Development of vaccinia virus based reservoir-targeted vaccines

A dissertation

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ABSTRACT

One of the strategies to control vector-borne diseases is to eradicate or limit the causative agent from its zoonotic reservoir. We have developed reservoir-targeted oral vaccines against two diseases: plague and Lyme disease. We have used vaccinia virus (VV) as the vector for delivery of the vaccine antigens. The plague vaccine is a recombinant VV expressing F1-V fusion antigen of *Yersinia pestis*. A single dose of the vaccine generates a strong immunogenic response in mice and confers long-term protection against a lethal challenge of *Y. pestis*.

The Lyme vaccine is a recombinant VV expressing *Borrelia burgdorferi* OspA antigen and was previously shown to be effective in protecting C3H mice against *B. burgdorferi*-infected tick bite. Here, we have shown development of an effective bait formulation to deliver the vaccine in the environment. The stability of the vaccine in the bait formulation was tested under simulated environmental conditions.

We have shown that the VV-ospA baited vaccine is efficacious in protecting *Peromyscus* mice against a bite by an infected tick bite. Also, the vaccine is effective in decreasing acquisition of *B. burgdorferi* by uninfected larval ticks after feeding on infected mice.

Further, we have used a live, *in vivo* imaging technology to determine the localization and distribution of VV in mice after oral infection via gavage administration and bait feeding. Using the *in vivo* imaging technology, we have attempted to optimize the bait composition for increased uptake of the virus during feeding.

In summary, we have developed orally deliverable baited vaccines against two vector-borne pathogens with the aim of reducing infection in animal reservoirs that in turn will reduce the number of human cases. The vaccines may be an important part of an integrated management program to reduce the incidence of plague and Lyme disease.

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CHAPTER 1

INTRODUCTION

Reservoir-Targeted Vaccines for disease control

One of the strategies to control vector-borne diseases is to reduce or eradicate its etiological agent from the zoonotic reservoir. Such a strategy is particularly useful to control diseases caused by pathogens which are more prevalent in a zoonotic reservoir than in humans. While human vaccination is possible, it may not be cost-effective to vaccinate an entire population if only a small proportion is at risk of infection. Human vaccination also carries the risk of side-effects.

One potential strategy is to target reduction of the infected vector. An obvious approach is to reduce the number of infected members of the vector population that are critical in maintaining the infectious cycle of the pathogen. One of the well-known examples employing vector-reduction approach was the U.S. campaign to eradicate yellow fever and malaria during the construction of the Panama Canal [1]. In that case, plans were implemented towards elimination of mosquitoes by draining standing water pools, oiling of ponds and swamps to eradicate larvae, cutting bushes and grass and capturing indoor mosquitoes. This resulted in successful eradication of yellow fever and a substantial drop in the number of malaria cases. Similar strategies toward mosquito control were also applied in the southeastern United States, as part of a program of the Tennessee Valley Authority during the 1930s and 1940s [1]. It again led to a significant decrease in malaria cases in U.S.

Reservoir-targeted vaccine strategies involve immunizing the zoonotic reservoir of a pathogen in order to reduce or eliminate the infection from the reservoir so that disease transmission to human beings can be limited. Such an approach may also cause fewer

disturbances to the ecological balance than eradicating a species. Another advantage of a reservoir-targeted vaccine would be that such an immunization program can be implemented by the government and does not require participation of every individual of a community to undergo a specific regimen.

Previous work has shown that vaccination of wild reservoirs successfully eradicated rabies virus from endemic regions around the world [2, 3]. The rabies vaccine was a recombinant vaccinia virus expressing rabies virus glycoprotein (V-RG) [4]. The vaccine was used to immunize raccoons and foxes and was the first environmental release of a recombinant virus. The vaccine was aerially distributed in the form of oral baits, and almost 28 million vaccine baits were used in this period. Immunization of foxes with the vaccine in the Czech Republic led to complete eradication of rabies between 1989-2006 [5]. Use of rabies vaccine in Germany and Belgium was also highly successful and led to a drastic reduction in number of cases over the last decade [6, 7]. In Belgium, the reduction in the number of rabies cases was close to 100% after vaccination of raccoons and foxes, which started in 1990s.

A number of other vector-borne diseases are candidates to be targeted by similar strategies using reservoir-targeted vaccines. Such pathogens like Babesia, *Yersinia pestis*, *Borrelia burgdorferi*, Anaplasma/Ehrlichia, Hantavirus, etc. are widely prevalent in the wild and humans are incidental hosts in the infectious cycle of the disease. In this study, we have shown development of reservoir targeted vaccines against two such pathogens: *Y. pestis* and *B. burgdorferi*.

Vaccinia virus as vectors for vaccine delivery

Viruses and bacteria have been widely used as delivery vectors for a number of vaccines and therapeutics [8, 9]. A number of viruses have been used as delivery vector for different purposes. Commonly used viral vectors include Adenoviruses, Adeno-associated viruses, Alphaviruses, Herpesviruses and Poxviruses. The bacterial vectors include *Salmonella*, *Shigella*, BCG, *Listeria*, and *Lactobacillus*. The choice of vector depends on the type and target of vaccine, route of delivery and their mechanism of action.

Vaccinia virus (VV) is a cowpox virus that has been extensively used for expression of recombinant proteins [2, 3, 10, 11]. It was first used as a vaccine against human small pox infection, famously discovered by Edward Jenner in 1796. Over the years, VV has been used as a vector in a number of vaccine constructs developed for animals and human use against various pathogens including human immunodeficiency virus, influenza virus, rabies virus, Japanese encephalitis virus, Newcastle disease virus, *Plasmodium falciparum*, hepatitis B virus, as well as used as a delivery system for vaccination against cancer [3, 12-15]. The VV genome consists of a 190 Kb double stranded DNA, which replicates in the cytoplasm of host cells [11]. It encodes all the enzymes and proteins required for replication and transcription in a eukaryotic cell (Figure 1.1). Vaccinia gives high protein expression from stable inserts and also results in high titer antibody responses as it is known to trigger both humoral and cell-mediated immune responses [16]. In addition, the virus has a wide host range as it has been shown to deliver protective immune responses in a variety of animals from rodents to primates [12]. The safety aspects of vaccinia virus are well documented. recombinant VV

expressing rabies virus glycoprotein (V-RG) has already been released into the environment as oral bait for controlling rabies in wild animals [3, 7] and has been highly successful throughout Europe and the U.S. Although non-target animals also ingested vaccinia-laden baits and developed an antibody response, no toxicity was reported as a result of vaccinia uptake by any of the wild species [7]. In addition, laboratory testing to determine safety of vaccinia was carried out on 50 vertebrate species [7]. No lesions or illness was recorded in any of the species upon vaccinia intake regardless of dose, route or life history stage of immunocompetent hosts.

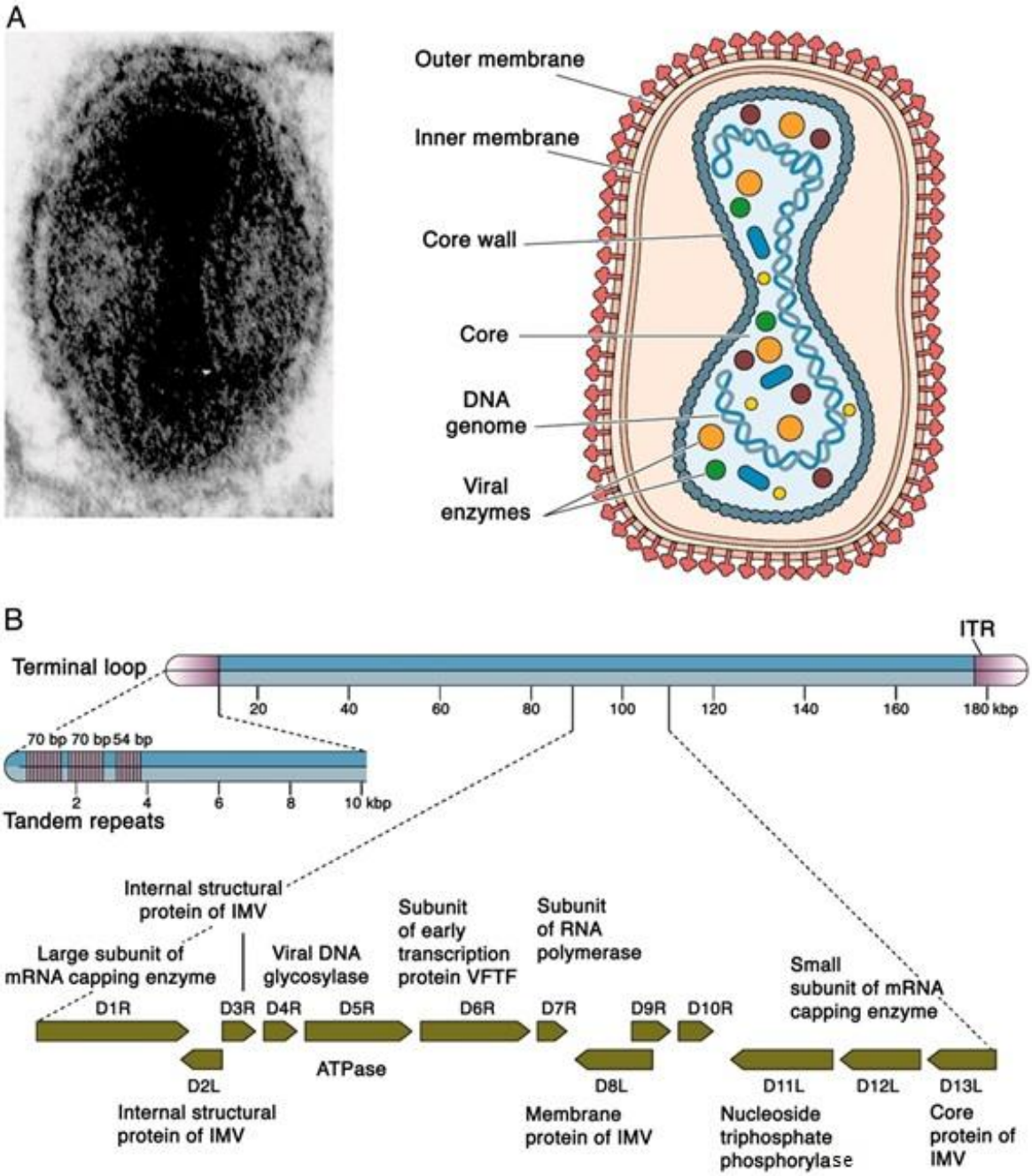


Figure 1.1: A representative image of virion structure (A) and genetic organization (B) of Poxviruses (reproduced with permission from Flint *et al.* 2004. *Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses*. ASM Press, Washington DC. 2nd Ed.).

Tissue tropism and persistence of VV in host

Orthopoxviruses disseminate through the lymphatic system and bloodstream of the host animal [17]. Although the virus can infect a wide range of hosts, it cannot be maintained in a latent state for a prolonged period of time. This is mainly due to a large genome size which leads to clearance of the virus by phagocytic cells and reticuloendothelial system of the host [18]. In addition, early modification of host plasma membrane with virus specific polypeptides triggers antibody-dependent cell-mediated cytotoxicity (ADCC) or cytotoxic T-lymphocyte (CTL) killing of infected cells.

Tissue tropism of VV varies among different strains and also on the route of inoculation. The difference in tropism is due to differences in genome sequence between different vaccinia strains. While the central part of DNA of vaccinia genome is highly conserved, there is variability in the terminal regions of DNA. Studies have suggested that the left hand and right hand terminal DNA regions of orthopoxvirus genome determine host range and virulence [19].

A number of studies have reported tropism and dissemination kinetics of different strains of VV infection via different routes of inoculation [17, 20-23]. Some strains of VV including a few attenuated strains have damaging effects when inoculated via certain specific routes. For example, VV-Copenhagen infection in inbred mice via intravenous or intraperitoneal route but not subcutaneous infection resulted in virus replication in ovaries leading to decreased fertility [21]. In another study, an attenuated thymidine kinase negative (TK^-) strain of vaccinia resulted in viral replication in murine ovarian tissue; however it was less pathogenic in mice, rabbits and chimpanzees [24, 25].

There is limited knowledge about dissemination of VV given via oral route. In a previous study, VV could be recovered for up to 48 h in the tonsils and retropharyngeal lymph nodes of raccoons and foxes fed with V-RG [26, 27]. However, no adverse clinical signs or gross histopathological lesions were observed [26, 27]. In athymic nude mice, parenteral administration of virus resulted in virus dissemination to liver, spleen and intestinal tissues, suggesting altered tropism of the virus in immunodeficient hosts [10, 28]. Oral administration of V-RG bait vaccine was shown to be non-detrimental to immuno-compromised mice as compared to parenteral administration, which resulted in systemic and progressive infection [10]. Intragastric inoculation of an attenuated vaccinia strain (MVA) resulted in limited infection in nasal associated lymphoid tissue (NALT) as observed using a luciferase reporter activity [23]. In another study, the vaccinia virus western reserve strain (VV-WR) carrying a luciferase reporter was showed to express reporter protein in gut associated lymphoid tissue (GALT) and spleen within 1 day post infection by oral gavage [22]. All these studies indicate progression of virus infection via lymphatic system of the host animal during initial stages after inoculation with subsequently clearance by the host immune response.

Most of the studies to determine the localization of vaccinia infection were carried out by direct titering of the virus in specific tissues or using a biochemical reporter assay. Luciferase reporter assays have been used in a number of studies; it has a high level of sensitivity (about 1000 times more sensitive than β-gal). A recombinant virus carrying a luciferase reporter is used for infection and luminescence produced as a result of luciferase activity is measured to determine the level of infection in specific organ or tissue type [29]. More recently, live animal bioimaging has been used to visualize the

localization of infection in an animal. A bioluminescence imager works on the same principle of luciferase reporter and transforms signals into visual images. Dissemination of VV-WR following intranasal inoculation in mice has been shown by live imaging technology [30]. Bioluminescence was measured to determine presence of virus in various organs in mice and luciferase activity correlated with the viral titers as determined in lung, brain, liver, and spleen [30].

***Yersinia pestis* and Plague**

Yersinia pestis is a gram-negative, facultatively anaerobic, cocco-bacillary, non-spore forming bacterium and is a member of the family *Enterobacteriaceae*. It is the etiological agent of the disease plague, which has claimed an estimated 200 million lives throughout history [31]. Plague has led to three massive pandemics in recorded history including the infamous 'Black Death' in the mid fourteenth century that claimed about one-third of the European population and had a huge impact on society's economy, art and culture that shaped the development of modern civilization [32, 33]. Over the past two decades, outbreaks of plague have been recorded in a number of countries including India, Peru, and several parts of Africa [33]. According to recent estimates, the worldwide number of cases of human plague are about 1000 – 5000 each year with around 100 – 200 annual deaths reported to the World Health Organization [33]. However, as a result of underreporting and poor diagnosis in many parts of the world, the actual number of cases may be much higher [33, 34]. In fact, due to an increase in the number of plague cases during the 1900s, it has been categorized as a re-emerging disease [33, 35, 36]. *Y. pestis* has also been identified as a potential threat to be used as a weapon for bioterrorism. This has led to a renewed search for strategies to prevent spread of the disease.

Infection with *Y. pestis* may give rise to three different manifestations of plague: bubonic plague (infection of lymph nodes), septicaemic plague (infection of blood with *Y. pestis* leading to sepsis), or pneumonic plague (occurring through direct inhalation of the pathogen from a diseased human or animal) [37, 38]. Bubonic plague is the most common form of plague which occurs following bacterial infection due to a flea bite or

exposure of open wounds to the pathogen. It is characterized by sudden onset of fever, chills, headache, weakness and swollen lymph nodes (called buboes) within 2-8 days of infection [33, 37-39]. Primary septicaemia is characterized by presence of bacteria in the blood stream leading to severe clinical complication including intravascular coagulation, peripheral gangrene and purpura [38, 40]. The mortality rate as a result of septicaemic plague may be as high as 30-50% because the antibiotics used to treat undifferentiated sepsis are not effective against *Y. pestis* [38, 41, 42]. Pneumonic plague is a highly contagious and lethal form of the disease which can be spread to individuals in close proximity via respiratory droplets. The incubation period for pneumonic form of plague is between 1-3 days and can be fatal unless treated within 1 day of onset [37, 38]. Administration of antibiotics, mainly streptomycin, tetracycline and sulfonamides, are the standard methods to treat plague. However, the challenge remains in rapid diagnosis of the disease especially in the underdeveloped parts of the world and if the disease is left untreated it may result in 30-100% mortality rate.

The transmission cycle of *Y. pestis*

Y. pestis is maintained in the wild by its transmission between different reservoir species by hematophagous adult fleas (Figure 1.2) [42, 43]. Rodents and certain lagomorphs form the main reservoir of plague pathogen [32, 43, 44]. *Y. pestis* can infect almost all mammalian species including Carnivora, Insectivora, Marsupialia, Artiodactyla and Primates, which are considered to become incidental hosts during the infection cycle [44].

