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An improved *Francisella tularensis* Live Vaccine Strain (LVS) is well tolerated and highly immunogenic when administered to rabbits in escalating doses using various immunization routes

Marcela F. Pasetti^{1,*}, Lilian Cuberos², Thomas L. Horn³, Jeffry D. Shearer⁴, Stephen J. Matthews⁴, Robert V. House⁴, and Marcelo B. Szein^{1,2}

¹ Center for Vaccine Development, Department of Pediatrics, University of Maryland School of Medicine, 685 West Baltimore St., Room 480, Baltimore, MD 21201

² Department of Medicine, University of Maryland School of Medicine, 685 West Baltimore St., Room 480, Baltimore, MD 21201

³ IIT Research Institute, 10 West 35th Street, Chicago, IL 60616

⁴ DynPort Vaccine Company LLC, 64 Thomas Johnson Drive, Frederick, MD 21702

Abstract

Tularemia is a severe disease for which there is no licensed vaccine. An attenuated *F. tularensis* live vaccine strain (LVS) was protective when administered to humans but safety concerns precluded its licensure and use in large scale immunization. An improved *F. tularensis* LVS preparation was produced under current Good Manufacturing Practice (cGMP) guidelines for evaluation in clinical trials. Preclinical safety, tolerability and immunogenicity were investigated in rabbits that received LVS in escalating doses (1×10^5 to 1×10^9 CFU) by the intradermal, subcutaneous or percutaneous (scarification) route. This improved LVS formulation was well tolerated at all doses; no death or adverse clinical signs were observed and necropsies showed no signs of pathology. No live organisms were detected in liver or spleen. Transient local reactogenicity was observed after scarification injection. Erythema and edema developed after intradermal injection in the highest dose cohorts. High levels of *F. tularensis*-specific IgM, IgG and IgA developed early after immunization, in a dose-dependent fashion. Scarification elicited higher levels of IgA. Antibodies elicited by LVS also recognized *F. tularensis* Schu-S4 antigens and there was a significant correlation between antibody titers measured against both LVS and Schu-S4. The ELISA titers also correlated closely with those measured by microagglutination. This is the first report describing comprehensive toxicological and immunological studies of *F. tularensis* LVS in rabbits. This animal model, which closely resembles human disease, proved adequate to assess safety and immunogenicity of *F. tularensis* vaccine candidates. This new LVS vaccine preparation is being evaluated in human clinical studies.

Keywords

Tularemia and vaccines; *F. tularensis* vaccines; *F. tularensis* and antibody responses; tularemia and rabbits

*Corresponding author. Marcela F. Pasetti. Center for Vaccine Development. 685 West Baltimore St. Room 480. Baltimore, MD 21201. Tel: (410) 706-5328; Fax: (410) 706-6205; E-mail: mpasetti@medicine.umaryland.edu.

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1. Introduction

Francisella tularensis, the causative agent of tularemia, is a highly infective microbial pathogen with the capacity to cause acute and fatal disease [1]. Two major subspecies pathogenic for humans have been identified: *F. tularensis* subspecies *tularensis* (also known as Type A) which predominates in North America, and subspecies *holarctica* (Type B), which produces a less severe disease and is prevalent in East Europe and Asia [2].

F. tularensis has gained attention in recent years as one of the six category A organisms identified by the Centers for Disease Control and Prevention that have the greatest potential to be deployed in biological warfare [1]. Civilians and military personnel massively exposed to this organism by aerosol or by the oral route are likely to develop Typhoidal tularemia, the most severe form of the disease, which would result in high mortality rates if left untreated. Survivors would require hospitalization and will suffer symptoms for several weeks with frequent relapses. *F. tularensis* released in such manner is also expected to establish enzootic reservoirs in wild animals which could result in subsequent outbreaks of disease in humans [3]. Although the disease that results from natural transmission (usually through insect bites or contact with infected animals and contaminated products) is less severe, it still carries a significant public health burden, especially in endemic areas.

