Characterization of a Cynomolgus Macaque Model of Pneumonic Plague Evaluating Vaccine Efficacy
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Abstract

Efficacy of a recombinant plague vaccine (rF1V) was evaluated in cynomolgus macaques (CMs) to establish the relationship between vaccine dosage, antibody titers and survival following aerosol challenge with a lethal dosage of Yersinia pestis (Y. pestis) strain Colorado 92 (CO92). CMs were vaccinated with a range of rF1V dosages using a three-dose schedule (Days 0, 56 and 121) to provide a range of survival outcomes. The humoral immune response following vaccination was evaluated in anti-rF1, anti-rV and anti-rF1V Bridge ELISAs. Animals were challenged via aerosol exposure on Day 149. Vaccine dosage levels and antibody response levels were each significantly associated with probability of survival (p<0.0001) in CMs. Vaccination also decreased signs of pneumonic plague in a dosage-dependent manner. There were statistically significant correlations between vaccine dosage and time to onset of fever (p<0.0001), time from onset of fever to death (p<0.0001), time to onset of elevated respiratory rate (p=0.0003) and time to onset of decreased activity (p=0.0251) after infection in animals exhibiting these clinical signs. Delays in the onset of these clinical signs of disease were associated with higher dosages of rF1V. Immunization with ≥ 12 μg rF1V resulted in 100% survival in the CMs. Since both vaccine dosage and anti-rF1V antibody titers correlate with survival, rF1V Bridge ELISA titers can be used as a correlate of protection.
Keywords: cynomolgus macaque, vaccine efficacy, pneumonic plague, *Yersinia pestis*

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Introduction

Plague is caused by the Gram-negative bacterium *Y. pestis*. While natural outbreaks of disease occur in wildlife populations, humans are most often incidental victims in the life cycle of the bacterium in rodents. Human disease may also result from contact with blood or tissues of infected animals or exposure to aerosolized droplets containing bacteria (1, 2). Three forms of the disease exist and are believed to be dependent upon whether the bacteria enter the lymph nodes (bubonic), the bloodstream (septicemic) or the lungs (pneumonic). Primary pneumonic plague is caused by inhalation of *Y. pestis*. It is the least common form of the disease (2%); however, it is the most serious form of the disease due to the high fatality rate and the potential for direct human-to-human transmission via spread of respiratory droplets (3). Due to the potential person-to-person transmission of pneumonic plague, the Centers for Disease Control and Prevention (CDC) considers this disease to be a serious potential threat and have classified *Y. pestis* as a Category A (Tier 1) bioterrorism agent (4).

Aerosol dissemination represents the most likely modern-day scenario for the use of *Y. pestis* as a biological weapon with a battlefield scenario or terrorist attack likely resulting in a significant number of pneumonic plague fatalities. In 1970, the World Health Organization modeled an attack scenario which predicted that the airborne release of 50 kg of *Y. pestis* over a city of 5 million would result in 150,000 cases of plague, of which 36,000 cases would be fatal (5).

Currently, no licensed vaccines against plague are available for human use in the U.S. The previously available U.S. Pharmacopeia (USP) vaccine was a killed, whole cell vaccine (KWC). Although its efficacy was never confirmed in controlled clinical studies, observations of
vaccinated humans and a number of animal studies suggested that the USP vaccine was effective against bubonic plague, but had limited efficacy against pneumonic plague (6-10). Further, this vaccine produced a number of moderate to severe side effects, ranging from mild headache to severe malaise and fever (9,10). Live-attenuated vaccines have been used in several countries, but were not licensed in the U.S. due to their reactogenicity (11). To overcome the limited efficacy and reactogenicity associated with KWC and live-attenuated plague vaccines, respectively, the *Y. pestis* F1 and V antigens were identified as promising components for a new generation of recombinant protein vaccines.

Early proof-of-concept studies investigated the immunogenicity and efficacy afforded by vaccination with the individual F1 and V antigens and found that each provides some level of protection from challenge. However, the combined use of the F1 and V antigen was found to have an additive protective effect and was more effective than single-antigen vaccines in mouse models of pneumonic plague (7,8,12).

A recombinant plague vaccine (rF1V) is currently in advanced development by the U.S. Department of Defense to provide pre-exposure prophylaxis to military personnel ages 18 to 55 years against battlefield exposures to aerosolized *Y. pestis* (13). The vaccine contains both the F1 and V antigens fused into a single protein which is adsorbed to aluminum hydroxide adjuvant to enhance the immunogenicity of the rF1V protein. Efficacy of rF1V cannot be determined directly in humans due to the ethical implications of conducting inhalational *Y. pestis* challenge studies. In addition, the incidence of pneumonic plague in the general population is extremely low, making “field studies” impractical. Therefore, licensure of rF1V will rely upon immunogenicity and efficacy data obtained in nonclinical studies, immunogenicity and safety data from clinical studies and meeting the requirements of the Animal Rule (14). The CM was
chosen as a suitable nonhuman primate model for pneumonic plague based upon previous studies which demonstrated that CMs exhibit a clinical course of disease similar to that described for humans (15). Further, proof-of-concept model development studies indicated that CMs responded to plague vaccines and were protected from disease following lethal aerosol challenge with *Y. pestis* (15,16).