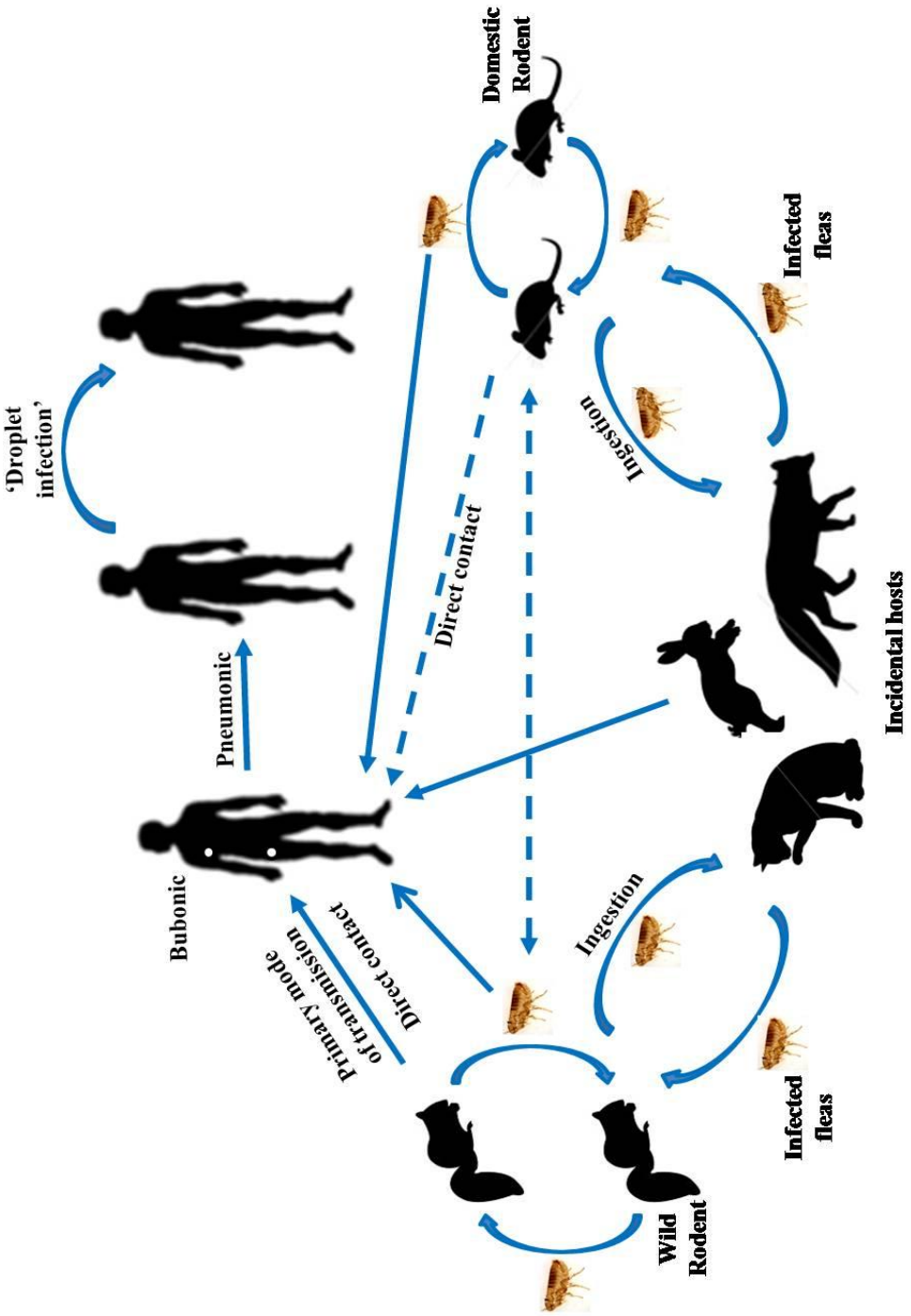


Figure 1.2: The transmission cycle of *Yersinia pestis* (adapted from Gage and Kosoy. 2005. *Annu. Rev. Entomol.* 50: 505-528). The solid lines indicate usual events during the transmission cycle of plague bacteria while the broken lines indicate rare events during the transmission cycle.

The susceptibility of these species to *Y. pestis* varies widely. Birds, reptiles and amphibians are believed to be resistant to *Y. pestis* infection.

Fleas acquire the pathogen from an infected blood meal and can ingest at least 300 organisms in a single intake [37, 45]. The bacteria multiply in the flea midgut and form clusters within a few days of an infected blood meal. The bacterial mass quickly causes blockage of proventricular and esophageal passage [46]. During subsequent feeding attempts by infected flea, the blood distends the esophagus, mixes with the pathogen and is regurgitated into the host during termination of feeding [46]. According to one estimate, regurgitation may lead to transmission of about 11000 – 24000 bacilli into the mammalian host [47, 48].

Plague cases in humans is a result of infection either by direct contact with infected animals [49, 50], eating infected animals like guinea pigs or camels [51-53], or from being bitten by infected fleas [32, 33]. At present, most of the human plague cases around the world have been classified as sylvatic plague, which are caused as a result of contact with rural wild animals.

Many parts of the world including a large part of Asia, Africa, and Americas have the plague pathogen prevalent in wild reservoirs and hence the threat of the deadly disease remains (Figure 1.3) [33]. In North America, the plague pathogen is prevalent in the wild in south-western United States and Mexico. The main zoonotic reservoirs of plague include squirrels, marmots, mice, rabbits, and gerbils [37, 54-57]. Prairie dogs are often infected with *Y. pestis* but are not a common cause of human plague since their fleas are not prone to feed on humans even in absence of their natural host [37].

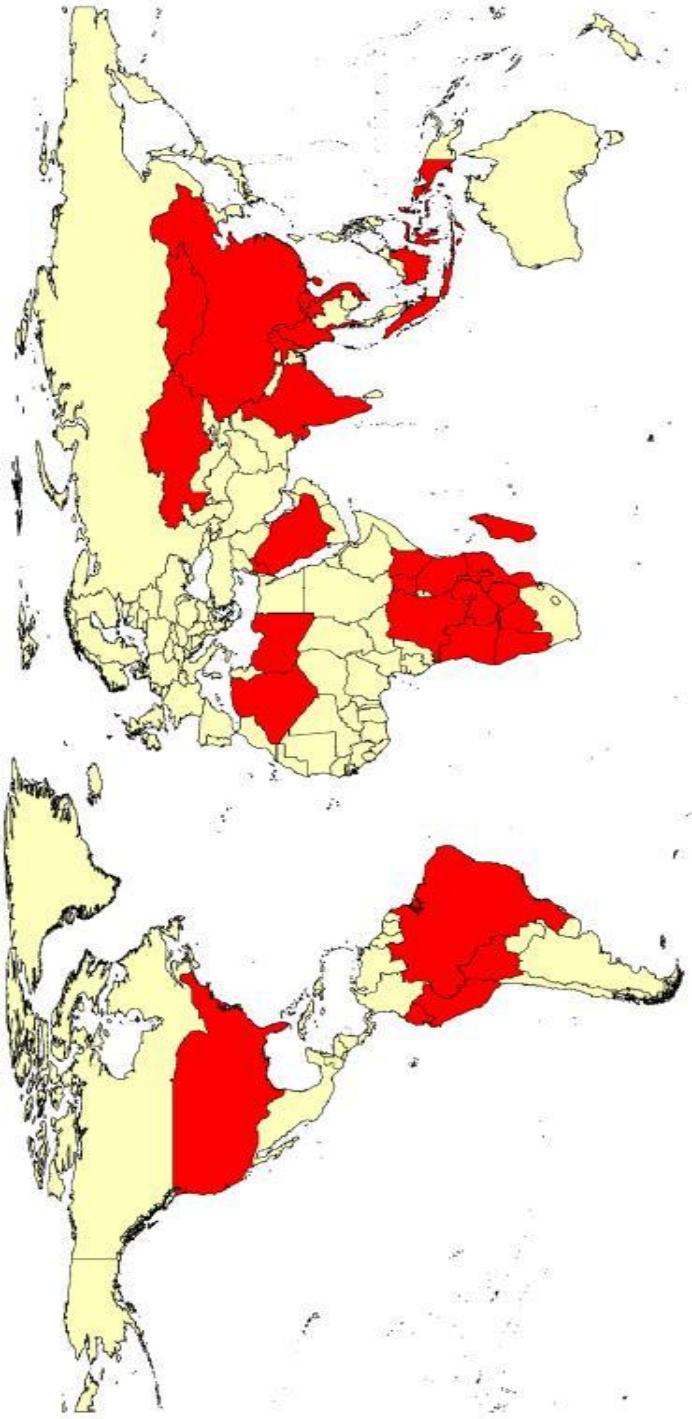


Figure 1.3: World map showing countries where presence of *Yersinia pestis* has been reported in wild reservoir species (from Stenseth *et al*. 2008. *PLoS Medicine*. 5: 9-13).

Vaccines against *Y. pestis*

Antibiotics are commonly used for treatment or prevention of *Y. pestis* infection. Although antibiotics are most successful against the bubonic form of plague, in case of septicaemic or pneumonic plague the disease develops rapidly post infection and treatment must start very quickly in order for antibiotics to be effective. In addition, there are reports of isolation of antibiotic resistant strains of *Y. pestis* [58] which further makes the use of antibiotics less certain.

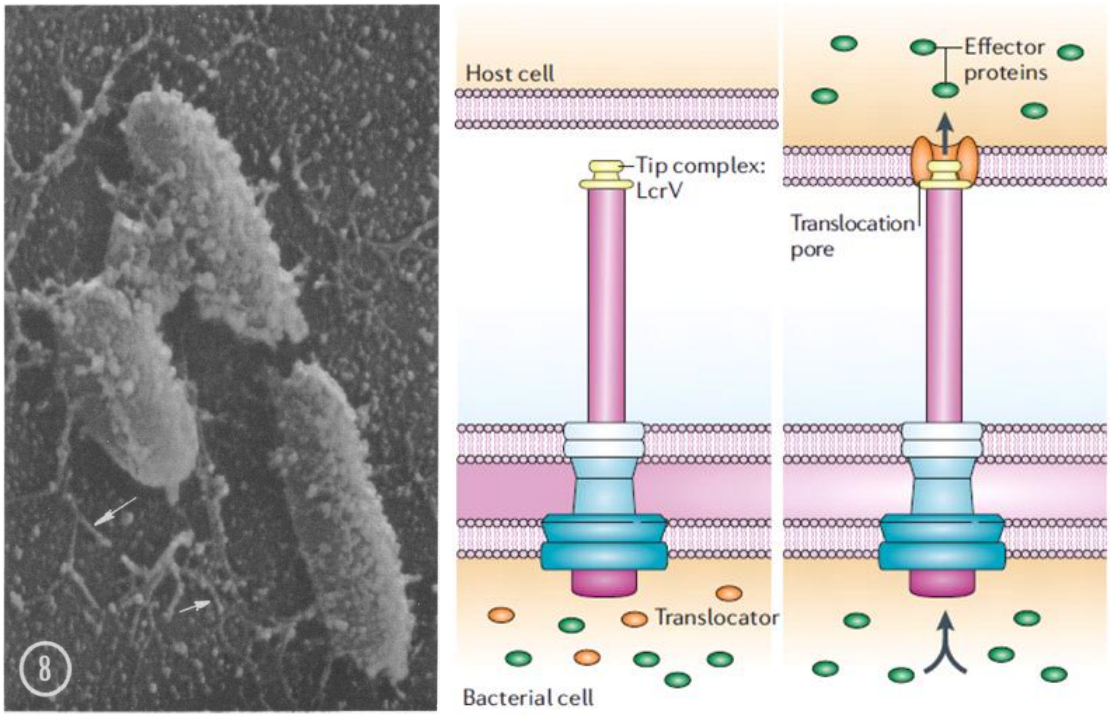
A number of vaccine strategies have been developed against plague over the past several decades. Killed whole cell vaccines in humans have been used since late 19th century and have been shown to protect against bubonic plague [59]. The vaccine was used on US servicemen stationed in Vietnam during 1961-1971 and was thought to be protective against plague in comparison to the incidence of the disease in unvaccinated Vietnamese people [60, 61]. However, the efficacy of this vaccine against pneumonic plague was less conclusive [61, 62]. Other vaccine candidates have included the use of a number of live-attenuated *Y. pestis* strains which have also been shown to protect humans and animals against virulent *Y. pestis* challenge [61]. Immunization with a live attenuated vaccine EV76, which lacked the pigmentation locus (Δ*pgm*) making it incapable of assimilating chromatophores, protected mice against virulent *Y. pestis* challenge [61, 63]. However, this strain was not fully attenuated for human use. In another study, an *aroA* mutant of *Y. pestis* was shown to be attenuated in guinea pigs but was found virulent in mice when administered subcutaneously [64]. The *aroA* gene encodes an enzyme that is essential towards biochemical synthesis of several bacterial compounds including aromatic amino

acids. Another attenuated strain of *Y. pestis* lacking the *dam* gene, which codes for DNA adenine methylase and has key roles in DNA replication and repair, protected mice upon vaccination against subsequent challenge with a sub-lethal dose of *Y. pestis* [65]. Immunization with mutants of *Yersinia pseudotuberculosis*, a less pathogenic variant in human beings, has been shown to protect mice against plague and may therefore provide new insights in plague vaccine development [66, 67].

In addition to live-attenuated vaccines, development of subunit vaccines that are based on various virulence factors of *Y. pestis* has also been attempted. A number of *Yersinia* outer effector proteins (Yops) including YopD, YopE, YopH, YopK, YopM and YopN as well as surface antigens including F1 and LPS have been tested for their immunogenic potential and vaccine efficacy [61, 68-70]. Although vaccination with many of these subunit antigens generated an antibody response only F1 (or caf1) and V-antigen (or LcrV) were found to be consistently protective in a mouse model and humans against a challenge [59, 71-78].

F1 and LcrV as vaccine candidates against *Y. pestis*

F1 and V are *Yersinia* virulence markers which are expressed after 1-4 hours of infection of macrophage cell line and start altering the host immune responses by 1-2 days post infection [37, 79]. F1 is a 15.5 Kda proteinaceous capsule protein which prevents engulfment of *Y. pestis* by macrophages and neutrophils presumably by obstructing receptor interaction involved in uptake of the pathogen (Figure 1.4A) [80].



A B

Figure 1.4: A. Scanning electron micrograph depicting presence of fraction 1 envelope antigen (or F1 capsular protein) (marked by arrows) on the surface of *Y. pestis* bacilli (reproduced with permission from Chen and Elberg. 1977. *Infect. Immun.* 15: 972-977).

B. Diagrammatic representation of *Y. pestis* type III secretion system (T3SS) (reproduced with permission from Cornelis. 2006. *Nature Rev. Microbiol.* 4: 811-825). LcrV forms the tip complex of the T3SS. The tip complex interacts with the host cell membrane, and helps form a translocation pore platform for secretion and translocation of effector proteins into the host cell.

LcrV is involved in formation of the tip component of the type III secretion system (TTSS) needle in *Yersinia* (Figure 1.4B) [81]. It is involved in secretion and translocation of effectors and delivery of Yops into eukaryotic cells [82, 83].

The protection conferred by F1 and V against plague has been shown to be mediated by induction of systemic humoral responses [84-87]. It was shown that passive immunization with anti F1-antibodies or anti-LcrV antibodies protected against challenge with *Y. pestis* [85-87]. One potential drawback of a single F1 or V protein vaccine is that their efficacy is questionable when challenged against a $F1^-$ or V variant strain of *Y. pestis* respectively [88]. It must be noted that naturally occurring F1 mutant strains of *Y. pestis* have been isolated, which may cause human disease similar to wild type strains [89, 90]. In addition, *Y. pestis* strains carrying variant LcrV-like proteins have been isolated in nature [91].

The immunogenicity of a combination vaccine can vary with differences in the ratio of the different antigens in a formulation. The individual immunogenicity against the each antigen may have an additive or inhibitory effect on the response against other antigens. Immunization with different combinations of F1 and V protein was previously reported to be more effective than a single F1 or V vaccine against *Y. pestis* challenge (Table 1.1) [92]. Also, a recombinant F1-V fusion protein has been shown to produce a strong antibody response in mice and protected against experimental pneumonic and bubonic models of plague [92]. In fact, the efficacy of F1-V fusion protein vaccine was greater as compared to F1 or V-antigen given alone or F1 + V antigen given together when challenged with lethal dose of virulent *Y. pestis* (Table 1.1A) [92]. In addition, F1-V

fusion protein protected against F1^{-} *Y. pestis* strain to the same extent as observed with V alone and was more effective than F1 + V combination. vaccine (Table 1.1B) [92].

In a recent study, subcutaneous injection of F1-V fusion protein has been shown to protect black-footed ferrets when challenged against *Y. pestis* [93]. All the vaccinated animals survived after feeding on *Y. pestis* infected mice as compared to control unvaccinated ferrets which succumbed to *Y. pestis* infection [93]. In addition, high antibody response in ferrets was observed as a result of booster doses, up to 2 years post vaccination [93]. In another study, a recombinant raccoonpox virus expressing the F1 antigen and a truncated V-antigen was used as baited vaccine to immunize prairie dogs. The vaccine conferred protection against lethal challenge of *Y. pestis* infected by intradermal inoculation [94]. Human immunization with F1-LcrV protein has also been reported and the vaccine in currently under clinical trials [77].

Table 1.1A

Treatment group	Strain	Challenge dose[b]	Survivors/total
Alhydrogel alone	$F1^+$ CO92	0.9×10^2	0/15
F1–V (35 μg)	$F1^+$ CO92	0.9×10^7	14/14
F1–V (35 μg)	$F1^+$ CO92	0.9×10^8	9/15
F1–V (35 μg)	$F1^+$ CO92	0.9×10^9	5/14
F1 (10 μg)	$F1^+$ CO92	0.9×10^2	14/15
F1 (10 μg)	$F1^+$ CO92	0.9×10^3	13/15
F1 (10 μg)	$F1^+$ CO92	0.9×10^4	3/15
V (25 μg)	$F1^+$ CO92	0.9×10^7	10/15
V (25 μg)	$F1^+$ CO92	0.9×10^8	5/15
V (25 μg)	$F1^+$ CO92	0.9×10^9	0/15
F1+V (10 μg+25 μg)	$F1^+$ CO92	0.9×10^7	12/15
F1+V (10 μg+25 μg)	$F1^+$ CO92	0.9×10^8	3/14
F1+V (10 μg+25 μg)	$F1^+$ CO92	0.9×10^9	5/15

[a]Animals were immunized on day 0 and challenged on day 42 as indicated in Section 2.
[b]Number of LD_{50} administered.

Table 1.1B

Treatment group	Strain	Challenge dose[b]	Survivors/total
Alhydrogel alone	$F1^-$ C12	1×10^7	0/15
F1–V (35 μg)	$F1^-$ C12	1×10^7	8/15
F1–V (35 μg)	$F1^-$ C12	1×10^8	5/15
F1–V (35 μg)	$F1^-$ C12	1×10^9	0/15
V (25 μg)	$F1^-$ C12	1×10^7	8/15
V (25 μg)	$F1^-$ C12	1×10^8	2/15
V (25 μg)	$F1^-$ C12	1×10^9	0/15
F1+V (10 μg+25μg)	$F1^-$ C12	1×10^7	5/15
F1+V (10 μg+25μg)	$F1^-$ C12	1×10^8	3/15
F1+V (10 μg+25μg)	$F1^-$ C12	1×10^9	1/15

[a]Animals were immunized on day 0 and challenged on day 43 as indicated in Section 2.
[b]Number of LD_{50} administered.

Table 1.1: Protective efficacy of various combinations of F1 and LcrV protein is shown (reproduced with permission from Heath *et al.* 1998. *Vaccine*. 16: 1131-1137). A. Relative efficacy of different proteins against a lethal subcutaneous challenge with $F1^+$ *Y. pestis*. B. Relative efficacy of different combinations of F1 and V against a lethal subcutaneous challenge with $F1^-$ *Y. pestis* is shown.

***Borrelia burgdorferi* and Lyme disease**

Borrelia burgdorferi is a gram-negative bacterium that belongs to the class of *Spirochaetaceae*. It is the etiological agent of Lyme disease, which is the most common vector-borne disease in United States with approximately 30,000 confirmed cases of human Lyme reported in 2009 (Center for Disease Control, Division of Vector-Borne Diseases). The disease is transmitted to humans via bite of infected *Ixodes scapularis* ticks. During early infection, Lyme disease is characterized by a transient localized skin rash called erythema migrans (Figure 1.5) [95]. During the early stage of disease, the symptoms in infected individuals may include fever, chills, fatigue, headache, and body and joint aches [95]. If the disease remains untreated, the spirochetes disseminate to distal tissues and organs such as joints, heart and nervous system (Figure 1.5) [95]. Interaction of the bacteria with the host at these sites results in various clinical manifestations of the disease including arthritis, carditis, or meningitis [95].