There is presently no licensed vaccine adequate to protect against tularemia (Reviewed in [4; 5]). In the 1930s, a live attenuated Type B strain was given to over 50 million people living in endemic areas in the Soviet Union by the subcutaneous or scarification route [6;7]. An attenuated *F. tularensis* live vaccine strain (LVS) derived from the Soviet vaccine was used for a number of years in the Western world as an investigational new drug (IND) for prophylaxis of laboratory workers and military personnel. A series of human studies using the LVS NDBR101 Lot 4 sponsored by the Department of Defense showed that this vaccine was well tolerated, with only mild to moderate (mostly local) adverse events, and highly immunogenic; more than 90% of vaccinees responded with microagglutination titers $\geq 1:20$ [8]. Although this early vaccine met the expectations of reducing the incidence of natural and laboratory-acquired disease, it was never licensed by the Food and Drug Administration (FDA), mainly due to limited data on safety and efficacy in humans. Major concerns were the unknown basis of attenuation and the possibility of reversion to virulence. In addition, this vaccine was produced using research-quality procedures that would not meet the current quality standards of vaccine production for use in humans. New approaches in the development of tularemia vaccines include the use of rational attenuating strategies to produce safer and better characterized strains and the identification and purification of protective antigens [4;5]. Despite the progress made, no leading candidates have yet been identified for evaluation in humans. Therefore, there has been renewed interest in efforts to improve the existing LVS for the foreseeable future. DynPort Vaccine Company LLC (DVC), under contract to the Joint Vaccine Acquisition Program (JVAP), developed and improved the manufacturing process for *F. tularensis* LVS in compliance with good manufacturing practice (cGMP) guidelines. A new formulation derived from LVS NDBR101 Lot 4 was produced using standardized fermentation, purification, and formulation processes. This new vaccine formulation was subjected to extensive characterization and rigorous lot release requirements.

The purpose of this study was to evaluate the preclinical safety and immunogenicity of this newly manufactured LVS vaccine prior to its evaluation in human clinical trials. Acute and definitive toxicology studies were conducted in rabbits using different dosage levels and routes of immunization; such studies were designed in collaboration with the Centers for Biological Evaluation and Research at the FDA to support the IND of this new vaccine for use in humans. Taking advantage of these studies, we performed an in depth analysis of the host humoral

responses to *F. tularensis* LVS measuring the kinetics and class-profile of antibodies recognizing LVS and Schu-S4 antigens and a comparison of vaccine-induced antibody titers measured by ELISA and microagglutination.

2. Materials and methods

2.1. Pedigree of the LVS vaccine

The vaccine used in this study was derived from LVS NDBR101 Lot 4 and manufactured by Cambrex Biosciences (Baltimore, MD) for DVC. The original culture was obtained from the Gamaleia Institute, USSR, in 1956 [9;10]. This culture produced “blue” and “gray” colony variants; the blue variant, which was more virulent and immunogenic in mice and guinea pigs, was selected for vaccine production. Subsequently, the strain was serially passed through mice by Eigelsbach and Downs at the U.S. Army Medical Unit, Fort Detrick, MD and the organism recovered from a moribund animal was designated LVS [11]. Fort Detrick produced eight initial lots of vaccine and the vaccine manufacturing process was transferred to the National Drug Company (NDC) in Swiftwater, PA. Eleven lots, designated NDBR101 Lots 1–11, were prepared by NDC at Swiftwater from 1962 through 1964. The LVS NDBR101 Lot 4 was used as the starting material to manufacture an initial DVC Product Development Cell Bank (PDCB) and the Master Cell Bank (MCB). Subsequently, the MCB was used to manufacture the Working Cell Bank (WCB), which was used as a starting material for the vaccine preparation evaluated in this study. The LVS vaccine used in these studies was found to be 100% blue phenotype.

2.2. Preparation of vaccine inoculum and dose formulation

F. tularensis LVS (WCB) was grown to logarithmic phase in modified casein partial hydrolysate medium and purified via tangential flow filtration against 10 mM potassium phosphate and 10% sucrose buffer. After purification, the vaccine was formulated in 10 mM potassium phosphate, 10% sucrose and 1.3% gelatin. The final product was lyophilized and stored at 2 to 8°C (for the acute study) or at –10 to –30°C (for the definitive toxicology study) until used. For the acute toxicity study, multiple vials of lyophilized LVS (Lot # 703–1202–014) were reconstituted in Water for Injection (WFI; Abbott Laboratories, North Chicago, IL) and pooled to yield a working stock of 1×10^{10} CFU/ml. This stock was diluted in Dulbecco's Phosphate Buffered Saline (PBS; BioWhittaker, Walkersville, MD) to yield target doses of 10^5 to 10^9 CFU/animal. For the definitive toxicity study, multiple vials of LVS (Lot # 703–0603–017) were reconstituted in WFI to yield a stock solution of 1×10^9 CFU/ml. Log-fold dilutions of this stock were prepared in Sterile Saline for Injection USP (SSI, Baxter Healthcare Corp., Deerfield, IL) to yield final doses of 10^6 and 10^8 CFU/animal. Plate counts of the inocula were performed before and after vaccination to confirm dose levels. The microbial titer (CFU/ml) was determined by enumerating colonies on triplicate cystine heart agar containing 5% defibrinated sheep blood cells plates (acute toxicology study) or on commercially available triplicate glucose cysteine blood agar plates (definitive toxicology study; PML Microbiologicals, Wilsonville, OR) after approximately 73–93h of incubation at 37° C.