We initially developed and characterized the CM model of pneumonic plague (17) following recommendations described in FDA draft guidance titled, “Essential Elements to Address Efficacy Under the Animal Rule, Draft Guidance for Industry (January 2009)” (18). The results of this initial model development study showed that a post-exposure rise in temperature, loss of the temperature diurnal rhythm, bacteremia and increased heart and respiration rates, followed by a decrease in activity strongly correlated with a lethal outcome. The pathology in the lungs of all CMs that died included pneumonia, pulmonary congestion, fibrinous pleuritis and neutrophil infiltration. This is consistent with the pathology described for human pneumonic plague (16).

The vaccination and challenge studies described here were designed to further develop the CM model. The studies were designed to establish the relationship between rF1V vaccine dosage, Bridge ELISA titers and survival in CMs with the goal of assessing the utility of the Bridge ELISA as a correlate of protection, which is an important component in bridging clinical and nonclinical data to predict clinical benefit. Additionally, the effect of rF1V vaccine dosage on disease progression following aerosol challenge was evaluated. These studies complete the planned development studies characterizing the CM model of pneumonic plague. The results indicate the CM is an appropriate model for demonstrating efficacy of rF1V according to the Animal Rule.
Materials and Methods

Animals. Indonesian origin CMs, 80 male and 41 female, were procured from a licensed USDA approved vendor and weighed at least 2 kg at the time of vaccination. All animals tested negative for the presence of pre-existing antibody titers to the rF1V vaccine antigen. They were also tuberculin test negative and seronegative for Simian T-cell Leukemia Virus (STLV), Simian Immunodeficiency Virus (SIV), Simian Retroviruses (SRV-1 and SRV-2, SRV-3 and SRV-5 via PCR) and Herpes B. The study was approved by the Battelle Institutional Animal Care and Use Committee and the Animal Care and Use Review Office of the U.S. Army Medical Research and Material Command and conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (19).

Test and Control Articles. Current good manufacturing practices grade rF1V Drug Product (160 μg/mL), formulated in 5 mM Na/K phosphate buffer containing 150 mM NaCl and 1.3% (w/v) Alhydrogel,® was diluted in sterile phosphate buffered saline (PBS), without additional adjuvant, to prepare vaccine dosages ranging from 0.016 μg to 20 μg per CM. Vaccine dilutions were prepared on each day of vaccination. Control animals received sterile PBS.

Study Design. Three CM studies were planned and conducted in a stage-wise approach such that results from one study allowed for the design and selection of vaccine dosage for subsequent studies. The first two studies were dosage ranging studies (N=21 and N=44) and designed to generate a range of survival outcomes to evaluate the relationship between vaccine dosage, antibody response and survival. The third study (N=56), an expanded efficacy study, was conducted to further evaluate these relationships with the goal of increasing statistical confidence in the study data. Group sizes and vaccine dosages were selected for each study based upon statistical analysis of the cumulative results, and based upon probabilistic simulation of most
likely subsequent outcomes. For all studies, CMs were randomized into study groups according to body weight and age with males and females randomized separately. On Days 0, 56 and 121 CMs were administered vaccine or saline in a 0.5 mL volume by the intramuscular route. To control for bias, vaccine and control materials were labeled to identify each individual CM, and included vaccination day and date, but not vaccine dosage or study group information. Technicians performing CM vaccinations, observations, sample collections and gross necropsy were blinded regarding the vaccination status of the CMs. Blood samples were collected to evaluate antibody titers on Days 0, 14, 28, 56, 70, 84, 98, 121, 135 and 149. Blood samples drawn on the day of vaccination and the day of challenge were collected prior to conducting those activities. The target challenge dosage of 229 LD$_{50}$ (5.5 x 10$^3$ cfu) $Y$. pestis CO92 was selected for these studies based upon an earlier model development study that established an LD$_{50}$ of 24 cfu (17). Animals were challenged by aerosol inhalation on Day 149 and observed for 21 days post-challenge.

**Telemetry.** Animals in the two dosage ranging studies (N=65) were implanted with telemetry transmitter units (D70-PCT, Data Sciences International) for collection of body temperature, activity and respiration. The cardiac lead was not used and was trimmed and tied off according to the manufacturer’s instructions. Implantation surgery was performed as previously described (17). An approximate five week recovery period was given prior to placing the animals on study. To conserve battery life, telemetry units were not turned on during the vaccination period. Animals in the expanded efficacy study were not implanted with telemetry units.