Bacteria belonging to the class of *Spirochaetaceae* have a characteristic helical morphology with its inner and outer membranes surrounding a periplasmic flagella and a flexible cell wall (Figure 1.6) [95-98]. The flagellar bundles arise from either ends of the cell, winds around the flexible cellular protoplasmic cylinder of *B. burgdorferi* overlapping in the center, and rotate in opposite directions [96, 99, 100]. This gives the bacterium its typical corkscrew-like motility pattern [99], which facilitates penetration into mammalian tissues and establishment of infection [100]. *B. burgdorferi* also has a unique genetic make-up with a highly segmented genome composed of a small linear chromosome (~900 Kb) and more than 20 linear and circular plasmids [101, 102].

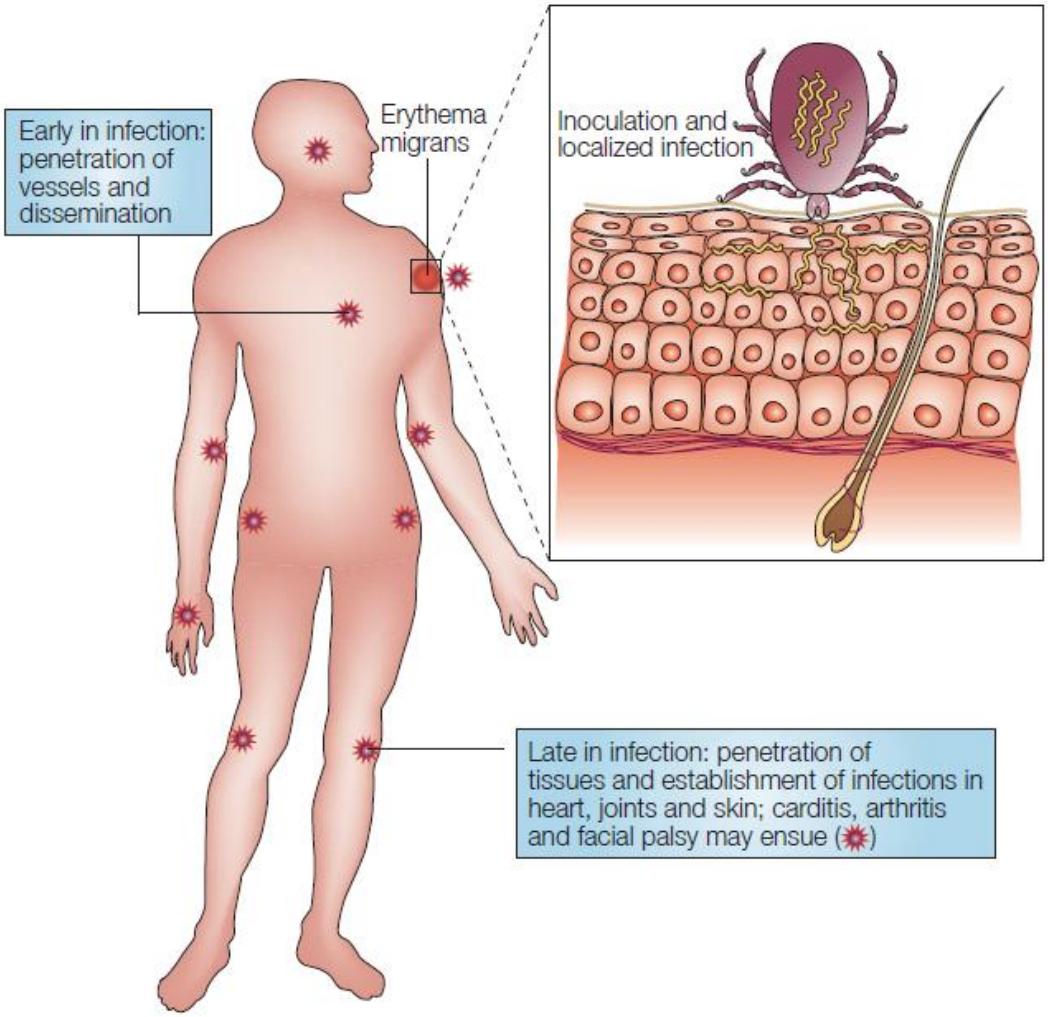


Figure 1.5: Different stages of Lyme disease (reproduced with permission from Rosa *et al.* 2005. *Nature Rev. Microbiol.* 3: 129-143). *B. burgdorferi* is introduced into humans upon bite by an infected *Ixodes scapularis* tick. During the early phase, localized infection leads to Erythema migrans. If the disease is left untreated, the bacteria disseminate and establish infection in distal tissues like hearts, joints and nervous system.

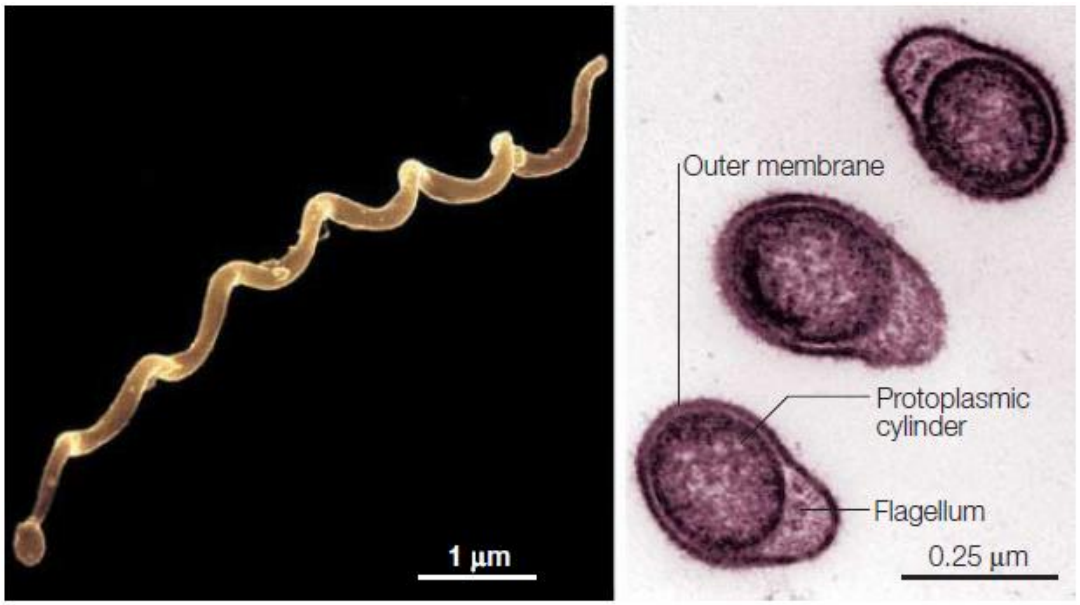


Figure 1.6: Scanning (left) and transmission (right) electron micrographs of *B. burgdorferi* (reproduced with permission from Rosa *et al.* 2005. *Nature Rev. Microbiol.* 3: 129-143).

The transmission cycle of *B. burgdorferi*

B. burgdorferi is an obligate pathogen that can infect a wide range of vertebrate mammals (Figure 1.7) [103-106]. *Ixodes scapularis* ticks are the main vector of the Lyme pathogen. The ticks have three life cycle stages: larvae, nymphs, and adults. Engorged adult females lay eggs which hatch into larvae. Uninfected tick larvae acquire the bacteria by feeding on infected small rodents like white-footed mouse (*Peromyscus* sp) [103, 107]. The infected larvae molt into nymphs which transmit the bacteria to new hosts during their subsequent feeding thus increasing number of infected hosts for perpetuation of infection [108]. Once the nymphs molt into adults, they feed primarily on large mammals that are not part of the *B. burgdorferi* endemic cycle [108]. Humans are incidentally infected by *B. burgdorferi* as a result of bites by infected nymph and adult ticks.

Role of OspA in *B. burgdorferi* transmission

In order to survive the tick-mammalian host cycle, *B. burgdorferi* has to adapt to varied environments of temperature and pH during its transmission cycle. They achieve this by regulating their gene expression pattern including a number of lipoproteins and non-lipidated outer membrane proteins (OMPs). One such protein, OspA (Outer Surface Protein A) is upregulated inside the tick host and helps the bacteria to persist in the tick between blood meals [109]. OspA binds to receptor of OspA in the tick midgut, TROSPA [110]. When infected ticks feed on a blood meal, the incoming blood triggers a change in spirochete gene expression pattern. During this time, the spirochete population multiplies [111-113], the expression of OspA is downregulated [109, 114] while they migrate

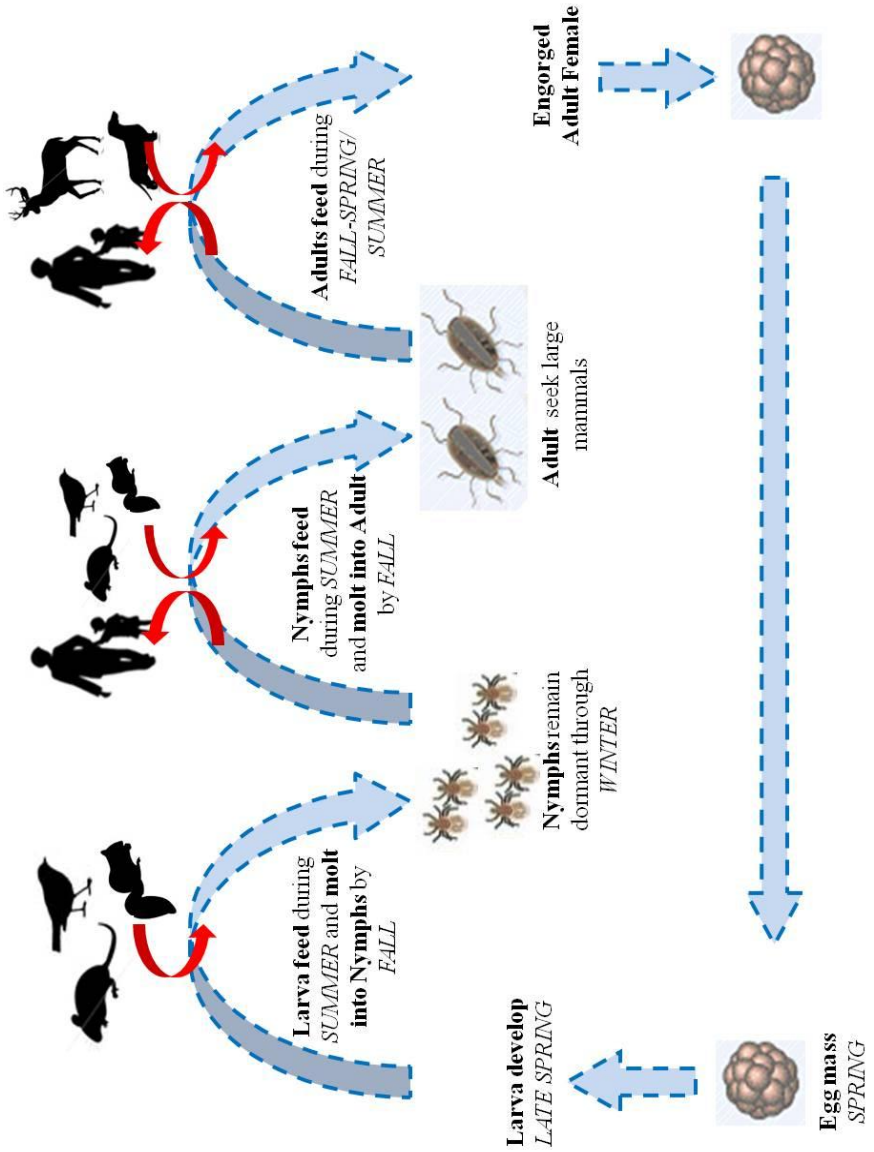


Figure 1.7: Diagrammatic representation of infectious cycle of *B. burgdorferi* (Adapted from Sood (Ed.). 2011. Lyme Borreliosis in Europe and North America – Epidemiology and Clinical Practice. *Wiley-Blackwell.* New Jersey). Blue arrows indicate the natural lifecycle of *Ixodes* ticks, the vector of Lyme spirochete, during which they feed on a variety of vertebrate mammals. During their feeding cycle, the ticks acquire or transmit *B. burgdorferi* (direction of bacterial transmission is depicted by red arrows).

towards salivary glands and are subsequently transmitted to the mammalian host. In the tick salivary glands, *B. burgdorferi* OspC protein is upregulated which plays a role in bacterial survival from the initial innate immune response of the mammals [115-117].

OspA as a vaccine candidate against Lyme disease

Previous studies have shown that immunization with recombinant OspA protects mice from *B. burgdorferi* infection during tick bite [118, 119]. In OspA vaccinated animals, anti-OspA antibody is taken up by infected ticks during feeding which kills the spirochete inside the ticks and thus prevents bacterial transmission to OspA-vaccinated hosts [120-123]. OspA vaccination of mice after establishment of infection does not clear the infection from mice [124]. This observation is not surprising since OspA is not expressed in a mammalian host, so anti-OspA antibody in mouse serum would not be effective in clearance of the infection. An OspA based human vaccine for Lyme disease was also previously approved by the United States Food and Drug Administration. The vaccine was a purified OspA protein adsorbed to aluminum hydroxide adjuvant in phosphate buffer saline (PBS) [125]. The vaccine was first released in 1998 and conferred protection to vaccinated individuals against *B. burgdorferi* infection. The vaccine was later removed from market by the manufacturer due to low sales. Contributing to the low sales were the incomplete protection, the need for frequent booster vaccinations and a concern, that was subsequently shown to be unfounded, that antibodies to OspA may be involved in the development of autoimmune arthritis.

Strategies for control of Lyme disease

A number of different strategies have been carried out towards controlling the spread of *B. burgdorferi* infection to limit Lyme disease. While most of these strategies aim to reduce the densities of tick vector from an endemic region and have shown initial promise, none of them have been totally successful in eradicating the disease. In one of the approaches, the population of white-tailed deer was reduced in Monhegan island in Maine with the aim to reduce spirochete infection [126]. Although deer are not important reservoir for *B. burgdorferi*, they are important hosts for adult ticks. Reduction in deer population led to reduction of ticks and hence subsequent reduction of *B. burgdorferi* carriage in ticks [126]. However, such practices of host eradication were effective only in isolated circumstances; other studies of deer reduction have not shown the same result. Other approaches have included spraying acaricides on vegetation or application of acaricides directly on tick hosts such as mice and deer with the aim to reduce tick numbers [127].

Since all these strategies have their own limitations, an effective plan to control Lyme disease would be implementation of an integrated management combining several strategies [127, 128]. Reservoir vaccination can be an important component of the integrated management protocol towards controlling Lyme disease. Tsao *et al*. have previously shown that vaccination of wild *Peromyscus* with an OspA based vaccine administered by injection can decrease the carriage rate of *B. burgdorferi* in ticks the following season, providing support for the concept of a reservoir targeted vaccine for Lyme disease [129, 130]. Practical application of a reservoir-targeted vaccine for Lyme

requires the development of an oral delivery system. An attenuated strain of *Salmonella enterica* serovar Typhimurium expressing OspA was used to vaccinate mice by oral gavage [131]. The vaccinated mice were shown to be protected against spirochete challenge. Similar studies with other bacterial vectors expressing OspA have also been shown to be efficacious in mice against spirochete infection [132, 133]. It has previously been shown that use of VV expressing OspA can be effective as a vaccine to target the reservoir [134]. A single dose of 10^8 pfu VV-ospA administered by oral gavage was immunogenic in inbred C3H mice. The vaccine conferred 100% protection to vaccinated mice against *B. burgdorferi* infection.

Vaccine distribution in oral baits

Designing appropriate bait is essential towards successful implementation of immunization of different zoonotic species and must be specific for the target animal. Some of the important considerations towards developing a suitable bait for the Raboral (V-RG) vaccine delivery were: (1) the bait must be attractive to the target species, (2) it must be effective in vaccine delivery and generating an immune response, (3) vaccine-laden bait should be stable under different environmental conditions like variation of temperature and humidity, (4) the bait preparation must be cost-effective and able to be produced in large quantity, and (5) distribution can be performed easily. In addition, it is important that the target animals will discover the baits in the wild and ingest them. Thus, identifying suitable bait attractants to maximize bait discovery by vector species is essential.

Understanding the behavior of target animal species is essential for design of a suitable bait. The currently used Raboral V-RG bait is a cube shaped polymer (2.0 cm × 3.5 cm × 3.5 cm) made of fish meal and is made to target raccoons and coyotes [135]. The vaccine is sealed in a small sachet and placed inside the bait capsule. The bait with vaccine weighs approximately 26 g and can be swallowed by larger mammals.

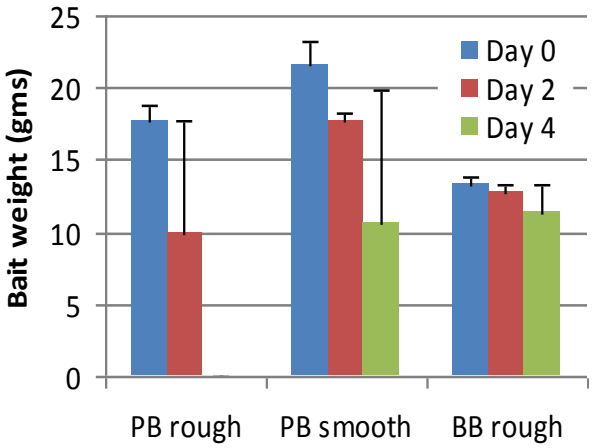
Studies were carried out to identify suitable flavors for mouse bait [136]. To determine palatability to mice, two different flavorings (peanut butter and blueberry) and two different textures (rough and brittle versus smooth and rubbery) were tested in caged wild mice (Figure 1.8). The caged mice were provided with blocks of bait in the presence of standard rodent chow and water. The mice were allowed to eat *ad libitum* and baits were

weighed at different time points to determine uptake. It was found that the rough formulation with peanut butter flavoring was eaten in great preference to rodent chow, the smooth peanut butter bait or either of the blueberry preparations (Figure 1.8A). The observations were further confirmed with wild mice in open environment.

In addition to bait composition, determining the optimum distribution frequency and mode of distribution is imperative to maximize bait discovery by the target animals. The Raboral V-RG baits were dropped from airplane flying at a specific speed at an optimum altitude to distribute the required number of baits per unit area. Each specification of bait distribution was tested during field trials to maximize the effect [135].

To test vaccination of small rodents in the wild, mouse nest boxes (25 cm x 20 cm x 20 cm) with a 2.5cm diameter entry hole were used [136]. A spacer was placed adjacent to the entry so that the box could be cable tied to a tree with the entrance facing the tree. Sample baits containing a rhodamine tracking dye were placed inside the nest box. The dye was used to identify mice which had taken up the bait for statistical analysis. The method can be used to optimize distribution of mice nests and number of baits required to vaccinate target animals in a unit geographical area.

A.



B.

C.

