to inoculate 3 mL BHI-chlor (10 μg/mL) broth and passaged for 3 days with 1:1000 dilutions at 42°C with shaking. The passage at high temperature prohibits the temperature-sensitive pKSV7 plasmid from replicating and selects for plasmid integration into the Listeria chromosome.

VITA

Stephanie K. Norwood snorw001@odu.edu

Education

MS Biology, Microbiology and Immunology concentration, Expected December 2023
Biological Sciences Department, Old Dominion University, Norfolk, VA, 23529
BS Biology, Chemistry minor, May 2018
Virginia Wesleyan University, Virginia Beach, VA

Employment & Teaching Experience

Scientist, Eurofins Central Laboratory, March 2023 – present
- Development and validation of new methods within the clinical laboratory for various disciplines including ELISA, PCR, Flow Cytometry, PBMC isolation
- Assist in writing SOPs and training technical staff on validated methods
- Perform laboratory testing, QC procedures, and equipment maintenance using appropriate documentation

Graduate Teaching Assistant, ODU Biology, Jan 2020 – Dec 2022
- Instruct undergraduate laboratory classes of 15+ students
- Administer assignments and develop instructional materials
- Courses taught:
  - General Biology Lab I and II
  - Honors Biology Lab I and II
  - Introductory Microbiology Lab
  - Methods in Immunological Research Lab

Laboratory and Research Specialist, ODU Biology, August 2018 – Jan 2020
- Provide technical preparative assistance for academic and research laboratories
- Prepare culture media & chemical solutions to meet classroom needs
- Collect, treat, and dispose of regulated medical waste

Presentations

S. Norwood and L.M. Shollenberger. 2022, *Generation of Listeria Mutant Vaccine Vectors to Overcome Th1/Tc1 Type Vaccine Failure*. ASM Microbe. Washington DC **[Poster]**

S. Norwood and L.M. Shollenberger. 2022, *Lethal Dose (LD50) Determination in BALB/c Mice Using Mutant and Wild-type Listeria monocytogenes Vaccine Vectors*. Biological Graduate Student Organization (BGSO) Research Symposium. Norfolk, VA. **[Oral]**

S.K. Norwood, Z.J. Bement, L.M. Shollenberger. 2020, *Generation of clinically-relevant vaccine vectors for use in schistosome-endemic areas.* American Association of Immunologists (AAI) Annual meeting. Honolulu, HI **[Poster]**
Meeting canceled due to COVID19

Publications

S.K. Norwood, Z.J. Bement, L.M. Shollenberger. 2020, *Generation of clinically-relevant vaccine vectors for use in schistosome-endemic areas.* J Immunol. 204 (1 Suppl.) 167.15 (Abstr.)

Awards

Outstanding Graduate Teaching Assistant Award, ODU Biology, 2022
Runner-up, master's student talks, BGSO research symposium, 2022

Trainee Abstract Award, American Association of Immunologists (AAI), 2020