**Immune Response.** Bridge ELISAs were performed as previously described (20) to determine the levels of anti-rV, anti-rF1 and anti-rF1V antibodies elicited by vaccination. The Bridge ELISA uses an assay format that allows direct comparison across clinical and nonclinical
samples and is therefore considered species neutral. Antibody binding to the rF1V antigen coated on the plate is detected using biotinylated rF1V antigen rather than a biotinylated species-specific antibody. The assay is considered semi-quantitative because the standard curve is generated using chicken IgY antibodies to allow measurement of polyclonal antibodies to \( Y. \) \textit{pestis} antigens in serum. Results are calculated using a four parameter logistic (4PL) curve fit. The assay limits of detection are 5 and 320 U/mL with limits of quantitation between 10 and 160 U/mL.

**Challenge Material.** Four days prior to challenge a vial of CO92 Working Cell Bank was thawed and streaked for isolation on Congo Red agar (CRA). The plate was incubated at 26 ± 2°C for 36 to 72 hours. Several CRA pigmented colonies were selected and suspended in sterile heart infusion broth (HIB) until the optical density of the suspension at 600 nm (OD\(_{600}\)) was 0.200 ± 0.05. The suspension was diluted 1:100 into sterile HIB and the culture was incubated at 26 ± 2°C with shaking for approximately 24 hours. Bacteria were collected by centrifugation, washed and resuspended in PBS containing 0.05% (wt/vol) gelatin and 9.7% (wt/vol) \( \alpha-\alpha \)-trehalose (PBSGT), to an OD\(_{600}\) of 2.56 ± 0.2, which corresponds to a reference concentration of approximately 2.05×10\(^9\) cfu/mL. The bacteria were further diluted in PBSGT to the appropriate nebulizer concentration required to achieve the target aerosol challenge.

Challenge material was characterized for titer, phenotype, purity and Gram stain. Titer and phenotype were determined by plating dilutions of the challenge material in triplicate on Tryptic Soy agar (TSA) and CRA, respectively. The plates were incubated at 28°C ± 2°C for 36 to 96 hours. Colony counts were performed to determine titer and percent pigmentation. Purity was evaluated by streaking a sample for single colony isolation on the following media types: Tryptic Soy Agar with 5% Sheep Blood (TSAB), MacConkey Agar (MAC), Phenylethyl Alcohol Agar
(PEA), and Cefsulodin Irgasan Novobiocin Agar (CIN). A set of plates consisting of each media type were incubated at different three temperatures: 28°C ± 2°C, 37°C ± 2°C and 23°C ± 2°C. Colony morphology was recorded for plates incubated at the three temperatures daily for three days (72 ± 2 hours), with the exception of the MAC plates which were discarded after 48 hours. After 72 hours, the sets of plates incubated at 28°C ± 2°C and 37°C ± 2°C were also discarded. The remaining set of TSAB, PEA and CIN plates was incubated at 23°C ± 2°C for an additional 11 days and the colony morphology was recorded at 7 and 14 days. Gram staining was performed using routine procedures.

**Aerosol Challenge.** CMs were challenged on Study Day 149 by head-only aerosol exposure. Prior to exposure, each CM was anesthetized with Telazol (2-6 mg/kg), moved into a Class III biological safety cabinet and placed in a plethysmograph. Body plethysmography was performed real-time on each CM during aerosol exposure to measure tidal volume, total accumulated tidal volume and minute volume.

Exposure dosages were determined based on the calculated aerosol concentration of *Y. pestis* CO92 in the test atmosphere of the exposure chamber and the total volume of test atmosphere air inhaled by the CMs measured via real-time plethysmography using Buxco BioSystems software (Buxco Research Systems, Wilmington, NC). The aerosol concentration of *Y. pestis* CO92 in the exposure chamber was determined by viable plate counts performed on aerosol samples collected with the glass impinger (Model 7541, Ace Glass, Inc., Vineland, NJ). Samples were diluted as necessary and plated in triplicate on TSA plates. The aerosol particle size was determined during each exposure using an aerodynamic particle sizer spectrometer (Model 3321, TSI Incs., Shoreview, MN).
The preparation and use of *Y. pestis* challenge material, CM challenges and all post-challenge activities were conducted under BSL3/ABSL3 conditions in a facility registered with the CDC Select Agent Program. The facility also meets requirements of the DoD Biological Select Agent Transfer program.

**Clinical Observations.** Trained technical personnel observed the CMs throughout the study. CMs were observed twice daily throughout the vaccination period. Following challenge, the CMs were observed three times a day, at approximately 8 hour intervals, throughout the 21-day post-challenge observation period for clinical signs of disease. Time-to-onset of clinical signs, as measured by telemetry or signs of disease recorded by technical personnel was documented in the study records.