Figure 1.8: A. Determination of bait palatability for mice (Telford *et al.* 2011. Tick and Tick-borne diseases. In press) - Blocks of bait of two different flavorings (peanut butter and blueberry) and two different textures (rough and brittle vs. smooth and rubbery) were provided to caged wild mice in the presence of standard rodent chow and water. The baits were weighed at day 0, 2 and 4 to determine uptake. The rough formulation with peanut butter flavoring was preferred by caged mice. Error bars represent standard deviation of three independent experiments. B. A caged wild mouse with bait. C. Mouse nest boxes in use

Goals of the thesis

The goal of this thesis was to develop a suitable vaccination strategy to target the animal reservoir of two human pathogens: *Y. pestis*, the causative agent of plague, and *B. burgdorferi*, the causative agent of Lyme disease. Vaccinia virus (VV) has been characterized as the vector for delivery of the two vaccines.

The first aim was to construct the recombinant VV-based *Y. pestis* vaccine, characterize its protein expression and determine the immune response kinetics in an animal model. All these steps were described with the plague vaccine, which is a recombinant VV expressing F1-V fusion protein. Further, the protective efficacy of the vaccine was determined against a lethal challenge of *Y. pestis*.

The second aim was to determine the protective efficacy of a VV-based vaccine when delivered as oral baits. The suitability of a bait formulation was tested by determining the stability of the VV vaccines in baits and the protective efficacy conferred to reservoir animals by bait-delivered vaccines. We have described the efficacy of the baited Lyme vaccine in *Peromyscus leucopus* that is a natural reservoir of *B. burgdorferi*.

The third aim was to investigate the dissemination kinetics of VV in mice after oral infection. The differences in infection kinetics of VV delivered via gavage route vs. bait feeding were evaluated. In addition, I aimed to improve the composition of the oral bait for enhanced uptake of vaccinia virus to generate increased antigenicity.

CHAPTER 2

CONSTRUCTION AND EFFICACY TESTING OF A RECOMBINANT VV-BASED VACCINE AGAINST *YERSINIA PESTIS*

Note: This chapter has been published

Bhattacharya D, Mecsas J, Hu, LT. 2010. Development of vaccinia virus based reservoir-targeted vaccine against *Yersinia pestis. Vaccine*. 28: 7638.

Chapter 2 Abstract

Yersinia pestis, the causative organism of plague, is an endemic zoonotic pathogen with a worldwide distribution. Although the last plague epidemic occurred in early 1900s, human cases continue to occur. Most of the human infections of plague occur due to contact with infected wild animals. We have developed a vaccinia virus based vaccine against *Y. pestis* to target the zoonotic reservoir of plague pathogen. The vaccine is aimed to interrupt the transmission of disease from wild animals as a potential strategy for decreasing human disease.

A vaccinia virus vector was used to express the F1 capsular protein and the LcrV type III secretion component of *Y. pestis* as a fusion protein. Here we show that a single dose of this vaccine administered orally, generates a dose-dependent antibody response in mice. Antibody titers peak by 3 weeks after administration and remain elevated for a minimum of 45 weeks.

Protective efficacy of VV-F1-V vaccine was determined against *Y. pestis*. Vaccination provided up to 100% protection against intranasal challenge with 10 times the lethal dose of *Y. pestis*. Also, the protection lasted a minimum of 45 weeks post-vaccination. An orally available reservoir-targeted vaccine against *Y. pestis* may be a suitable strategy for the prevention of enzootic plague.

Chapter 2 Results

Expression of recombinant F1-Vprotein

We used VV-vRB12 for the construction of the recombinant virus. The vRB12 strain has a deletion in the *vp37* gene, which encodes a 37-KDa outer envelope protein that is essential for virus packaging, egress, and plaque formation [137]. The recombinant virus gains a copy of the *vp37* gene during crossing over with pRB21-tPA-F1-V, thus permitting selection of recombinants by plaque formation (Figure 2.1). A TPA signal sequence was added to the 5'-end of the vaccine construct to target the recombinant protein for export and improve antibody production [138].

Four rounds of neutral red plaque assay were carried out to select and purify recombinants. After construction and selection, confirmation that the selected viral clone contained the insert was performed by PCR (Figure 2.2A). PCR was also performed to determine purity of the final virus using primers specific for the irrelevant *gpt* insert that is lost during recombination (Figure 2.2B). Expression of the F1-V fusion protein was determined by western blotting (Figure 2.2C).

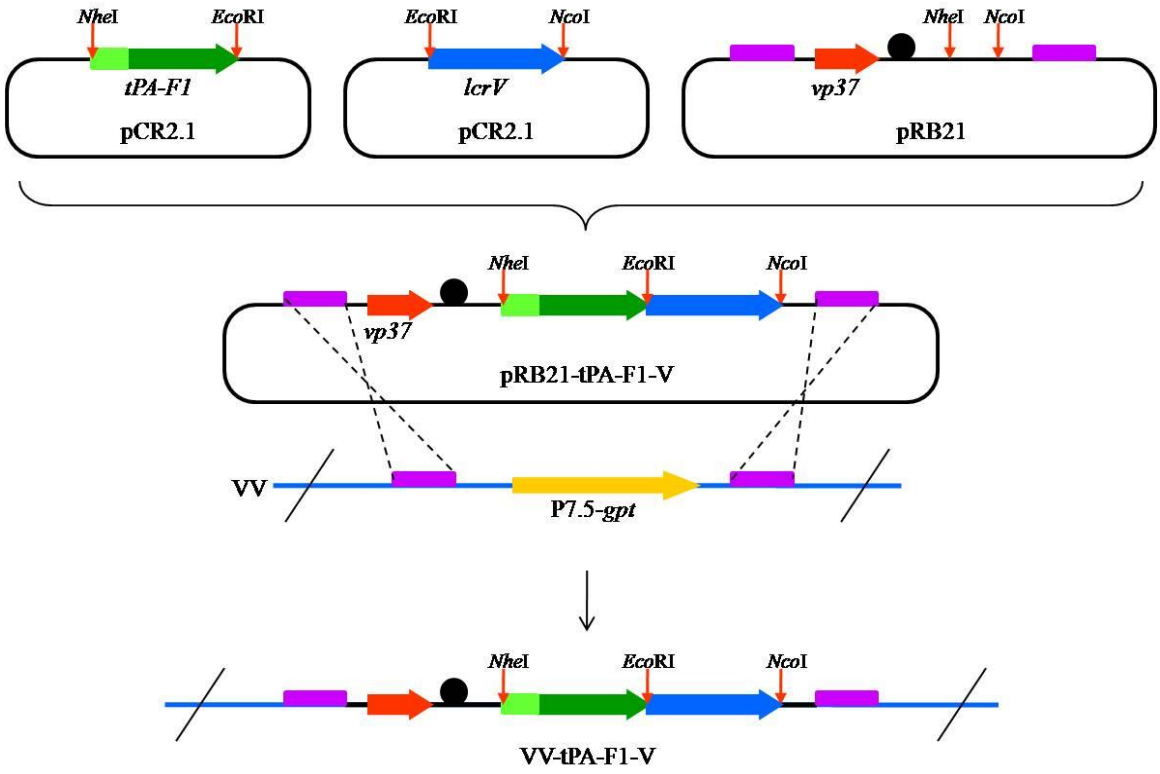
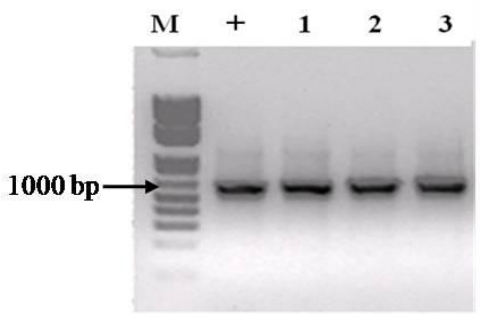


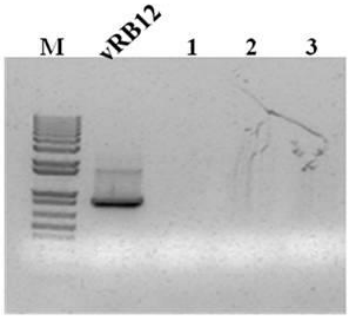
Figure 2.1: Strategy to construct VV-F1-V. F1 (green arrow) and LcrV (blue arrow) were amplified and cloned in pCR2.1 at specific restriction sites (marked by red arrows). A TPA signal sequence (green rectangle) was added to the 5'-end of F1 fragment. The tPA-F1 and LcrV fragments were fused by overlap PCR and ligated into vaccinia virus cloning vector, pRB21. Plasmid pRB21 has a vaccinia virus promoter (black circle), *vp37* gene (red arrow) for selection of recombinants, and VV homologous sites (purple box) for recombination with VV-vRB12. pRB21 carrying tPA-F1-V was transfected into VV-vRB12 infected CV-1 cells. Double cross over at homologous sites results in formation of recombinant virus, VV-F1-V. The recombinant virus gains a copy of *vp37* gene during crossing-over event which is essential for plaque formation, the feature used for recombinant selection.

Figure 2.2

A.



B.



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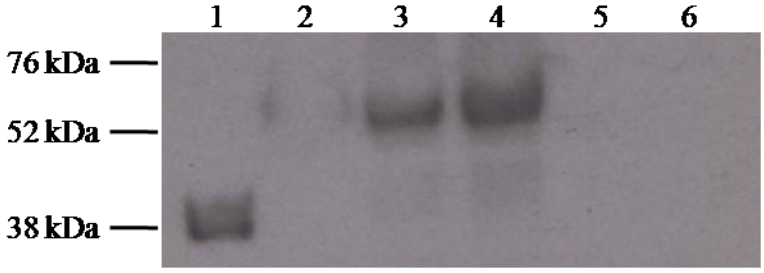


Figure 2.2: Construction of VV-F1-V vaccine

A. PCR showing presence of F1-V fusion insert in different clones of VV-F1-V obtained after four rounds of neutral red plaque selection. The selected viral clones were amplified by infecting HeLa cells. Cell lysate were prepared and PCR was performed using a pair of internal primers specific for F1-V. '+' is the positive control for PCR; 1, 2 and 3 are different clones of VV-F1-V; 'M' is 1Kb Plus DNA ladder (Invitrogen Inc.).

B. Negative PCR showing absence of vRB12 population in different clones of VV-F1-V. PCR was performed using primers specific for the irrelevant gpt insert that is lost during recombination to determine purity of the final virus. vRB12 was used as PCR control; 1, 2 and 3 are different VV-F1-V clones (same as described in figure 2.2A); 'M' is 1Kb Plus DNA ladder (Invitrogen Inc.).

C. Confluent HeLa cells were infected with VV-F1-V. After 3 days of infection, cells were collected and lysed by sonication. Cell lysates were subjected to SDS-PAGE and probed with an antibody to LcrV to test for expression of F1-V fusion protein. Lane 1: Positive control, *Yersinia* lysate (expressing only LcrV); Lane 2, 3 and 4: Different dilutions of VV-F1-V infected cell lysate; Lane 5, 6: Negative control, cell lysate infected by a non-specific strain of VV (VV-CD56) which lacks F1-V insert. The predicted sizes of LcrV and F1-V are 37 kDa and 53 kDa respectively.

Antibody response to VV-F1-V

We determined the antibody response in VV-F1-V-vaccinated mice against purified GST-LcrV protein. C57BL/6 mice were infected with 10^7 or 10^8 pfu of VV-F1-V by oral gavage. The doses were selected based on our laboratory's previous experience with VV-OspA vaccine [134]. Serum samples were collected and analyzed by western blotting using an anti-LcrV antibody. Serum antibody response was detected by western blotting in mice vaccinated with 10^8 pfu of VV-F1-V starting on week 4 post-vaccination (Figure 2.3A).

In addition, we quantified the anti-LcrV antibody titers in serum samples at various time points by endpoint ELISA. A dose-dependent antibody response to the VV-F1-V was observed (Figure 2.3B). Mice immunized with 10^8 pfu vaccine had antibody responses that were more than 10 fold higher than mice vaccinated with 10^7 pfu of the vaccine. The difference in antibody response between the two doses was maintained throughout the course of observation and the levels of antibody remained constant over this period.

Kinetics of antibody development

We evaluated the timing of development and the stability of the antibody response post-vaccination with VV-F1-V. For this purpose, serum antibody titers at various time points were determined by endpoint ELISA.

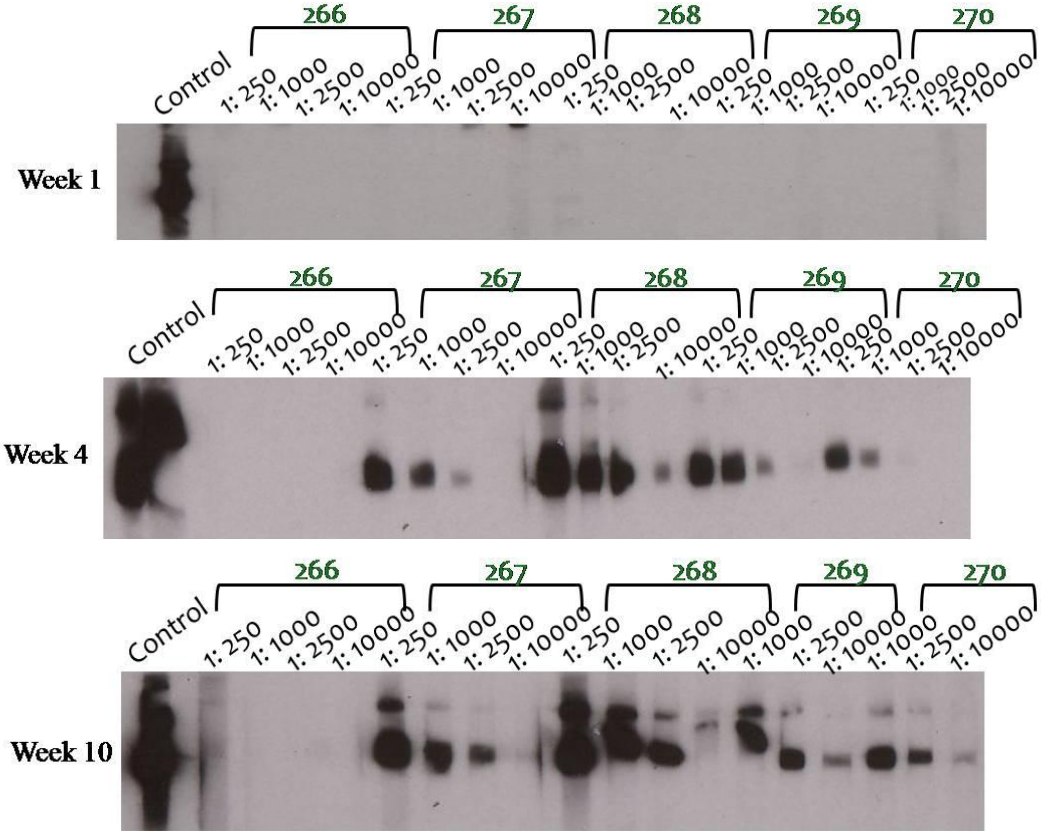
Mice immunized with 10^8 pfu of VV-F1-V developed an antibody response within 7 days post-vaccination and reached a peak titer by week 3 (Figure 2.3C). The antibody titers peaked at a dilution of approximately 1:256,000. The antibody response generated with a

single dose of 10^8 pfu of recombinant virus was stable for at least 45 weeks (the end of the observation period).

The kinetics of the antibody response in mice administered 10^7 pfu of vaccine appeared to be similar, but a lower titer was attained. Antibody titers remained stable for the 10 weeks these mice were followed (Figure 2.3B).

Figure 2.3

A.



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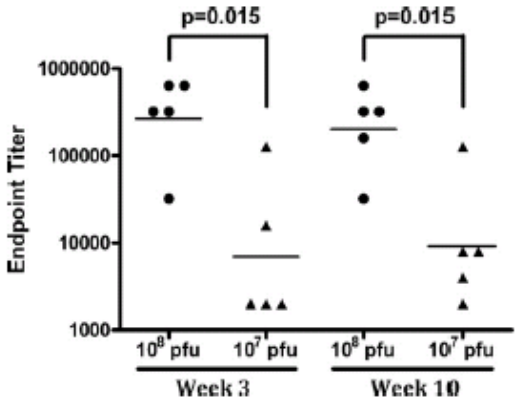

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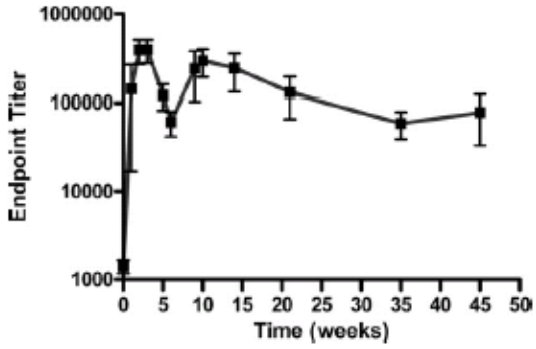


Figure 2.3: Antibody response kinetics in VV-F1-V vaccinated mice

A. Western blots showing anti-LcrV antibody response in VV-F1-V-vaccinated mice: Mice (numbered 266-270) were vaccinated with 10^8 pfu of VV-F1-V and serum samples were collected at various time points. GST-LcrV protein was run on a preparative denaturing gel and transferred to a membrane. The membrane was cut into narrow strips and each strip was incubated with appropriate dilutions of mouse serum. No antibody response was observed on week 1 post-vaccination. Anti-LcrV antibody on week 4 and 10 post-vaccination is shown. Mouse # 266 generated a week antibody response as detected by western blotting.

B. Dose-dependent antibody response in VV-F1-V vaccinated mice: C57BL/6 mice were administered either 10^7 (triangle) or 10^8 (circle) pfu of VV-F1-V by oral gavage. Antibody response in serum obtained at week 3 and 10 post vaccination is shown. Antibody titers against LcrV were measured by endpoint dilutional titering. Each data point represents antibody titer for one mouse. Black bars represent the geometric mean of antibody response for each group. The difference in antibody titers between the two dosage groups was significant ($p<0.05$) by Mann-Whitney U test.

C. Kinetics of antibody response: C57BL/6 mice were vaccinated with 10^8 pfu of VV-F1-V. Serum antibody response against LcrV was measured at various time points by endpoint dilutional titering. The reciprocal of highest dilution above the cut-off was defined as endpoint antibody titer of each serum sample. The mean of absorbance values of unvaccinated mouse serum was set as cut-off. Each square represents the mean antibody titer for at least 3 mice. Error bars represent standard deviation.

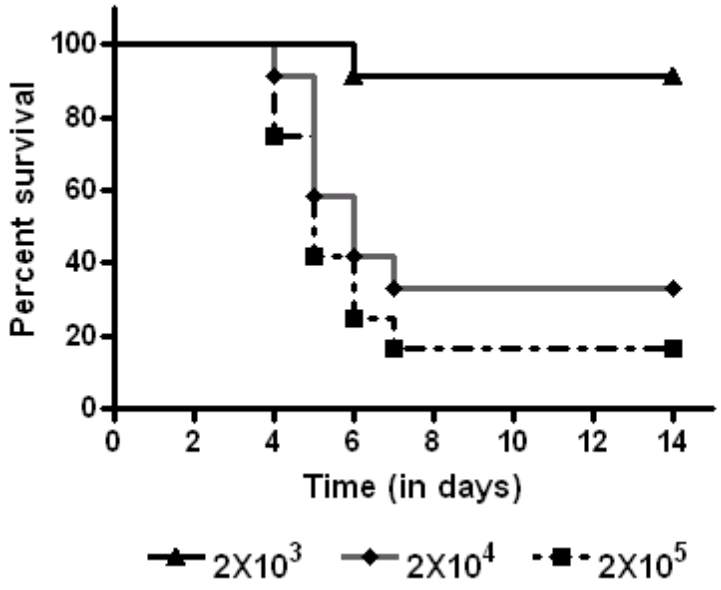
Efficacy of VV-F1-V vaccine

To determine the protective efficacy of the vaccine, we utilized a mouse intranasal infection model [92]. *Y. pestis* strain KIM D27 is an attenuated strain that lacks the pigmentation locus (*Δpgm*) that includes few virulence genes. Intranasal administration of *Y. pestis* KIM D27 has been reported to result in lethality to mice. We determined the LD_{50} dose of *Y. pestis* KIM D27 in C57BL/6 males by intranasal route of inoculation. Using Reed-Muench method [139, 140], the LD_{50} was calculated as 1.6×10^4 cfu (Figure 2.4A). A dose of 2×10^5 cfu was used in all subsequent challenge experiments.

To determine the efficacy of VV-F1-V vaccine, groups of mice were immunized with various doses of the vaccine to generate a range of antibody responses. A control cohort of mice was immunized with 10^8 pfu of a VV expressing an irrelevant protein, the outer surface protein A of *B. burgdorferi* (VV-ospA) [134]. Antibody titers in mice were determined by ELISA four weeks post vaccination. At week 5 post-vaccination, mice were challenged by intranasal administration of *Y. pestis* KIM D27. We observed titer-dependent protection against challenge of *Y. pestis* KIM D27 (Figure 2.4B). Mice with an endpoint titer more than 32,000 (n=15) were fully protected against the challenge. Mice with an endpoint titer in the range of 8000-16000 (n=5) showed a survival rate of 80% against challenge. In comparison, the control vaccinated mice (n=17) had a percentage survival of less than 20%.

Figure 2.4

A.


Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (in days)
2X10^3
2X10^4
2X10^5

B.

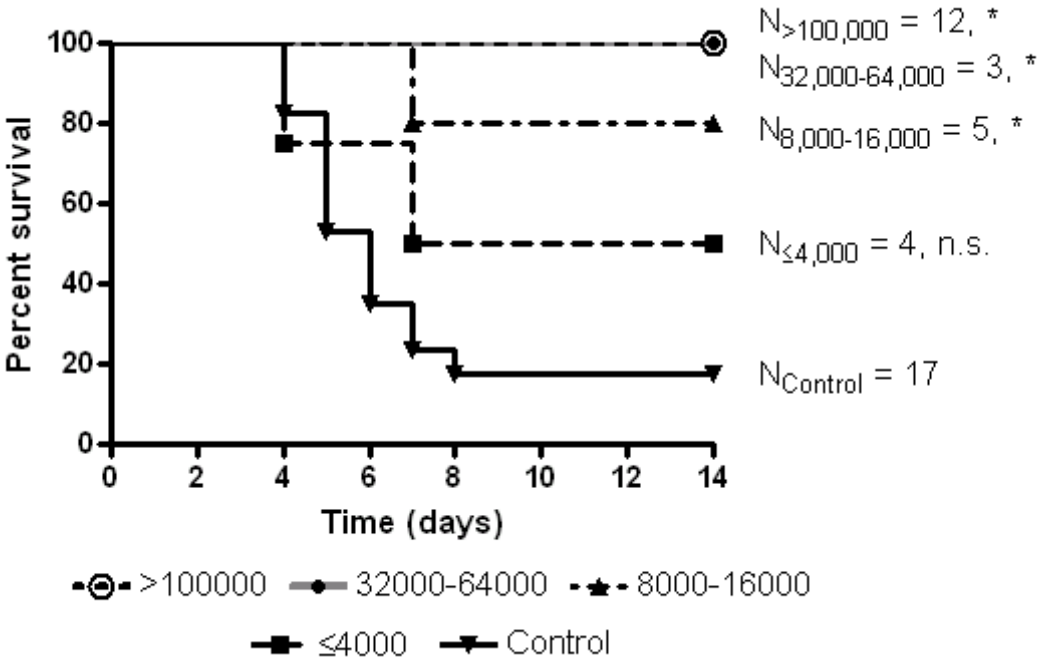

Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
N>100,000 = 12, *
N32,000-64,000 = 3, *
N8,000-16,000 = 5, *
N≤4,000 = 4, n.s.
NControl = 17
>100000
32000-64000
8000-16000
≤4000
Control

Figure 2.4: Protective efficacy of VV-F1-V

A. Determination of LD_{50} of *Y. pestis* KIM D27 in C57BL/6 mice by intranasal route of inoculation. Three groups of mice (n=12 in each group) were given $2X10^3$, $2X10^4$, or $2X10^5$ cfu of *Y. pestis* KIM D27 by intranasal inoculation. Survival rate of the groups of mice was determined over a period of 14 days. The method of Reed and Muench was used to calculate the LD_{50} dose [140].

B. Protective efficacy of VV-F1-V vaccine in mice. C57BL/6 mice were vaccinated with differing amounts of VV-F1-V to generate a diversity of antibody titers. VV-F1-V or control vaccinated mice were administered with a dose of $10XLD_{50}$ of *Y. pestis* KIM D27 by intranasal inoculation at 5 weeks post vaccination. Three independent experiments were performed (n=3). The survival rates of mice with differing anti-OspA antibody titers from the three experiments are shown. Log-rank test was performed to determine the significance by comparing survival percentage of each titer group to the control vaccinated mice. 'N' represents number of mice in each titer group, and asterisks indicate statistical significance obtained by log rank test.

The 'p' values obtained by log rank test are as follows: For titers>100000 vs. control, $p<0.0001$; for titers between 32000-64000 vs. control, $p=0.02$; for titers between 8000-16000 vs. control, $p=0.01$; for titers less than ≤ 4000 vs. control the difference was not significant (n.s).

Durability of protection by VV-F1-V

Our next aim was to determine the window of protection conferred by a single dose of VV-F1-V. For this purpose, two groups of mice were vaccinated with 10^8 pfu of VV-F1-V. One group was challenged on week 5 post vaccination as earlier described. The other group was challenged 45 weeks post vaccination. One hundred percent of vaccinated mice challenged either 5 weeks or 45 after vaccination survived (Figure 2.5). All mice in both groups had antibody titers that were greater than 1:32,000, the titer level at which 100% protection was observed in mice challenged earlier after vaccination (Figure 2.4B).

Bacterial clearance in VV-F1-V-vaccinated mice

We determined bacterial clearance in vaccinated mice vs. control. For this purpose, the number of bacterial cfu in lungs was determined in VV-F1-V-vaccinated mice vs. control at two time points (Day 2 and Day 4) post intranasal infection with *Y. pestis* KIM D27. The bacterial count in lungs of vaccinated mice was at least a log lower as compared to unvaccinated controls (Figure 2.6).

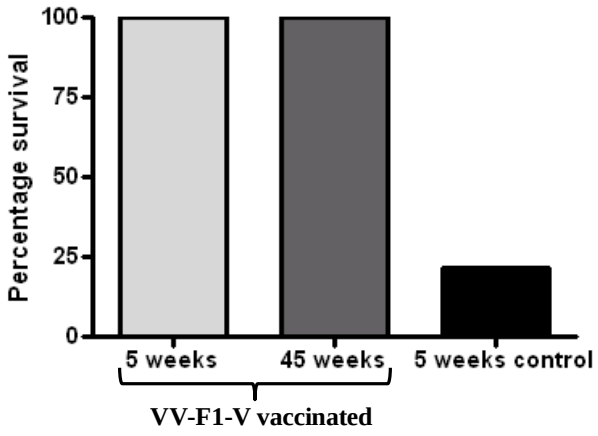


Figure 2.5: Durability of protection by a single dose of VV-F1-V vaccine. Mice were vaccinated with 10^8 pfu of VV-F1-V or control (n=14) and challenged with *Y. pestis* KIM D27 intranasally at a dose of 10 X LD_{50} dose at either 5 weeks (n=10) or 45 weeks (n=5) post vaccination. The mice were observed for 14 days post challenge. All VV-F1-V vaccinated mice in both the 5 week and the 45 week group had endpoint dilutional titer of more than 1:32,000.

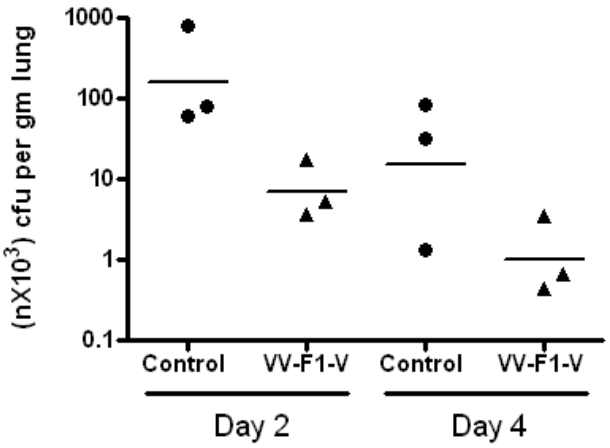


Figure 2.6: Clearance of *Y. pestis* KIM D27 in vaccinated mice. C57BL/6 mice were vaccinated with 10^8 pfu VV-F1-V or control vaccine (VV-ospA). The mice were infected with $2X10^5$ cfu of *Y. pestis* KIM D27 by intranasal inoculation at 5 weeks post vaccination. Bacterial count in vaccinated (triangles) and control mice (circles) was determined on day 2 or day 4 post challenge by plating appropriate dilutions of lung homogenate on tryptose blood agar plates. Each data point represents bacterial count of a single mouse and bars represent the geometric mean of bacterial count. The difference in the bacterial cfus between the vaccinated and control group were not statistically significant.

CHAPTER 3

EFFICACY OF RECOMBINANT VV VACCINES DELIVERED VIA ORAL BAITS

Note: This chapter has been accepted for publication

Bhattacharya D, Bensaci, M, Luker, KE, Luker, G, Wisdom, S, Telford, SR and Hu, LT. 2011. Development of a baited oral vaccine for use in reservoir-targeted strategies against Lyme disease. *Vaccine*. In press.

Chapter 3 Abstract

Lyme disease is a major human health problem which continues to increase in incidence and geographic distribution. As a vector-borne zoonotic disease, Lyme disease may be amenable to reservoir targeted strategies for control. It has been previously shown that a recombinant vaccinia virus (VV) expressing outer surface protein A (OspA) of *B. burgdorferi*, the causative agent of Lyme disease, protects inbred strains of laboratory mice against infection by feeding ticks and clears the ticks of infection when administered by gavage. Here we extend these studies to develop an effective bait formulation for delivery of the VV based vaccine and test its characteristics under simulated environmental conditions. We show that the VV-ospA vaccine is efficacious in decreasing acquisition of *B. burgdorferi* by uninfected larval ticks as well as in decreasing transmission from infected ticks to its natural reservoir, *Peromyscus leucopus*, when fed to mice in oral baits. We also show the efficacy of VV-F1-V vaccine when delivered as oral baits. The VV-F1-V vaccine baits were immunogenic in C57BL/6 mice and conferred protection against a lethal dose of *Y. pestis*.

Chapter 3 Results

Stability of VV in oral bait

Although baits for reservoir-targeted use of vaccinia virus-based vaccines have been developed for use with raccoons and foxes, significant differences between these animals and rodents, the target animals for Lyme or Plague required us to redesign the bait formulation. The vaccine for raccoons and foxes wraps bait material around a small sachet containing the vaccinia virus in a liquid vehicle. Because mice are significantly smaller and cannot swallow the sachet intact, the sachet method is unlikely to result in significant uptake of vaccine. As a result, we tested a vaccine fabrication process that incorporated VV directly into a liquid-retaining bait formulation. We then tested this formulation under a variety of conditions likely to be encountered during deployment in the wild. The stability of VV in oral bait was determined at 25°C, at 37°C, and under freeze-thaw conditions (Figure 3.1). At 25^{o}C, titers of VV-ospA declined by approximately 1 log over 6 weeks when kept in a sealed conical tube (Figure 3.1A). Titers in the bait could be easily maintained above the minimum dosage required to generate a protective antibody response for this duration of time. To test the effects of desiccation on the vaccine titer, we allowed the baited vaccines to remain exposed to ambient, unhumidified air. Under these conditions, the bait became harder within a few days due to loss of moisture. However, the VV titers were not significantly different compared to conditions where moisture was maintained (Figure 3.1B).

We also tested the effects of multiple freeze-thaws on viral titers. Because larval ticks feed in early Spring, the vaccine is likely to be deployed beginning in late Winter/early

Spring when temperatures in endemic areas frequently still reach freezing. The VV-ospA titers were similar to those kept at 25^{o}C till at least 21 freeze-thaw cycles spanning over 3-week period (Figure 3.1C). By the end of 28 freeze-thaw cycles, the VV titers fell to approximately 10% of the initial titer in oral bait. Conversely, the titers of VV-ospA were not maintained at high temperatures. When the baits were kept at a constant 37°C temperature, the VV titer in bait decreased to 1% of its initial titer within one week (Figure 3.1D).

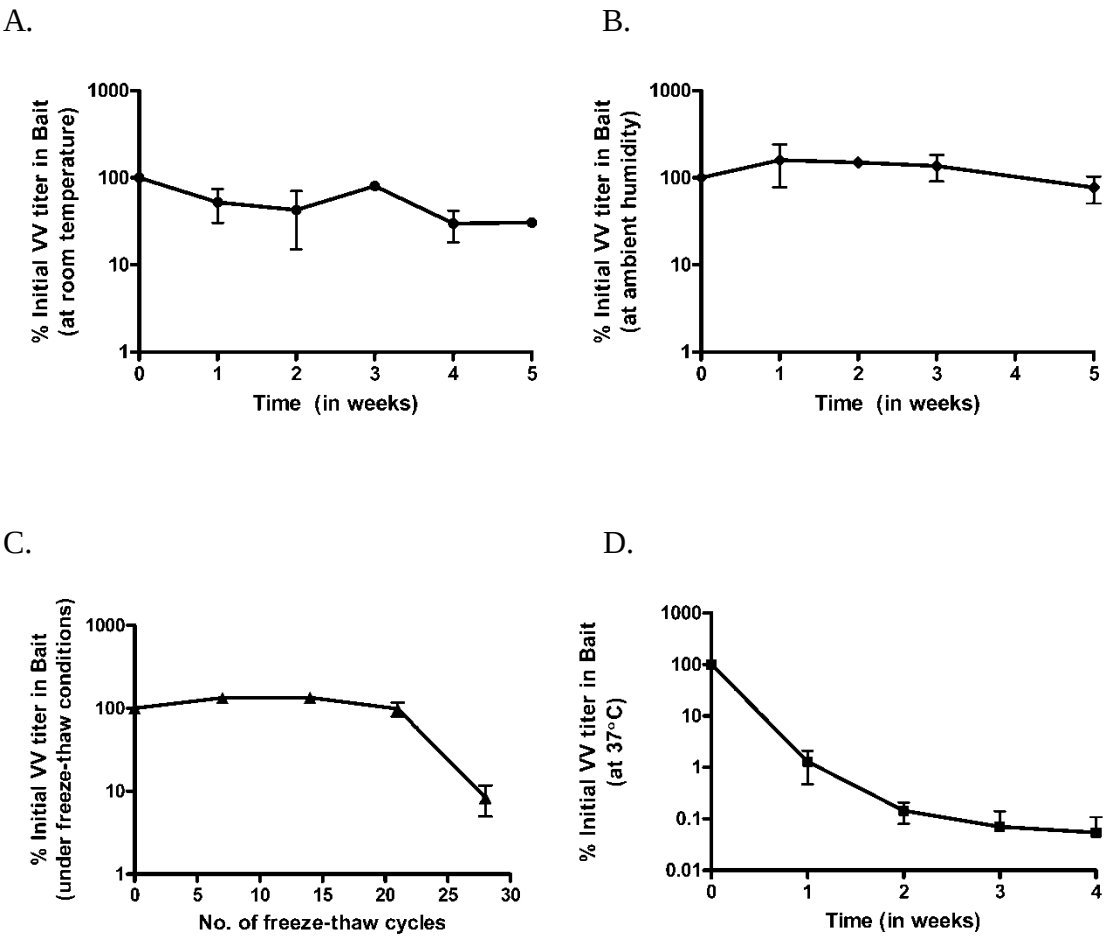


Figure 3.1: Stability of VV in oral baits is shown. The stability was determined by titering VV in baits by crystal violet plaque staining at different time points. Titers per unit weight of bait were measured at each time point. VV stability in baits was determined under following different conditions: constant room temperature (A), ambient humidity (B), effect of freeze-thaw (C) and 37°C (D). Figures show result of 3 independent experiments. Error bars represent standard error of the means. In figure 3.1D, the lower error bars for weeks 3 and 4 (not shown) extend to 0% as virus infection was not observed in two experiments.

Antibody response against VV-ospA in *Peromyscus* mice

We have previously reported the efficacy of VV-ospA when administered to *Mus musculus* inbred mice by gavage. To determine whether VV-ospA was effective when administered orally to its natural host, *Peromyscus leucopus* mice were immunized with 2×10^8 pfu of VV-ospA administered by oral gavage or offered *ad libitum* for ingestion as a 2-3 gm oral baits. The dose was selected based on our previous experience with VV-based vaccine [134, 141]. 30% of the mice given the baited vaccine consumed the vaccine within 24 hrs and 90% had consumed the entire vaccine by 48 hrs. Serum samples were collected at different time points and antibody titer to OspA was determined by endpoint ELISA. Anti-OspA antibody titers were not significantly different in mice vaccinated either via oral gavage or bait fed at 4 weeks post-vaccination (Figure 3.2A).

We determined the stability of anti-ospA antibody response in *Peromyscus* mice immunized via feeding with VV-ospA laden baits or control baits. In *Mus musculus* mice given VV-ospA by gavage, we have observed a peak of the anti-OspA antibody titer at between 5-6 weeks with a steady decline to baseline over 10 weeks [134]. The VV-ospA orally vaccinated Peromyscus mice reached protective antibody levels by week 4 post-vaccination, with antibody titers peaking at 12 weeks and remaining stable for at least 10 more weeks before beginning to decrease (Figure 3.2B). Antibody levels remained above control through week 35 (furthest time point tested).