**Bacteria in Blood and Tissues.** Approximately 2 mL of blood was collected from either the femoral artery or vein of the CMs for bacteriological culture. In the first dosage ranging study, samples were collected approximately 6 hours following the onset of fever and continued daily for 7 days, whereas samples in the second study were collected on post-challenge days 2 through 9 regardless of onset of fever. In the expanded efficacy study, blood was collected from the control CMs at the time of death or euthanasia and from survivors at the end of the post-challenge observation period (Day 21). Blood was collected in tubes containing sodium polyanethol sulfonate as an anticoagulant and mixed thoroughly. Within 4 hours of collection, the entire sample volume was inoculated into 40 mL BACTEC blood culture bottles (Becton Dickinson Peds Plus/F). The bottles were incubated at 28°C ±2°C for 24 to 48 hours. Samples from the blood culture bottles were streaked onto CRA and CIN agars and incubated at 28°C ± 2°C for 36 to 96 hours. Following incubation, the agar plates were visually inspected for colony morphology consistent with *Y. pestis*.
To determine bacterial burden in tissue, samples of spleen, liver, kidney, and lung were collected at the time of necropsy and prior to tissue fixation in all studies. The samples were homogenized, plated on CIN agar and incubated 28°C ± 2°C for 36 to 96 hours to determine concentration of *Y. pestis* bacteria per gram of tissue.

**Necropsy.** Gross necropsies were performed on all CMs that succumbed or were found moribund and euthanized following aerosol exposure. Necropsies were also performed on CMs that survived to the end of the 21 day post-challenge observation period. Tissues collected included the liver, lungs, spleen intrathoracic lymph nodes and any gross lesions. Sections of lung, intrathoracic lymph nodes and any gross lesions were examined by histopathology.

Tissues were processed and approximately 5-micron sections were made and stained with hematoxylin and eosin stain for microscopic examination.

**Statistical Analysis.** Data collected from all three studies were combined for statistical analysis using Stata Statistical Software, Release 12. Logistic regression models were used to evaluate the relationship between Bridge ELISA titers and survival as well as between vaccine dosage and survival. One-sided Fisher’s Exact tests were performed to assess survival proportions in the vaccinated groups compared to controls. Time-to-death data combined with survival data were analyzed to determine if there were differences in protection for the study groups based on a time-to-death model.
Results

Challenge Material. Characterization of the challenge material for all three studies indicated that the average concentration of the overnight cultures was $1.65 \times 10^9$ cfu (range $8.83 \times 10^8$ to $5.53 \times 10^9$ cfu). Further, the cultures were Gram negative, pure cultures of *Y. pestis* with >99% pigmented colonies.

Aerosol Exposures. The mean estimated inhaled dosage of *Y. pestis* across all three studies was $5.54 \times 10^3$ cfu (range $3.97 \times 10^2$ to $2 \times 10^4$ cfu) which represents 231 LD$_{50}$ (range 17 to 833 LD$_{50}$). The mass median aerodynamic diameter of the particles across all aerosol exposures ranged from 1.65 to 2.87 μm. Aerosols with these particles sizes are capable of reaching into the alveoli of the lung which was the target area for deposition in the lung (21).

Antibody Response. Data from all three studies were combined to evaluate the antibody response following vaccination. Since the kinetics of the anti-rF1, anti-rV and anti-rF1V antibody responses following vaccination were similar; the results presented herein focus on the anti-rF1V antibody response as the representative response (Figure 1). Detection of anti-rF1V antibody titers was observed in CMs vaccinated with dosages ≥0.4 μg beginning two weeks following the first vaccination. The geometric mean concentration (GMC) of anti-rF1V antibody titers decreased prior to administration of the second dose on Day 56, but was above baseline levels. A robust antibody response was observed in all groups that received >0.4 μg of rF1V two weeks (Day 70) following the second vaccination with increases in the GMC anti-rF1V antibody titers compared to those at Day 56 of approximately 2 logs. A decrease from peak antibody titers was observed by Day 84; however, antibody titers did not decline to the levels observed just prior to the second vaccination. Administration of the third vaccination on Day 121 increased GMC anti-rF1V antibody titers slightly higher than the second vaccination. Anti-rF1V antibody
titers were not detected in CMs vaccinated with rF1V dosages ≤0.08 μg or sham vaccinated control CMs.

**Survival.** A summary of vaccine dosage, percent survival, mean time-to-death and GMC anti-rF1V antibody titers at the time of challenge are shown in Table 1. Administration of the rF1V vaccine to CMs using a three-dose schedule with dosages ranging from 0.0016 to 20 μg demonstrated antibody dependent survival and elicited a range of survival outcomes following lethal aerosol challenge. A significant association between vaccine dosage and survival (p<0.0001) was observed, with higher vaccine dosages associated with higher probabilities of survival. The proportion of CM survival in groups vaccinated with ≥ 4μg was significantly greater than that of the control group in which 2 of 20 CMs survived following challenge.