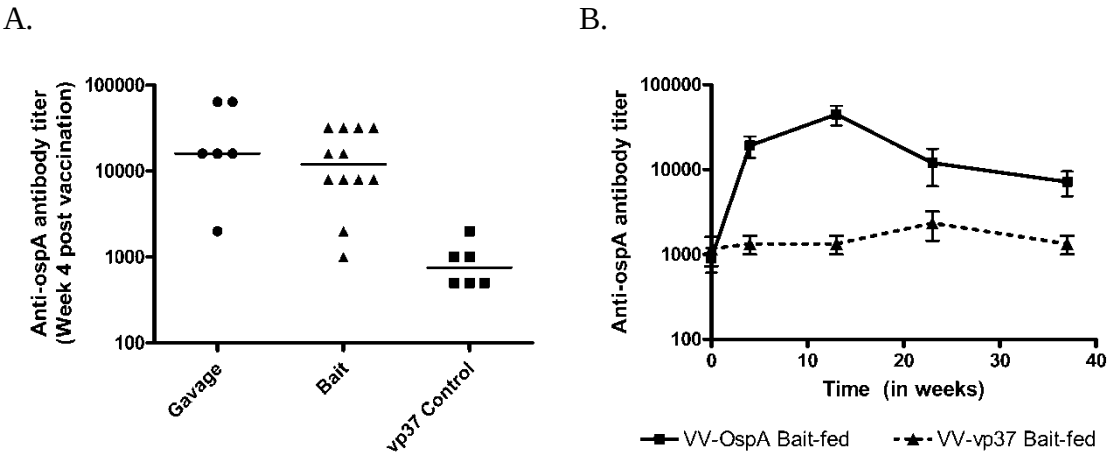


Figure 3.2: Anti-OspA antibody response in *Peromyscus* mice.

A. Endpoint antibody titer in *Peromyscus* mice on week 4 post vaccination is shown. VV-ospA was administered to mice via oral gavage (circles) or fed as oral baits (triangles). The control mice (squares) were vaccinated with an irrelevant VV strain (VV-vp37) either via gavage or bait-fed. Each data point represents the antibody response in a single mouse and the horizontal bars represent geometric mean antibody response of the group.

B. Kinetics of anti-OspA antibody response post-bait immunization is shown. *Peromyscus* mice were fed with baits laden with 2×10^8 pfu VV-ospA or control (VV-vp37). Mean and standard error of antibody titer of at least five VV-ospA-vaccinated (square, solid line) and three control-vaccinated mice (triangle, dotted line) are shown.

Efficacy of VV-ospA in prevention of *B. burgdorferi* transmission to uninfected mice

To determine the efficacy of VV-ospA administered by oral baits in protecting uninfected mice against transmission of *B. burgdorferi* from infected ticks, *Peromyscus* mice were vaccinated with VV-ospA or control (VV-vp37) via bait feeding. The antibody response in mice was determined 4 weeks post-vaccination to ensure successful vaccination of the mice. Six *B. burgdorferi*-infected nymphs were allowed to feed on each mouse. The mice were tested for spirochete infection by culturing serial ear punch samples and by culturing of the heart, bladder and joints at the time of sacrifice. We have previously found that culture of multiple organs is more sensitive than PCR in detecting infection in our hands [134] . Only about 16 % of VV-ospA vaccinated mice became infected with *B. burgdorferi* compared with 75% of VV-vp37 vaccinated mice ($p < 0.005$) (Table 3.1).

As expected, protection was correlated with antibody titer. The two VV-ospA-vaccinated mice that acquired *B. burgdorferi* infection upon tick feeding had low anti-OspA antibody titers (endpoint titer < 8000) post-vaccination.

Efficacy of VV-ospA in prevention of *B. burgdorferi* acquisition by larvae ticks from infected mice

A major mechanism of action of a reservoir targeted OspA vaccine is through the prevention of acquisition of *B. burgdorferi* by uninfected larval ticks from infected mice. Because OspA is not expressed by *B. burgdorferi* in the mouse host, antibodies to OspA do not clear an infected mouse of *B. burgdorferi* infection; however, antibodies to OspA have been shown to prevent transmission from the infected mouse back to uninfected

ticks [118, 119, 124]. Peromyscus mice were infected with *B. burgdorferi* by feeding of *B. burgdorferi* infected nymphal ticks. 1-2 weeks after feeding, mice were administered VV-ospA or control (VV-vp37) via oral baits. Four weeks after consumption of the baits, uninfected *Ixodes* larvae were allowed to feed on the vaccinated infected mice. The fed larvae were recovered and then cultured for *B. burgdorferi*. We observed a significant reduction in *B. burgdorferi* transmission from infected mice to tick larvae during feeding upon VV-ospA vaccinated mice compared to control vaccinated mice (Table 3.2).

Table 3.1: Efficacy of VV-ospA bait vaccine in prevention of *B. burgdorferi* transmission to *Peromyscus leucopus*. Mice were fed with baits containing 2×10^8 pfu VV-ospA or control (VV-vp37). The mice were challenged by feeding 5-6 *B. burgdorferi*-infected *Ixodes scapularis* nymphs, 5 weeks post-vaccination. Spirochetes transmission to mouse was tested by culturing ear punch, bladder, and heart at various time points. Statistical analyses (Fisher's exact test*) showed a significant degree of protection from spirochete infection in VV-ospA vaccinated mice vs. controls.

	Total Mice	# Mice Infected	% Mice Infected*
VV-ospA	12	2	16.16
VV-vp37	12	9	75

*$p < 0.005$

Table 3.2: Efficacy of VV-ospA vaccine in prevention of acquisition of *B. burgdorferi* by tick larvae. *B. burgdorferi*-infected *Peromyscus* mice were vaccinated with VV-ospA or control vaccine via bait-feeding. Uninfected tick larvae were fed on these mice to repletion. Presence of the spirochete in fed larvae was tested by culturing crude extracts of larvae in BSK. Statistical analyses (Fisher's exact test[†]) showed a significant difference in acquisition of the infection by larvae feeding on vaccinated mice vs. control vaccinated mice.

	Total Fed larvae	# Larvae Infected	% Larvae Infected[†]
VV-ospA	43	10	23.29
VV-vp37	53	45	84.90

[†]$p < 0.0001$

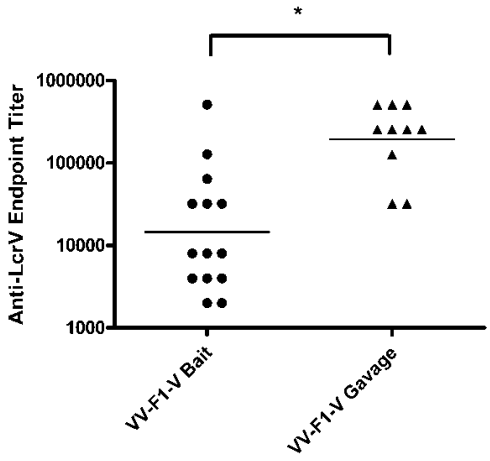
Antibody response against VV-F1-V bait vaccine

We have earlier shown generation of a robust antibody response after oral immunization of C57BL/6 mice with VV-F1-V vaccine. To compare the level of antibody response via gavage vs. bait feeding, we vaccinated C57BL/6 mice with 2×10^8 pfu of VV-F1-V either by oral gavage or offered *ad libitum* for ingestion as a 2-3 gm oral baits. Serum antibody response was determined by endpoint ELISA at 4 weeks post-vaccination (Figure 3.3A). The antibody titers in C57BL/6 mice after bait vaccination was at least a log lower as compared to the titers generated in gavage immunized mice. This is unlike the anti-OspA antibody response generated in *Peromyscus* mice where we observed a comparable antibody response administered via gavage or oral baits.

Efficacy of VV-F1-V bait vaccine against *Y. pestis*

We determined the efficacy of VV-F1-V bait vaccine in C57BL/6 mice. The VV-F1-V and control vaccinated mice were challenged intranasally against a 10 x LD_{50} dose of *Y. pestis* KIM D27. The lower antibody response following bait-fed immunization resulted in a decreased protection against the challenge. The VV-F1-V bait fed mice had a survival rate of 50% against the challenge as compared to gavage immunized mice where more than 80% were protected (Figure 3.3B). Of note, we have previously shown that protective efficacy of VV-F1-V vaccine is dependent on the level of antibody titer generated [141], thus the decreased efficacy by bait vaccine was not surprising.

A.



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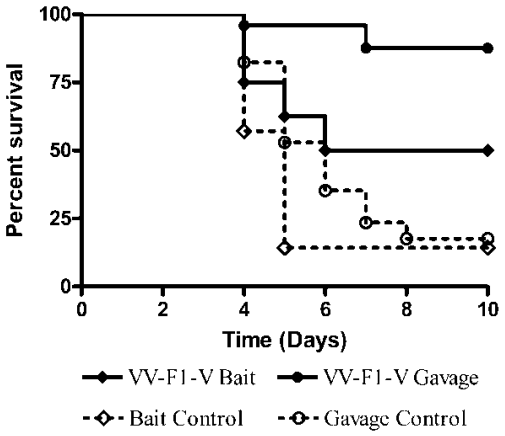


Figure 3.3: Immunogenicity of VV-F1-V bait vaccine in C57BL/6 mice. A. Serum antibody response against VV-F1-V vaccine in C57BL/6 administered as oral baits (circles) or gavage route (triangles) is shown. The difference in antibody titers between the two groups was significant as analyzed by Mann-Whitney U test ($p < 0.05$).

B. Survival rate of mice vaccinated with VV-F1-V administered as oral baits (closed diamonds) or by oral gavage (closed circles) against 10 x LD_{50} dose of *Y. pestis* KIM D27 is shown. Control vaccinated mice are shown as dotted lines: control bait-fed (open diamonds) and control gavage (open circles). The difference in vaccinated and control groups are significant as analyzed by log-rank test.

CHAPTER 4

DISSEMINATION KINETICS OF VV IN MICE VIA ORAL ROUTE

Note: This chapter has been accepted for publication

Bhattacharya D, Bensaci, M, Luker, KE, Luker, G, Wisdom, S, Telford, SR and Hu, LT. 2011. Development of a baited oral vaccine for use in reservoir-targeted strategies against Lyme disease. *Vaccine*. In press.

Chapter 4 Abstract

Dissemination of vaccinia virus in mice is dependent upon both the strain of virus and the route of inoculation. There is limited knowledge on the dissemination kinetics of vaccinia post-oral infection. Previous studies with the V-RG Raboral vaccine have shown localized infection of vaccinia virus in tonsils and retropharyngeal lymph nodes of raccoons and foxes within 48 hours post-feeding [26, 27]. The infection did not give rise to any adverse clinical signs or histopathological lesions. An attenuated strain of VV, modified vaccinia Ankara (MVA), resulted in limited infection in nasal associated lymphoid tissue (NALT) post intragastric inoculation.

For our vaccine, we have used a mouse adapted strain of VV called VV-WR as the delivery vector. In this study, we have determined the localization and dissemination kinetics of this strain of VV by using a live, *in vivo* imaging technology to track VV expressing a firefly luciferase reporter. We have compared the infection kinetics of VV after gavage vs. bait infection. Also, we have used the *in vivo* imaging technology to optimize the bait composition for increased uptake of the virus during feeding.

Chapter 4 Results

Dissemination of VV in mice

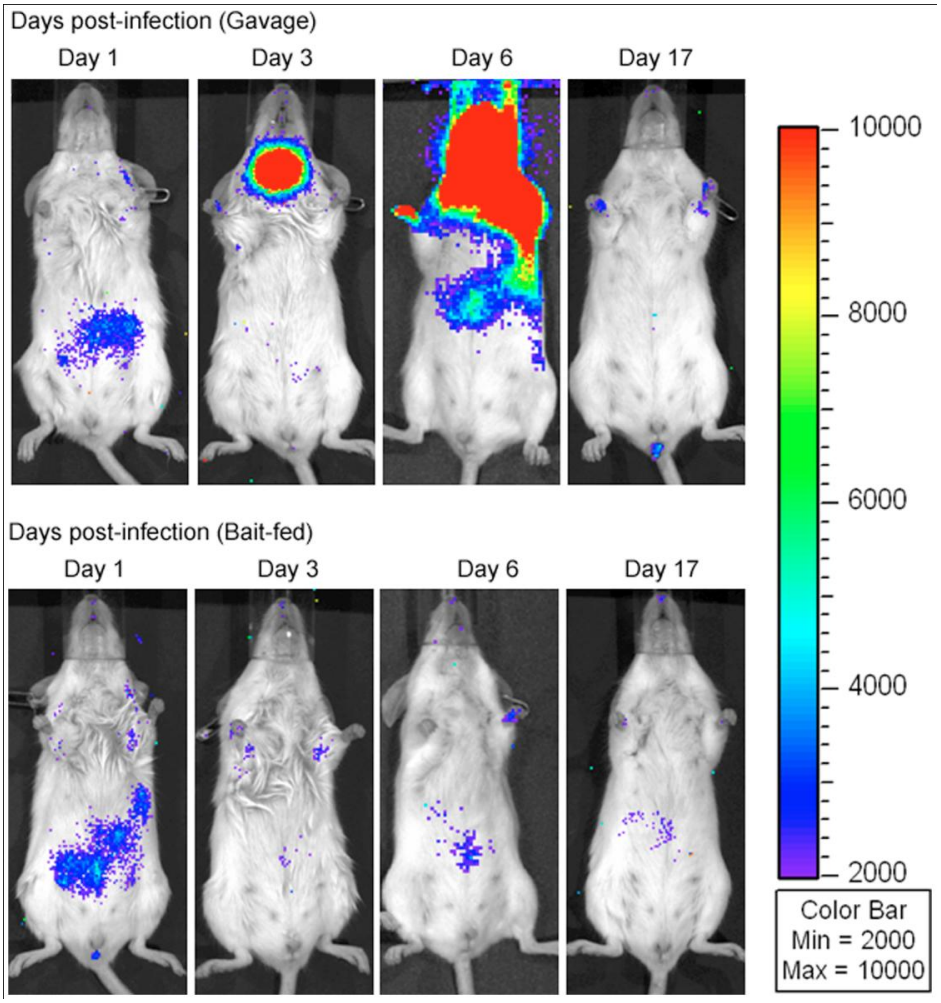
Dissemination of VV after oral inoculation has not been well established and may vary between different strains of VV. To determine the dissemination of VV in mice we used a recombinant VV (strain WR) with a luciferase reporter (VV-FL) inserted into the same vp37 locus that was used for generating the recombinant VV-ospA and VV-F1-V vaccines. Balb/c mice were used for imaging as the lighter coat color causes less interference with the luciferase activity signals. Mice were infected with 10^8 pfu of VV-FL given by oral gavage or fed with oral baits. After intraperitoneal injection of luciferase substrate, the bioluminescence signals were captured using an IVIS200 imager at multiple timepoints.

When VV-FL was administered via oral gavage, we observed a higher level of VV infection as compared to bait-fed mice. Although there was significant variability from mouse to mouse in the strength of the luciferase signal, gavage-infected mice generated robust signals from the region of the brain and nasal associated lymphoid tissues (NALT) by Day 3 which was never seen in orally infected mice (Figure 4.1A and 4.1B). The bioluminescence data were confirmed by direct titering of VV from specific organs. The mean VV-FL titer in brain and NALT was 6.7×10^3 pfu/g and 1.12×10^5 pfu/g respectively. However, no virus could be recovered from spleen, mesenteric lymph nodes or Peyer's patches. A proportion of the gavaged mice had no luciferase signals from the brain or NALT and only a small amount of signal from the region of the abdomen. In these mice we could not detect any VV by tissue culture in the brain or NALT.

In bait fed mice, we observed signals predominantly around the abdominal region (Figure 4.1C). These signals were far lower in intensity than those seen in the brain and NALT of gavaged mice. The abdominal signals also appeared to disappear sooner as observed by loss of luciferase activity. While in case of gavage infection the virus was cleared by day 17 post-infection (n= 6), in bait-fed mice we observed clearance of the virus by day 12-14 post infection (n=6).

Figure 4.1: Dissemination of VV in mice post oral infection

A.



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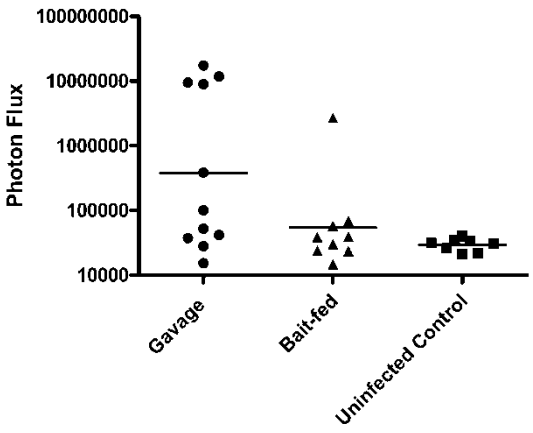

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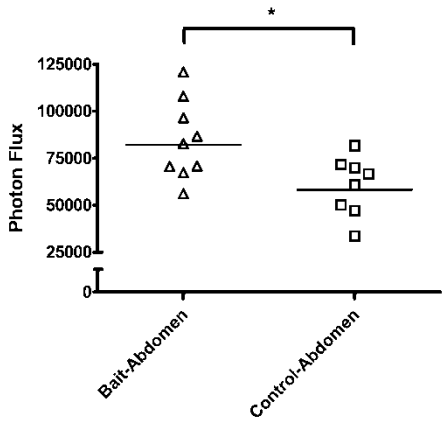


Figure 4.1: Mice were infected with 10^8 pfu of VV-FL by oral gavage or fed as oral baits and bioluminescence images were captured using IVIS 200 imager at different time points post-infection. All images were normalized to an uninfected negative control.

A. Representative panel of bioluminescence images describing kinetics of VV-FL dissemination in a Balb/c mice is shown. Luciferase activity in a single representative mouse is shown at different time points. During VV-FL infection via gavage (upper panel), a strong localization of virus was observed around brain and NALT by Day 3. These are representative images of the strong response phenotype post gavage route infection. The Bait-fed mice (lower panel) generated a low level of VV infection as compared to gavage infected mice. Localization of brain and NALT was rarely observed post-bait-feeding.

B. Quantitative comparison of luciferase activity in the brain and NALT region of mice infected via gavage (circles) or bait-fed (triangles) is shown. Luciferase activity was quantified using LiveImage software. Each data point represents photon flux in the region of interest (ROI) of a single mouse on Day 5 post-infection. For comparing gavage vs. bait-fed infection, ROI was drawn around the brain and NALT region of each mouse. During VV-FL administration via gavage route, almost half of the mice generated a robust infection around brain and NALT which was not observed via bait-fed infection. The differences in the luciferase signals were statistically insignificant.

C. Quantitative measure of luciferase activity in the abdominal region of mice infected via bait-feeding (open triangles) on Day 5 post-infection is shown. A significantly higher luciferase activity was measured in infected mice as compared to uninfected controls

(open squares) ($p < 0.05$, Mann Whitney U test). The ROI for quantification of bait-fed infection was drawn around the abdominal region.

Manipulation of bait formulation to improve infection with VV

Although we have observed similar antibody titers between gavaged and bait fed animals against VV-ospA vaccines (Figure 3.2A), the difference in productive infection as judged by live animal imaging and viral cultures of organs suggested that there may be opportunities to further optimize the bait formulation to increase productive infection—either resulting in the development of higher antibody titers or in allowing a decreased dose of VV to be used in the vaccines. One hypothesis for increased infection in the brain and NALT of gavaged mice is that local injury to the retropharynx during gavage administration may increase the VV uptake by mice. To determine whether we could achieve better infection with VV by oral administration if we created some oral trauma, we added broken nut shells to cause local mucosal injury to the oral cavity of mice during feeding. In the wild, mice frequently feed on hard substances that cause minor mouth trauma. However, we did not observe any significant increase in luciferase signal by IVIS imaging in mice fed baits containing nut shells versus those fed regular baits (Figure 4.2A).

Another possibility to explain the difference in signal between oral gavage and bait delivery is the gavage vaccine is delivered as a single bolus in a buffered solution that may protect the virus from inactivation by gastric acidity. VV is known to be inactivated at low pH [26].

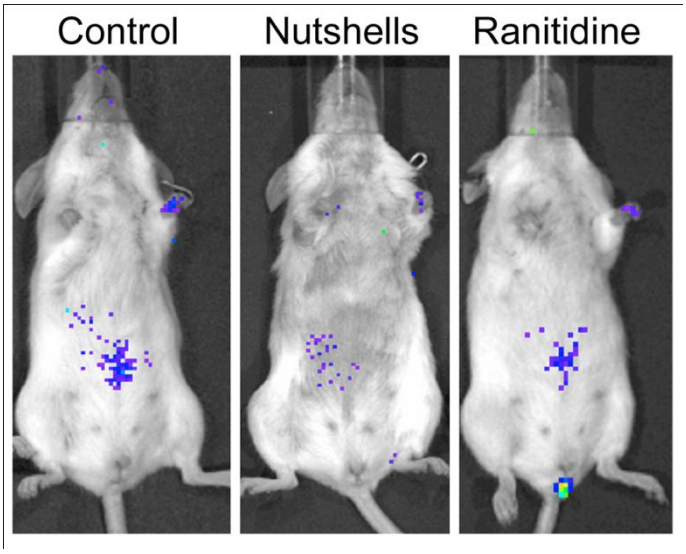
To reduce the gastric pH in bait fed mice, we administered the histamine 2 receptor antagonist, ranitidine, by intraperitoneal injection prior to bait feeding. The drug was administered twice daily and the bait feeding was monitored during this time. No change

in luciferase signal was observed in ranitidine treated mice versus control mice (Figure 4.2A and 4.2 B).

We also determined the effect of ranitidine administration during bait feeding on antibody response against VV-ospA vaccine. For this purpose, *Peromyscus* mice were administered with ranitidine and fed with VV-ospA baits. The control mice fed on VV-ospA baits without ranitidine administration. Anti-ospA antibody titers in mice sera were determined by endpoint ELISA 4-weeks post vaccination. No significant difference in anti-ospA antibody titers was observed between the two groups of mice (Figure 4.2C).

Figure 4.2: Effect of variations in bait feeding on uptake of VV

A.



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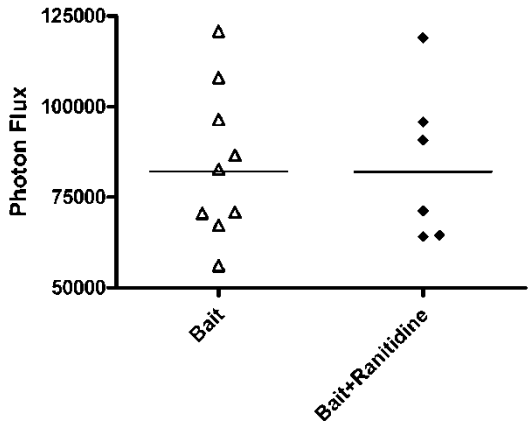

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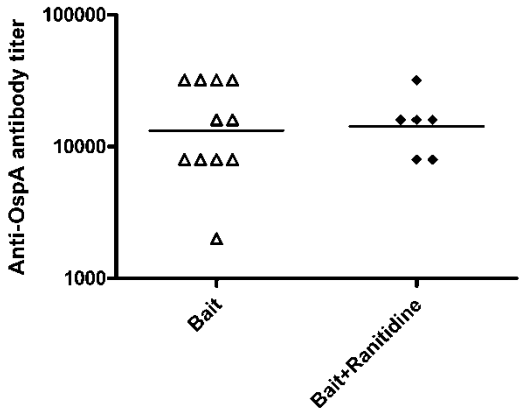

Figure 4.2: Effect of variations in bait feeding on uptake of VV

A. In one experiment, Balb/c mice were infected with VV-FL via feeding with baits that were mixed with broken nutshells. In another experiment, ranitidine was administered (i.p.) prior to offering mice with VV-FL laden ordinary baits to determine the effect of gastric pH neutralization on virus uptake via bait. Representative bioluminescence images of the two experiments on Day 5 post infection are shown. Control mouse (left) were fed with ordinary bait laden with VV-FL without ranitidine administration. No significant difference in mean intensity was observed either using altered bait formulation with nutshells (center) or by ranitidine administration (right) prior bait feeding.

B. Quantitative evaluation of VV-FL infection in Balb/c mice via ordinary bait-feeding (open triangles) vs. bait feeding with Ranitidine administration. VV-FL uptake on Day 5 post-infection is shown. No significant difference in luciferase activity was observed between the two groups of mice. The ROI for quantification of bait-fed infection was drawn around the abdominal region.

C. Effect of Ranitidine administration on anti-OspA antibody titer at week 4 post-vaccination is shown. *Peromyscus* mice were vaccinated with VV-ospA via bait-feeding with (closed diamonds) or without (open triangles) administration of ranitidine (i.p). Each data point represents the antibody response in a single mouse and the horizontal bars represent mean antibody response of the group. No significant difference in antibody response was observed between the two groups.

CHAPTER 5

DISCUSSION

In this study, we have developed vaccinia virus based oral vaccines that would be suitable for deployment targeting the zoonotic reservoir of two infectious pathogens: *Y. pestis* and *B. burgdorferi*. We have also shown the protective efficacy of the two vaccines when delivered by oral bait. The protective efficacy of the baited Lyme vaccine was shown in a natural reservoir of *B. burgdorferi*. We have further tested that we can create bait that maintains sufficient titers of vaccinia to successfully vaccinate for over 1 month when exposed to conditions simulating those that will likely be encountered during distribution.

In chapter 2, we described studies determining the efficacy of an oral VV-F1-V vaccine against a lethal challenge of *Y. pestis*. Expression of the *Y. pestis* F1 and V antigens as a fusion protein from vaccinia virus administered orally generates long lasting antibody response after a single dose and protects mice against lethal *Y. pestis* infection after intranasal challenge up to 100%. Based on studies with F1-V showing good correlation between the intranasal models and subcutaneous models using wild-type *Y. pestis* [92], it is likely that this vaccine would also protect against subcutaneous challenge with *Y. pestis*. The long term stability of the antigenic response observed with our vaccine appears to be a characteristic of the F1-V fusion antigen as antibody responses to other vaccinia virus expressed proteins in mice has not been as long-lived [134]. In studies of subcutaneous vaccination, administration of the F1-V fusion protein produced strong antibody response in black-footed ferrets for up to 2 years after two doses [93].

Although our data are promising, there are some caveats to our results. Although intranasal inoculation is an effective delivery mechanism, it does not faithfully reproduce

infection introduced by the feeding of infected fleas. Other studies comparing protection arising from F1-V antigens after flea infection have shown a good correlation with intranasal challenge, so we anticipate that protection will be good with the vaccinia virus based oral vaccine [142]. Nonetheless this clearly will require further testing with a fully virulent strain and natural delivery through infected fleas. Second, although we have shown excellent protection with the vaccinia virus expressed F1-V fusion protein, we have not tested the relative contributions of the two components. Theoretically, the combination of both antigens would provide protection against naturally occurring variants at these two loci. Capsule (F1) deficient *Y. pestis* does occur in the wild as a small minority of wild isolates and has the potential to cause disease [90]. Others have shown that the F1-V fusion protein protects against $F1^{-}$ *Y. pestis* to the same extent as observed with vaccination by LcrV alone [92]. Similarly, there have been reports of two variant populations of *Y. pestis* based on variations in LcrV sequence, designated as V-O:3 and V-Yp [91, 143]. While V-O:3 is expressed by *Y. pestis* serotype O:3 as well as by a few non-pestis strains of *Yersinia*, V-Yp is primarily expressed by *Y. pestis* alone [91, 143]. Protection by V-antisera is possible only if the immunizing V-Ag sequence is the same as carried by the challenge strain of *Y. pestis* [91, 143]. The prevalence of LcrV variants in wild populations of *Y. pestis* is not known. It will be of interest to determine whether antibodies to F1 provide sufficient protection for strains that express variant LcrV.

In chapter 3, we have shown the efficacy of the recombinant vaccinia vaccines in a bait formulation. The VV-OspA baited vaccine protected against both transmission of *B. burgdorferi* to vaccinated, uninfected *Peromyscus* mice as well as protected against

acquisition of infection by uninfected ticks feeding on infected, vaccinated mice. We have also shown that the vaccine can be delivered in a package which can easily be consumed by a mouse in hours. Also, the baited vaccine can be effective for greater than 4 weeks under expected conditions and provides protection after a single dose, giving it an advantage over non-virally vectored OspA vaccines which require multiple doses to develop protective immunity [129, 132, 144-146]. Of note, in previous studies, we have not seen an adverse effect on mice of administering doses with viral titers up to 2 logs higher than our target dose suggesting that there is unlikely to be significant harm to the animal for consuming a dose higher than is necessary to generate a protective antibody response [134]. We have also previously found that consumption of multiple doses over time does not result in adverse response and may in fact boost the antibody response [134]. In a separate study, this bait formulation has been shown to be palatable to wild mice as well as laboratory-reared animals [136]. A one-time placement of baits at 5 nest boxes over a 1 hectare area results in greater than 50% uptake of the bait in mice suggesting that penetration of the vaccine can be sufficient to provide to a significant proportion of the mouse population in the baiting area, particularly when baiting is carried out over an entire season. We anticipate that a distribution strategy will be based on replacement of bait vaccine into nest boxes on a 2-4 week schedule during peak tick feeding season.

Protection with the baited vaccine was not as high as we have previously reported for gavage administration of the VV-OspA in inbred C3H mice, which was up to 100% [134]. Failures of the vaccine in this study were all related to failure to develop sufficient antibody responses to OspA. The reason for the failure to develop an antibody response

to OspA in some of the bait fed animals is unknown. We did not observe significant differences in the time it took mice to completely consume the vaccine between responders and non-responders although more subtle differences in the timing of uptake may have been missed by our observations. It is also possible that distribution of VV-OspA in the baits was uneven. Although random sampling of the baits did not show appreciable differences in viral concentration in different baits, it is certainly possible that these baits, which were fabricated under non-commercial laboratory conditions, had significantly lower vaccine dosages in some baits due to insufficient mixing. Genetic differences between responders and non-responders are a less likely explanation given that we tested mice from two separately maintained sources of *Peromyscus* and found no differences in the rate of response in each.

We have also determined the efficacy of VV-F1-V vaccine in the bait formulation. One major drawback that we encountered with the VV-F1-V baited vaccine was that the level of antibody response in C57BL/6 mice was at least a log lower as compared to gavage vaccination. Although the VV-F1-V baits were still protective against *Y. pestis* challenge (~50% protection rate), a lower antibody response resulted in a 30% drop in the efficacy as compared to gavage inoculation. The lower antibody response against VV-F1-V bait-feeding was surprising as the same bait formulation used to deliver VV-ospA vaccine in *Peromyscus* mice generated a comparable antibody response to gavage route. One possible explanation for the difference in our observation may be due to the difference in animal strains. Further experiments will be needed to investigate the difference in antibody response between gavage vs. bait vaccination of VV-F1-V. One possibility will be to compare the antibody response against VV-ospA vaccine administered via gavage

vs. bait-feeding in C57BL/6 mice. Further studies will also be required to determine the efficacy of VV-F1-V baited vaccine in target reservoirs of plague such as prairie dogs, squirrels, or ferrets.

There are multiple hurdles to be considered in the development of a reservoir targeted vaccine. Although the use of a live, replicating viral vector allows for oral administration that results in high antibody titers, primary consideration must be for the concern for human contact with the vaccine and the potential for accidental infection. Raccoonpox has previously been reported to be used as a delivery system for development of a similar reservoir targeted vaccine against *Y. pestis* [147]. The F1-V antigen delivered using a raccoonpox viral vector is protective against infection with *Y. pestis* delivered by intramuscular injections [147]. The use of raccoonpox as a delivery vector has some potential advantages and disadvantages compared with the use of vaccinia virus. One potential advantage to raccoonpox is that it may be less infectious for humans, although this has not been studied in a systematic way. There has been a single report of a laboratory worker that was accidentally inoculated with raccoonpox that had a very mild, self-limited disease [148]. Among the disadvantages are that, in contrast to vaccinia virus, there is no experience with raccoonpox in environmental releases to determine its safety profile both for target and non-target animals. Another concern is the potential for recombination with other circulating pox viruses. Raccoonpox is endemic in North America. Although originally isolated from raccoons, the true reservoir remains uncertain and the rates of infection in animals that would be target species for a *Y. pestis* vaccine (e.g. prairie dogs, other small rodents) is unknown. The prevalence of prior infection could affect safety and efficacy of the vaccine in several ways. First, the

presence of a circulating wild virus increases the risk of recombination and loss of control of the vaccine. Although a replicating vaccine that could be passed between animals would be advantageous for penetration of a vaccine into a susceptible population, the ability of the vaccine to replicate, spread and potentially recombine would pose risks to our ability to control damage should the vaccine have unanticipated adverse effects. Both a strength (from a safety standpoint), and a weakness (from an efficacy standpoint), of vaccinia based oral vaccines is that vaccinia does not appear to be passed from animal to animal [134] and is reportedly shed for only 48 hrs after ingestion of Raboral vaccine by raccoons and foxes [17]. Thus, cessation of bait deployment of a vaccinia virus vaccine should be sufficient to halt wild transmission. The second issue is that prior infection with raccoonpox may inhibit the development of a protective response to a raccoonpox based vaccine. This disadvantage may not be limited to raccoonpox as prior infection with raccoonpox may affect responses to the vaccinia virus based rabies vaccine [149]. However, this is likely to be less of an issue with poxviruses from different genera and we have found that re-infection can occur with mice administered vaccinia virus vaccine [134].

The current experience with vaccinia virus based reservoir targeted vaccines has been excellent to date. Over 75 million doses of the Raboral vaccine for rabies prevention that targets raccoons and foxes have been distributed in Europe and the U.S. [5, 7]. To date, there have been two reports of human infection resulting from contact with the vaccine [150, 151]. In both cases, inoculation of the virus resulted from removal of the vaccine-laden bait from a pet dog. Both case patients had some degree of immunosuppression (pregnancy in one case and immunosuppressive medications for inflammatory bowel

disease in the other). Both cases resulted in the development of skin lesions of vaccinia infection with one patient undergoing excision and drainage of a suspected abscess.

As part of the approval process for the vaccinia virus based rabies vaccine, vaccinia virus has been tested in multiple "non-target" species [7]. Although vaccinia virus is capable of infecting a wide range of species, no long-term detrimental effects were seen on any of the tested species [7]. The occurrence of horizontal gene transfer between vaccinia virus and other Orthopox viruses has been documented in the laboratory. Genetic recombination occurs between viruses of the same genus but not between separate genera [152]. No evidence of recombination with endogenous viruses has been shown with distribution of Raboral. There is no known wild reservoir of orthopox viruses of the same genus as vaccinia virus circulating in the U.S. although in various parts of Europe, related cowpox viruses are found in bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) [153-156].

In chapter 4, we have described the use of *in vivo* imaging to track the virus as it established infection provided several interesting insights into the vaccine. The strain of VV used to construct both VV-OspA and VV-FL is a mouse adapted, neurotropic strain of VV. Administered by gavage, it has previously been shown to localize in gut associated lymphoid tissue (GALT) and spleen within 1 day post infection [22]. Intranasal administration of the VV-FL strain used in our experiments revealed dissemination to lung, brain, liver, and spleen [30]. Our studies with the luciferase expressing VV confirmed that mice administered this strain by gavage developed productive infection of the brain and NALT. However, when ingested orally from baits,

the virus showed no propensity to involve the brain and NALT and was limited to a low grade infection of the intestinal tract. Despite the fact that mice taking oral baits showed similar antibody titers as those receiving gavage, the viral titers were undetectable by culture in NALT, brain, mesenteric lymph nodes and Peyer's patches. This low grade infection may have advantages for the use of this vaccine in the wild as low viral titers would be much less likely to be passed to other animals (e.g. predators that consume an animal soon after bait ingestion).

We also used *in vivo* imaging to assist in rapidly assessing the impact of various manipulations of the vaccine baits on productive viral infection. Based on the differences between responses in gavaged and bait fed mice, we tested changes to the consistency as well as the effects of decreasing gastric acidity. Although none of our manipulations resulted in improved antibody titers to OspA, the *in vivo* imaging system allowed us to rapidly assess the impact of our manipulations on productive viral infection and correlate it to the antibody response. As our goal was the development of improved vaccine formulations and not in discovering the mechanisms of infection, we did not extensively pursue the study of the effects of gastric pH on viral infection as, if the administration of ranitidine intraperitoneally at concentrations known to inhibit gastric acidity did not improve infection, it seemed unlikely that incorporation of an antacid into the bait would be successful. However, it remains possible that other, more effective means of neutralizing the effects of pH on the virus (e.g. microencapsulation of the vaccine) may prove effective in allowing a lowered dose of the vaccine to be effective.

In summary, we have developed VV vectored vaccines against Plague and Lyme disease. Previous approaches to limit Lyme disease have included reducing tick numbers by spraying acaricides on vegetation as well as application of acaricides directly on tick hosts such as mice and deer [127]. While each of these approaches has been shown to be successful in limited settings, none is effective enough to be likely to significantly reduce the incidence of human Lyme disease as a stand-alone intervention. Integrated management strategies combining several different approaches are currently being examined and may show promise in reducing carriage in wildlife reservoirs. Successful development of a reservoir targeted vaccine against *B. burgdorferi* would be an important tool in an integrated strategy for the control/eradication of Lyme disease.

Despite the small incidence of human plague, targeting of *Y. pestis* is valuable because of the severity of the infection. However, the relative paucity of human cases suggests that antigens targeting *Y. pestis* may be best included as part of a vaccine "cocktail" that targets multiple human diseases that utilize the same reservoir to improve the cost/benefit ratio of vaccine deployment. Since vaccinia virus is relatively large and can accommodate multiple inserts, it is an ideal vector to pursue a multi-target reservoir vaccine strategy. The antigens against multiple diseases can also be expressed using individual expression vectors mixed in an appropriate ratio and delivered via single bait. Antigens that would protect against other pathogens like Hantavirus, Babesia, Anaplasma/Ehrlichia or their vectors would be ideal for inclusion in a combined vaccine. Future studies will be needed to determine the design and efficacy of such combination vaccines and also strategies to deploy such multivalent reservoir-targeted vaccines. Also, field studies will be necessary to determine the efficacy and the optimal distribution

strategies of the vaccine and to answer questions about how nesting and communal behaviors of the animals in the wild may affect the uptake of the vaccine.