Further, a statistically significant (p<0.0001) vaccine dosage-dependent response was observed in all three Bridge ELISAs (rF1, rV and rF1V). Similarly, the association between Bridge ELISA titers (anti-rF1, anti-rV and anti-rF1V) on Day 149 and survival was statistically significant (all p-values <0.0001). Logistic regression curves illustrating these relationships, using anti-rF1V as the representative response, are shown in Figure 2. Pairwise comparisons assessing time-to-death and overall survival between the groups shows a significantly delayed mortality between groups vaccinated with ≥ 4 μg and the controls (Table 2).

**Clinical Observations.** Clinical signs associated with pneumonic plague, such as fever, coughing, changes in respiration, lethargy and hunched posture were observed during the post-challenge period. There was a statistically significant difference between survivors and non-survivors (p<0.05) in the frequency of clinical signs as determined by Fisher’s Exact Test (Table 3). Increased incidence in clinical signs of hunched posture and lethargy trended with lower dosages of rF1V (data not shown).
Telemetry. An increase in body temperature was the first telemetry parameter exhibiting a change following challenge. Fever was identified as an increase in body temperature ≥1.5 °C above the animal’s baseline for > 2 hours. Mean temperature changes from baseline plotted over time-post challenge are presented for all vaccine dosage groups in Figure 3a. Thirty-three (25 vaccinated and 8 controls) of 65 CMs in the dosage ranging studies presented with fever and 29 (21 vaccinated and 8 controls) succumbed to challenge. The mean time to onset of fever was 63 hours post-challenge (range 46.2 to 155.3 hours) with a mean time-to-death following onset of fever of 55.7 hours (range 33.3 to 87.8 hours). Statistically significant correlations between vaccine dosage and time to onset of fever (p<0.0001) and time from onset of fever to death (p<0.0001) were observed. None of the CMs vaccinated with 16 or 20 μg of rF1V developed fever. Beginning 49 hours post-challenge, group mean temperatures of control CMs and those vaccinated with ≤0.4 μg of rF1V were elevated >2°C. A similar increase in temperature was delayed 24 hours in CMs vaccinated with 2 μg of rF1V. Four vaccinated CMs developed transient fever post-challenge. Two of the CMs received vaccine dosages 0.016 and 0.4 μg with onset of fever observed 54.5 and 47.4 hours, respectively, following challenge. The other two CMs were vaccinated with 12 μg and developed fever 82.3 and 155.3 hours post-challenge. Body temperature in three of the four CMs returned to baseline by Day 6 post-challenge. Fever in the fourth CM (155.3 hours) resolved to baseline by Day 9. All four of these CMs survived to the end of the post-challenge observation period.

Statistically significant dosage-response relationships were observed between vaccine dosage and onset of respiratory changes such as increased respiratory rate (p≤0.0003). As vaccine dosage increased, hours to onset of increased respiratory rate became longer (Figure 3b).
A statistically significant relationship was also observed between increasing vaccine dosage and a delay in decreased activity (p=0.0251). Activity levels in the CMs post-challenge were generally reduced relative to baseline levels in the controls and groups vaccinated with ≤4 μg of rF1V (Figure 3c). The reduction in activity relative to baseline was especially evident in the 0.08 μg vaccine dosage group. Group mean activity levels generally returned to pre-challenge baseline levels by day 6 post-challenge, although some changes in activity continued in surviving CMs until the end of the post-challenge observation period.

**Bacteremia and Tissue Burden.** All CMs that succumbed to challenge had positive blood cultures (data not shown). Two vaccinated CMs (0.4 and 0.016 μg) had a positive blood culture for one day (Day 3), but survived. The two surviving controls were not bacteremic. Increasing dosages of the rF1V vaccine reduced the numbers of CMs that were bacteremic and in those vaccinated CMs that succumbed, delayed the onset of bacteremia. The proportion of CMs vaccinated with 4 μg (2/9), 8 μg (0/10), 12 μg (0/9) or 16 μg (0/6) of rF1V that became bacteremic by Day 3 post-challenge and succumbed were statistically significantly lower compared to the proportion of control CMs (8/8) with positive blood cultures (p-values all <0.05). Blood cultures collected from survivors at the end of the 21-day post-challenge observation period were all negative.