CHAPTER 6

MATERIALS AND METHODS

Viral, bacterial and mouse strains

VV strain vRB12, which is a mouse adapted WR strain of VV with a deletion of *vp37* gene, was a kind gift of Dr. Bernard Moss (National Institute of Health)[157]. VV-WR, strain vRB12, was used for construction of all recombinant VV vaccines. Vaccinia virus expressing OspA (VV-ospA) was constructed as previously described [134]. VV carrying firefly luciferase reporter gene (VV-FL) was constructed as described [30]. All strains of VV were grown and maintained in HeLa cells as previously described [158].

Y. pestis strain KIM D27 (*Y. pestis ΔpgmΔ*) was used for challenge experiments to test the efficacy of VV-F1-V vaccine. This is a pigmentation negative strain that has a 102-Kb deletion in the pigmentation (*pgm*) locus resulting in attenuation of *Y. pestis* when administered via subcutaneous route [37]. Plasmid pCD1 of *Y. pestis* was used for amplification of LcrV (or V) [159]. *B. burgdorferi* (strain N40) was used for testing all Lyme vaccine experiments. The spirochetes were grown in Barbour-Stoenner-Kelly II (BSK) media at 37°C as previously described [160].

C57BL/6 male mice, 6-8 weeks old, were purchased from Charles River Laboratories (Boston, MA). *Peromyscus leucopus* were obtained from the Peromyscus Genetic Stock Center (Columbia, SC) or from a colony of mice that has been outbred at Tufts University School of Veterinary Medicine. The mice from Tufts University School of Veterinary Medicine were derived from a single breeding pair collected on Martha's Vineyard, MA and were in their 12^{th} generation. These mice were maintained by random inbreeding within cages containing 6-18 animals. All Lyme vaccine tests were performed on 6-8 week old *Peromyscus* mice. For *in vivo* imaging the dissemination of VV in mice,

Balb/c mice were used. All Balb/c mice, 6-8 weeks old, were purchased from Charles River Laboratories (Boston, MA).

Ticks

Ixodes scapularis tick larvae were obtained from National Tick Research and Education Center, Oklahoma State University (Stillwater, OK). *B. burgdorferi*-infected nymphs were generated as previously described [134]. Briefly, uninfected larvae were allowed to feed on *B. burgdorferi*-infected SCID mice to repletion. The engorged larvae were collected and allowed to molt into nymphs in 4-6 weeks at room temperature and 95% relative humidity. Prevalence of *B. burgdorferi* infection in fed larvae was determined by culture and PCR of a portion of the recovered ticks from each batch [134]. Only batches with greater than 85% infection rates were used for subsequent studies.

Construction of VV-F1-V

The F1 gene was amplified from DNA purified from *Y. pestis ΔpgmΔ* using primers caf1-F (5'-ATGAAAAAAATCAGTTCCGTTATC) and caf1-R (5'-GTAGGCTCTAATCATGAATTCTTGGTTAGATACGGTTAC). The V gene was amplified from plasmid pCD1 of *Y. pestis* using primers LcrV-F (5'-ACCGTATCTAACCAAGAATTCATGATTAGAGCCTACGAACAA) and LcrV-R (5'-CCATGGTCATTTACCAGACGTGTCAT). A tissue plasminogen signal sequence was added upstream of F1 fragment by series of PCRs using specific overlapping primers, namely caf1 TPAovrlp (5'-GCTTTCCCATTGCCTGACCAGGGAATACATGGGAGGTTCAGAAGAATGAAAA

AAATCAGTTCCGTTATC) and caf1 TPAovrlp2 (5'-GCTAGCATGAAGAGAGAGCTGCTGTGTGTACTGCTGCTTTGTGGACTGGCTTTCCCATTGCCTGACCAG). The TPA-F1 and V products were cloned into pCR2.1 (Invitrogen Inc., Carlsbad, CA) as per the manufacturer's instructions (Figure 2.1). Clones containing appropriate insert were selected and confirmed by sequencing at the Tufts University Sequencing Facility. TPA-F1 and V DNA were purified from the selected clones using QiaPrep Spin Column (Qiagen Inc.). The TPA-F1 DNA was digested using *Nhe*I and *Eco*RI, while V DNA was digested with *Eco*RI and *Nco*I. The TPA-F1 and V restricted fragments were gel purified using Qiaquick column (Qiagen Inc.) and fused by PCR using primers caf1 TPAovrlp2and LcrV-R. The two fragments were separated by an *Eco*RI restriction site. The fused TPA-F1-V DNA was cloned into pCR2.1 and appropriate clones with the insert were selected and sequenced. The TPA-F1-V DNA was digested with *Nhe*I and *Nco*I. The restricted fragment was ligated to *Nhe*I and *Nco*I digested viral vector pRB21 (kind gift of Dr. Bernard Moss) to form pRB21-TPA-F1-V. The plasmid was transfected into *E. coli* TOP10 cells. Appropriate clones containing the correct insert were selected by restriction mapping and DNA sequencing.

The recombinant vaccinia virus was generated by transfecting pRB21-TPA-F1-V into infected cells as described [157], with the exception that Lipofectamine 2000 (Invitrogen Inc.) was used for transfection as per the manufacturer's instructions in place of $CaCl_2$. Briefly, confluent CV-1 cells (American Tissue Culture Collection, Manassas, VA) were infected with VV-vRB12 for 2 h at 37°C. 10 µl of Lipofectamine reagent was added to 500 µl of serum-free MEM. 5 µg of pRB21-TPA-F1-V DNA was added to prediluted Lipofectamine reagent and incubated for 20 min at room temperature. After removing the

viral inoculum from CV-1 cells, the DNA-Lipofectamine mix was added to the monolayer and incubated at 37°C for 30min. After 30 min, MEM supplemented with 2.5% FBS (MEM-2.5) was added and incubated at 37°C for 3 h. After 3 h, the media was replaced with fresh MEM-2.5 and incubated overnight at 37°C. The cells were then collected and prepared for neutral red plaque selection.

Recombinant virus was selected using neutral red plaque selection assay as described previously [157]. Briefly, virus harvested from CV-1 cells was plated on BS-C-1 cells (American Tissue Culture Collection, Manassas, VA). After 3 days of infection, well-separated plaques were isolated by pipeting infected cells from agarose plugs, while scraping the cells from plaque regions. Each plaque isolate was transferred to fresh tube containing MEM-2.5. The tubes were subjected to 3 freeze/ thaw cycles and trypsinized followed by infection into additional cells. Four rounds of plaque selection assay were performed to ensure isolation of purified recombinant virus.

The recombinant virus was maintained and amplified by infecting HeLa cells. Briefly, monolayers of HeLa grown in multiple 225 cm^2 tissue culture flasks (Corning Inc., USA) were infected with recombinant VV in MEM-2.5. After 3 days of infection, the cells were harvested in minimal volume of MEM. The cells were subjected to 3 freeze/thaw cycles to harvest the virus.

The virus titer was determined using crystal violet staining as described previously [134]. Briefly, confluent BS-C-1 cells are infected with serially diluted virus in MEM-2.5. After 2 days of infection, the media is removed and plaques are visualized by staining with 0.1% crystal violet dye to facilitate counting.

The presence of TPA-F1-V insert in recombinant VV was analyzed by PCR at an annealing temperature of 55°C using primers specific to F1-V insert, namely cafLcrVInt-F (5'-TCAGGATGGAAATAACCACCA) and cafLcrVInt-R (5'-ATGCATTACTGCCATGAACG). To determine presence of any unrecombined vRB12 contaminating the final selection, a PCR was performed using primers specific to *E. coli gpt* segment, namely vRB12Int-F (5'-TTTTAGCGATATAGCCGATGA) and vRB12Int-R (5'-GATGAAGCCTTCGCCATC) at an annealing temperature of 54°C. The *E. coli gpt* segment present in vRB12 is lost during the recombination event.

Preparation of oral baits

The bait formulation was developed by Foodsource Lures Corp. (Alabaster, AL). The bait formulation consists of a proprietary compound natural product and does not require plasticizers or catalysts that may inactivate the vaccine. The formulation has been found durable in wet and arid conditions. The bait matrix withstands rain and will maintain scent attractants for weeks. The bait can be formulated with minimal heating compared with other similar baits. The palatability of the bait has been tested on wild *Peromyscus* mice in the field [136]. Dry, powdered formula was mixed with peanut butter flavoring as an attractant, with or without nut shells and water at 50^{o}C. The ingredients were thoroughly mixed and allowed to cool to 40°C. The desired dose of recombinant VV was added to the semi-solid bait mix and cooled at room temperature to make solid bait cakes.

Stability of VV in oral baits

The stability of VV in the baits was determined under various conditions of temperature, humidity and freeze-thaw environment arising due to temperature variation between day and night. After exposure to the test condition, portions of VV-ospA laden bait cake were processed at different time points to determine the stability of virus. Different regions of the bait were tested (outer surface, inner core) and results combined. Of note, no consistent differences were seen between different areas of the bait under any condition. VV titers per unit weight of bait was measured by crystal violet plaque assay as previously described [141]. Briefly, VV-laden baits were flash frozen in liquid nitrogen, powdered using a pestle and mortar and resuspended in MEM supplemented with 2.5% FBS. The suspension was trypsinized, serially diluted and appropriate dilutions were plated on BS-C-1 cells in a six-well plate. The titers were calculated by crystal violet staining after 2 days of incubation at 37°C.

Mouse vaccination and Determination of antibody response

Cohorts of C57BL/6 male mice were infected by gavage with a dose of 10^7 or 10^8 plaque-forming units (pfu) of VV-F1-V. Cohorts of control C57BL/6 male mice were infected with 10^8 pfu of VV carrying a non-specific insert (VV-ospA) [134].

Peromyscus mice were vaccinated with VV-ospA or a control (VV-vp37) vaccine delivered via oral gavage or offered by oral baits that were placed in cages for the mice to ingest *ad libitum*. For vaccination with oral baits, only one mouse was kept per cage and each cage was supplied with approximately 2g of bait containing 2×10^8 pfu of VV-ospA

or control virus (VV-vp37). All mice represented in the experiments consumed the baits within 48 hrs. Gavaged mice were administered 2×10^8 pfu of VV-ospA or control vaccine (VV-vp37) suspension in MEM.

Serum from C57BL/6 mice was collected by tail-vein bleeding. For *Peromyscus* mice, serum was collected via submandibular bleeding. Serum antibody response was analyzed by western blotting and /or endpoint ELISA.

Western Blotting

The expression of F1-V fusion protein by recombinant VV was analyzed by western blotting. Briefly, a whole cell lysate of infected HeLa cells was obtained as described above. After lysis, the cell debris was removed by spinning cells at slow speed (300 X *g*). The supernatant samples were collected and separated on 10% SDS-PAGE followed by transfer of separated proteins on to a polyvinyldifluoride (Immobilon-P) membrane (Millipore, Bedford, MA). Western blotting was performed as previously described [134], with the exception of an anti-LcrV primary antibody used at a dilution of 1:15,000.

Serum antibody response in VV-F1-V vaccinated mice was also determined by western blotting. Briefly, GST-LcrV protein was run on a denaturing polyacrylamide preparative gel and transferred on to a polyvinyldifluoride membrane. The membrane was incubated with blocking buffer (TBS with 5% milk) at room temperature. The membrane was cut into narrow strips and each strip was incubated overnight with appropriate dilutions of mouse serum at 4°C. The membrane strips were washed three times with TBS buffer plus 0.1% Tween 20 and incubated with a secondary anti-mouse IgG HRP-linked antibody

(1:2000 dilution in TBS). The strips were again washed as earlier described and developed to visualize protein antibody.

Endpoint ELISA

Endpoint ELISA was used to determine the antibody titers generated in mice post-vaccination with VV-F1-V or VV-ospA. Hi-bind 96-well plates (Costar, Corning, NY) were coated with the appropriate protein. For VV-F1-V ELISA, plates were coated with 3µg/ml of GST-LcrV diluted in PBS and incubated overnight at 4°C. In case of VV-ospA ELISA, 2 µg/ml of MBP-ospA in PBS was used. The proteins were removed and the plates were blocked with 1% BSA dissolved in PBS for 1 hour at room temperature. Serum samples were dissolved in PBS to appropriate dilutions. After removing the blocking reagent, the top well of each lane was loaded in duplicate with diluted serum. The samples were serially diluted in equal volume of PBS. Following overnight incubation at 4°C, the samples were aspirated and the plates were washed three times with 0.1% Tween 20 in PBS. The plates with C57BL/6 mice serum were incubated with anti-mouse HRP-linked IgG secondary antibody (1:2000 dilution in PBS) for 1 hour at room temperature. In case of *Peromyscus* mice serum, an anti-*Peromyscus* HRP-linked IgG secondary antibody (1:25000 dilution in PBS) (KPL, Inc. Gaithersburg, MD) was used for the incubation. After incubation, the secondary antibody was aspirated and plates were washed as described above. Plates were incubated with SureBlue TMB peroxidase substrate (KPL, Gaithersburg, MD) for 5 min followed by addition of equal volume of TMB stop solution (KPL, Gaithersburg, MD). Absorbance was measured at 450nm. Unvaccinated mouse serum was used as negative control. The mean of absorbance values

of unvaccinated mouse serum was set as cut-off. The reciprocal of highest dilution above the cut-off was defined as endpoint antibody titer of each serum sample. To minimize variability between plates, a standard sample consisting of a mixture of serum from 5 different VV-F1-V or VV-ospA vaccinated mice that was aliquoted and frozen, was used to normalize readings. For quality control, any plates that showed variations of more than ±20% from the standards were discarded. The serum mix control was serially diluted as described above in each plate.

C57BL/6 infections and challenge with *Y. pestis*

The LD_{50} of *Y. pestis* KIM D27 in C57BL/6 mice given by intranasal route was determined. Three groups of 12 mice each were infected with doses of $2X10^3$, $2X10^4$ and $2X10^5$ cfu bacteria diluted in PBS. Briefly, *Y. pestis* KIM D27 (*Y. pestis ΔpgmA*) was grown overnight in Heart Infusion medium (Difco, Inc.) at 26°C with constant shaking. The overnight culture was diluted in the same medium to an absorbance of 0.2 at 600 nm. The diluted culture was grown as described above to an OD_{600} of approximately 0.8. The bacteria were diluted to selected doses in 30 µl PBS. Mice were anesthetized by isofluorane and infected intranasally with 30 µl dose of bacterial suspension. The mice were observed until moribund or 14 days post challenge, whatever comes first. The LD_{50} was calculated using Reed-Muench method [139, 140].

To determine protective efficacy of the vaccine, VV-F1-V- and control-vaccinated mice were challenged with a dose of 10 times LD_{50} of *Y. pestis* KIM D27 as described above. Dilutions of the bacterial inocula were plated on Heart Infusion agar to determine the actual dose administered. Three independent experiments were performed to determine

the vaccine efficacy. All the procedures were reviewed and approved by the Tufts University Institutional Animal Care and Use Committee.

Determination of VV-ospA efficacy

Efficacy of the VV-ospA was determined for both inhibition of tick acquisition by feeding larvae from infected mice and for inhibition of spirochete transmission to uninfected mice during feeding by infected nymphal ticks.

To test the efficacy against transmission to uninfected mice, mice were provided VV-ospA baits or control (VV-vp37) baits. Antibody response was determined 4 weeks post vaccination. On week 5 post-vaccination, mice were challenged by allowing 6-7 *B. burgdorferi* infected tick nymphal *Ixodes* ticks to feed to repletion as previously described [134]. The fed nymphs were collected and assayed for *B. burgdorferi* infection by culturing their homogenate in BSK media at 37°C. The mice were assayed for *B. burgdorferi* infection by culture of an ear punch in BSK on week 2 and cultures of the ear, bladder and heart at week 4 post-challenge. Presence of *B. burgdorferi* was determined by observing the cultures under microscope. A mouse was defined as infected with *B. burgdorferi* if one or more organ cultures were found positive by darkfield microscopy.

To determine the vaccine efficacy in preventing *B. burgdorferi* acquisition by larvae, we infected *Peromyscus* mice with *B. burgdorferi* by allowing 6-7 infected nymphal ticks to feed on these mice. Infection was confirmed by culture of an ear punch in BSK at week 2-3 after tick infestation. The infected mice were vaccinated with VV-ospA or control by

bait feeding and serum antibody response was determined 4 weeks post-vaccination. To determine VV-ospA efficacy, uninfected *Ixodes* larvae were fed on VV-ospA and control vaccinated mice. The fed ticks were collected and observed for 5 days. After 5 days, the ticks were homogenized and cultured in BSK media [134]. The presence of *B. burgdorferi* was determined by observing the cultures under microscope. All animal procedures were reviewed and approved by the Tufts University Institutional Animal Care and Use Committee.

Bioimaging of VV infection

Balb/c mice were given a single dose of 10^8pfu of VV-FL [30] via oral gavage or fed as oral bait. At various time points, mice were administered luciferin substrate (150 mg/kg per mouse) by intraperitoneal injection and bioluminescence was measured using an IVIS200 imager (Xenogen Corp.). All images were captured after 5 minutes of exposure at a binning parameter of 8 (medium). The images were normalized to a uniform photon flux scale to minimize the variability between images taken at different time points.

To determine the effect of gastric pH on viral uptake via bait, the mice were administered ranitidine (125 mg/kg mouse, every 12 h) by intraperitoneal injection concurrent with placement of bait in the cage. Injections were continued twice daily until the bait was consumed (about 2 days). Bioluminescence at various time points was measured using IVIS200 imager.

Determination of viral titers in mouse tissue

Crystal violet plaque assay was performed to determine the localization of VV infection in various mice tissues. The infection was tested in nasal-associated lymphoid tissue (NALT), brain, spleen, Peyer's patches and mesenteric lymph nodes based on reports about spread of VV virus given through other routes. Briefly, the organs were dissected from a sacrificed mouse, homogenized and resuspended in 1 ml of MEM medium. The tissue homogenate was incubated with equal volume of 0.5% trypsin for 30 min at 37°C and appropriate dilutions were plated on BS-C-1 six well plates. The VV titers were determined by crystal violet titering assay as previously described [141].

Statistics

Kaplan and Meier survival estimates were used for data analyses of mice survival experiments. Log rank test was used to calculate statistical significance between groups wherever applicable.

Fisher's exact test was used to determine if significant differences in experiments that resulted in discrete data points (e.g. positive and negative results for *B. burgdorferi* infection of mice and ticks).

The Mann-Whitney U test was used to calculate statistical differences in experiments that resulted in continuous variables (e.g. luciferase intensity between control and infected mice). The tests used for each experiment is listed in the figure legend.

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