All CMs that succumbed to challenge had >10^3 bacterial cfu/g in lung, spleen, liver and kidney tissue samples. The lungs consistently exhibited the highest quantities of bacteria, followed by the spleen, liver and kidneys (Table 4). The differences in tissue burdens between vaccinated and control CMs that succumbed was not statistically significant as determined by t-test (p=0.8612).
Necropsy and Histopathology. Gross lesions in animals that died following aerosol challenge included mottled lung discoloration and/or discolored intrathoracic lymph nodes and were present regardless of treatment (vaccinated or controls). These lesions correlated with microscopic observations of suppurative inflammation, fibrin exudation, hemorrhage, necrosis and edema in the airways, and interstitium of the lungs and within the intrathoracic lymph nodes, as well as intralesional rod-shaped bacteria with bipolar staining, all consistent with *Y. pestis* (27). There was a trend between frequency of gross lung lesions and vaccine dosage. The majority of lesions were found in the lungs of CMs vaccinated with dosages ≤ 8 μg (Table 5). Among CMs that succumbed, there were no major differences in lesion incidence and/or severity between the vaccinated and control groups of CMs. Lesions were not observed in CMs that survived to the end of the post-challenge observation period.
Discussion

The rF1V efficacy studies described here were conducted to further develop the CM model for use in testing the efficacy of rF1V according to the requirements of the FDA Animal Rule. Vaccination with rF1V elicited an immune response that provided protection from lethal aerosol challenge with *Y. pestis*. Our study results are similar to those reported by other groups conducting proof-of-concept studies showing that rF1 and rV based vaccines against plague elicit protective immune responses in CMs following aerosol challenge (15,22,23). However, in contrast to these proof-of-concept studies, that most often evaluated only one vaccine dosage, our studies evaluated a broad range of vaccine dosages. This provided the opportunity to evaluate the relationship between vaccine dosage and Bridge ELISA titers across a range of survival outcomes and allowed us to identify statistically significant correlations between vaccine dosage, anti-rF1V antibody titers and probability of survival. Combining all study data provided a statistically precise estimate of these relationships supporting the use of the rF1V Bridge ELISA antibody titers as a correlate of protection.

Our earlier model development study (17) identified fever, as measured by telemetry, as the first clinical sign of disease following aerosol challenge with CO92. Following onset of fever, increased respiration and decreased activity level (as measured by telemetry), hunched posture and bacteremia were observed in CMs that succumbed following challenge. Therefore, in the current studies we sought to determine whether rF1V vaccination delayed the onset or ameliorated signs of pneumonic plague in CMs.

Four vaccinated CMs in the dosage ranging studies developed transient fever, but survived challenge. Two of these CMs received low vaccine dosages (0.0016 and 0.4 μg) and were bactermic on Day 3, but tested negative on all subsequent days. At the time of challenge,
the CM vaccinated with 0.0016 μg rF1V had no detectable anti-rF1V antibody titers, whereas the CM vaccinated with 0.4 μg rF1V had an anti-rF1V antibody titer of 127 U/mL. Given the low anti-rF1V antibody titer in one CM and lack of titers in the other, survival of these two CMs is believed to be associated with individual animal variability. The other two surviving CMs were vaccinated with 12 μg rF1V and never developed bacteremia. Anti-rF1V antibody titers in these two CMs were 3,584 and 7,290 U/mL at the time of challenge which suggests the immune response elicited by rF1V limited infection and thus, prevented bacteremia.

Post-challenge clinical observations were normal in two of the four CMs with transient fever, whereas the other two CMs presented with diarrhea and/or occasional hunched posture. In the CM vaccinated with 0.4 μg, hunched posture was coincident with a transient fever. The occurrence of diarrhea in the other CM was observed periodically during the vaccination period as well as the post-challenge observation period and therefore not considered study related. This CM also presented with occasional hunched posture post-challenge, but was not associated with transient fever. The relevance of these clinical observations in the four surviving CMs is not known. Hunched posture, most often observed just prior to death, was accompanied by other clinical signs such as respiratory distress, lethargy, cough or not eating and was consistent with observations in our model development study (23). Vaccine dosage dependent responses were associated with delays in other parameters measured by telemetry in the dosage ranging studies, such as elevated respiratory rate and decreased activity. This provides further support that rF1V vaccination can provide protection against pneumonic plague.

All CMs in the dosage ranging and expanded efficacy studies that became bacteremic, with the exception of the two animals discussed above, succumbed to challenge. There was no significant difference in the proportions of animals having positive blood cultures in the low
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385 dosage vaccine groups (< 2 μg) compared to the control group suggesting these low vaccine
386 dosages provided little benefit in ameliorating disease. Further, the concentration of Y. pestis in
387 lung, liver, spleen and kidney of vaccinated and control CMs that succumbed to challenge were
388 not different between these groups. This suggests that once the bacteria reach the bloodstream
389 and disseminate to these organs, low level vaccine dosages did not induce an immune response
390 sufficient to combat infection.

The observed survival in 2 of 12 control CMs in the expanded efficacy study was
391 surprising given the estimated inhaled dosages of 272 LD$_{50}$ (6.52 x 10$^3$ cfu) and 301 LD$_{50}$ (7.22 x
392 10$^3$ cfu) in these animals and survival of control animals was not observed in the two dosage
393 ranging studies. An examination of the aerosol exposure system parameters indicated the system
394 operated as expected during the challenge of these CMs. Neither animal had anti-rF1V titers at
395 baseline, or at any of the collection points throughout the vaccination period. However, Bridge
396 ELISA titers measured at the end of the 21-day post-challenge observation period in these CMs
397 yielded anti-rF1V antibody titers of 376 and 1480 U/mL indicating the animals were exposed to
398 Y. pestis and mounted an immune response following challenge. Other groups have also
399 reported observations of control survival during vaccine efficacy studies conducted in CMs. In a
400 study by Cornelius et al. (22), 1 of 4 controls CMs survived following challenge with an inhaled
401 dosage of 1.45 x 10$^4$ cfu (216 LD$_{50}$) of CO92, while three animals that succumbed to challenge,
402 received inhaled dosages ranging from 1.27 x 10$^4$ to 2.3 x 10$^4$ cfu (189 to 343 LD$_{50}$). Quenee et
403 al. (24) reported 25 and 50% survival in control CMs following exposure to 250 and 50 LD$_{50}$,
404 respectively. Individual challenge data and assessments of rF1V antibody titers pre and post-
405 challenge are not provided for all the control animals in these studies, making interpretation of
406 these data difficult. Based on these observations, study designs for efficacy studies in CMs

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should consider low-level control survival in determining group size to ensure the study is appropriately powered to determine survival differences between vaccinated and control groups.

Our studies describe the further development of the CM model to evaluate rF1V vaccine efficacy. Data generated in these studies determined that Bridge ELISA titers and vaccine dosage were highly statistically significant predictors of protection (p<0.0001) and support the use of the anti-rF1V Bridge ELISA titers as a correlate of protection. Vaccination with rF1V was shown to ameliorate disease in a dosage dependent manner. The data support the use of the CM model and provide important information that will be used to plan the design of future pivotal efficacy studies that will play an important role in vaccine licensure using the FDA Animal Rule.
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14. New Drug and Biological Drug Products; Evidence Needed to Demonstrate Effectiveness of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible, Federal Register, Vol 67, No. 105, Friday May 31, 2002.


FIGURE LEGENDS

Figure 1. Group GMC rF1V Bridge ELISA titers showing the kinetics of the immune response following vaccination on Days 0, 56 and 121 and just prior to challenge on Day 149. The vaccine dosages (μg) are shown in the legend on the right side of the graph. Detectable antibody responses were not observed in CMs vaccinated ≤0.08 μg and data at or below this level overlap in the graph. The error bars represent the standard error of the mean.

Figure 2. Logistic regression curves showing the relationship between a) anti-rF1V Bridge ELISA titers and probability of survival and b) vaccine dosage and probability of survival. Both were highly statistically significant predictors of protection (p<0.0001).

Figure 3. Telemetry data for a) temperature, b) respiratory rate and c) activity are shown as group means over eight hour intervals. Each plot shows the mean change from baseline; the zero ordinate represents no change from normal baseline values.
FIGURE 1

[Graph showing geometric mean rF3V Bridge ELISA Titer, U/mL over study days for different concentrations.]
FIGURE 2

a)

b)
### TABLE 1 Summary of GMC (GSDC) rF1V Bridge ELISA Values and Associated Survival

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine Dosage, µg</th>
<th>GMC (GSDC) rF1V Bridge ELISA titers, U/mL</th>
<th>Mean Time-to-Death, hours</th>
<th>Percent Survival (Survival/Total)</th>
<th>p-value Survival Comparison to the Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>11,880 (1.168)</td>
<td>NA</td>
<td>100 (4/4)</td>
<td>0.0071&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>8,090 (1.306)</td>
<td>NA</td>
<td>100 (6/6)</td>
<td>0.0009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>5,262 (1.552)</td>
<td>NA</td>
<td>100 (9/9)</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>4,156 (2.060)</td>
<td>81.3</td>
<td>88 (22/25)</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2,027 (2.264)</td>
<td>102.8</td>
<td>63 (15/24)</td>
<td>0.0023&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>886 (2.542)</td>
<td>125.2</td>
<td>29 (6/21)</td>
<td>0.5375</td>
</tr>
<tr>
<td>7</td>
<td>0.4</td>
<td>50 (9.164)</td>
<td>111.1</td>
<td>20 (1/5)</td>
<td>1.0000</td>
</tr>
<tr>
<td>8</td>
<td>0.08</td>
<td>&lt;10 (NA)</td>
<td>118.8</td>
<td>0 (0/3)</td>
<td>1.0000</td>
</tr>
<tr>
<td>9</td>
<td>0.016</td>
<td>&lt;10 (NA)</td>
<td>102.5</td>
<td>25 (1/4)</td>
<td>1.0000</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>&lt;10 (NA)</td>
<td>88.7</td>
<td>10 (2/20)</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>Survival is significantly greater than that of the control group as determined by Bonferroni-Holm adjusted one-sided Fisher’s Exact Test p-value <0.05; NA=Not Applicable
**TABLE 2** Bonferroni-Holm Adjusted p-values from the Pairwise Log-rank Tests Comparing of Time-to-Death and Overall Survival between Groups

<table>
<thead>
<tr>
<th>Vaccine Dosage, μg</th>
<th>0.08</th>
<th>0.4</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.016</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.8089</td>
<td>0.0019*</td>
<td>0.0755</td>
<td>0.3212</td>
<td>0.8089</td>
<td>1.0000</td>
</tr>
<tr>
<td>0.08</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.0864</td>
<td>0.0001*</td>
<td>0.0059*</td>
<td>0.0621</td>
<td>0.2717</td>
<td>1.0000</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>1.0000</td>
<td>0.5937</td>
<td>0.0002*</td>
<td>0.0361*</td>
<td>0.2054</td>
<td>0.6294</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.7044</td>
<td>0.0009*</td>
<td>0.0411*</td>
<td>0.2262</td>
<td>0.6757</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.8089</td>
<td>0.8089</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.0010*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>&lt;0.0001*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.0014*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.0221*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1421</td>
<td></td>
</tr>
</tbody>
</table>

*Comparisons of time-to-death and overall survival between the groups were significantly different at the Bonferroni-Holm adjusted 0.05 level of significance. The survival rate for vaccine dosages of 4, 8, 12 and 16 μg were all significantly different than that of the control group. The survival rate in the 8 μg dosage group was significantly different than vaccine dosages ≤ 2 μg. Similarly, the survival rate in the 12 μg dosage group was significantly different than vaccine dosages ≤ 2 μg, except for the 0.0016 μg where the p-value is close to significance. The survival rates in the 16 and 20 μg vaccine dosage groups despite having 100% survival, with one exception (20 μg and control group), were not statistically different than groups vaccinated with dosages ≤ 2 μg. This appears to be the result of smaller samples sizes in the highest vaccine dosage groups.
TABLE 3  Incidence of Clinical Signs

<table>
<thead>
<tr>
<th>Clinical Signs</th>
<th>Survivor (n=66)</th>
<th>Non-Survivor (N=55)</th>
<th>Vaccinated (n=37)</th>
<th>Controls(^a) (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunched Posture</td>
<td>23% (15/66)</td>
<td>62% (23/37)</td>
<td>100% (18/18)</td>
<td></td>
</tr>
<tr>
<td>Fever(^b)</td>
<td>11% (4/35)(^c)</td>
<td>97% (29/30)(^d)</td>
<td>100% (18/18)</td>
<td></td>
</tr>
<tr>
<td>Lethargy</td>
<td>2% (1/66)</td>
<td>78% (29/37)</td>
<td>72% (13/18)</td>
<td></td>
</tr>
<tr>
<td>Changes in Respiration(^e)</td>
<td>0% (0/66)</td>
<td>30% (11/30)</td>
<td>17% (3/18)</td>
<td></td>
</tr>
<tr>
<td>Cough</td>
<td>5% (3/66)</td>
<td>30% (11/30)</td>
<td>6% (1/18)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Two control animals survived challenge and had no clinical signs of disease.

\(^b\)Fever was monitored in the two dosage ranging studies only.

\(^c\)This represents 4 of 35 CMs with transient fever that resolved.

\(^d\)Onset of fever was not determined in one vaccinated CM due to a malfunction with the telemetry transmitter.

\(^e\)Changes in respiration included wheezing, labored breathing and changes in respiratory rate.
TABLE 4  Concentration of *Y. pestis* in Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean Tissue Burden (cfu/g) by Vaccine Dosage (μg/mL) Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.15x10^7</td>
</tr>
<tr>
<td>Liver</td>
<td>1.79x10^7</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.37x10^7</td>
</tr>
<tr>
<td>Lung</td>
<td>5.4x10^5</td>
</tr>
</tbody>
</table>

Mean concentrations of *Y. pestis* in tissues determined at the time of necropsy.
### TABLE 5  Incidence of Microscopic Lesion in the Lungs and Lymph Nodes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of CMs with Lesions/Total</th>
<th>rF1V Dosage (μg) Group (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0.016 0.08 0.4 2 4 8</td>
</tr>
<tr>
<td>Lung</td>
<td>18/20 (100)</td>
<td>3/4 3/3 4/5 15/21 9/24 3/25</td>
</tr>
<tr>
<td>Bronchial Lymph Node</td>
<td>11/20 (55)</td>
<td>1/4 1/3 1/5 12/21 5/24 1/25</td>
</tr>
<tr>
<td>Mediastinal Lymph</td>
<td>12/20 (60)</td>
<td>0/4 0/3 0/5 8/21 5/24 1/25</td>
</tr>
<tr>
<td>Node</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Animals vaccinated with ≥12 μg rF1V survived challenge and lesions were not observed at the time of necropsy (end of the post-challenge observation period). Lesions were not observed in the two surviving controls.*