2.3. Animals

The study protocols were reviewed and approved by the IIT Research Institute Animal Care and Use Committee. New Zealand white rabbits (12–14 wks old) were purchased from Covance Research Products (Kalamazoo, MI). Males only were used for the acute toxicity study, and equal numbers of male and female rabbits were used for the definitive toxicity study. Animals were housed individually in stainless steel cages, and husbandry was in accordance with the *Guide for the Care and Use of Laboratory Animals* [12]. Certified Rabbit Diet #5325 (PMI Nutrition International, Brentwood, MO) and City of Chicago tap water were provided *ad libitum*. Rabbits were acclimated for two weeks prior to dosing.

2.4. Study design

For the acute toxicology study, 90 rabbits were divided into three cohorts of 30 animals each and administered Dulbecco's PBS (BioWhittaker) as control vehicle or five escalating doses (1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 CFU) of LVS by the subcutaneous (SC), intradermal (ID) or percutaneous/scarification (PC) route (Table 1). Animals were monitored for 34 days after dosing and they were euthanized by sodium pentobarbital overdose (~150–200 mg/Kg administered intravenously) for necropsy studies on day 35. For the definitive toxicology study, 72 rabbits were divided into two cohorts of 36 rabbits each (3 groups per cohort) and administered SSI as control vehicle or LVS vaccine in two dosage levels (1×10^6 CFU or 1×10^8 CFU) by the SC or PC route (Table 2). Animals were observed for 15 and 49 days post-dosing and euthanized and necropsied on days 16 and 50. Both studies were performed in accordance to 21 Part 58 of the Code of Federal Regulations and current industry practices for Good Laboratory Practice.

2.5. Administration of vaccine and controls

LVS or control article were administered in or below the skin of the dorsum in a volume of 0.1 ml regardless of route and dose. All administration sites were shaved, wiped with 70% ethanol and allowed to dry thoroughly prior to inoculation. The injection site was identified with an indelible marker following inoculation. SC, ID and PC injections followed standard procedures. The SC injection was performed using a 25 gauge needle attached to a syringe that was inserted under the animal's loose skin (held between the index finger and the thumb). For ID injection, the skin was gently stretched, a syringe attached to a 26 gauge needle was held parallel to the animal's body and the tip of the needle was inserted into the dermis layer. For PC injection, the inoculum was applied to the skin with a micropipette and pricked into the skin 15 times using a bifurcated needle with sufficient pressure to elicit a serosanguinous exudate. The inoculation site was allowed to air dry. Although specified vaccine doses (1×10^5 to 1×10^9 CFU) were applied to the skin's surface and pricked into the skin, the actual number of microorganisms internalized is unknown.

2.6. Safety assessment

Animals were observed twice a day for clinical evaluation and assessment of morbidity/mortality. Inoculation sites were examined daily and reactogenicity was scored as indicated in Table 3, using a scale similar to that described by Draize [13]. Body weights were determined at study initiation, weekly during the observation period, and at the time of euthanasia. Food consumption was determined twice weekly. Physical examinations were performed weekly. Ophthalmic examinations were performed for the definitive study only before dosing and prior to necropsies on days 16 and 50. In the acute toxicology study, spleens were removed at necropsy and cultured on triplicate cystine heart agar plates containing 5% defibrinated sheep blood cells for approximately 117h at 37° C to assess vaccine clearance. In the definitive toxicology study, approximately 40 organs were harvested; adrenal glands, brain, heart, kidneys, liver, lungs, spleen, and testes/ovaries were weighed at the time of necropsy, and macroscopic and microscopic evaluations were performed. Microscopic histopathology was performed on the brain, kidneys, liver, lungs (with bronchi), lymph nodes (mesenteric and popliteal), ovaries, skin (covering the injection site), spleen and testes in the control, low and high dose groups. Portions of the spleen and liver were cultured on triplicate glucose cysteine blood agar plates (PML Microbiologicals) for approximately 74–122h at 37° C to assess clearance of vaccine organisms. Prior to necropsy, blood samples were collected from a marginal ear vein on days 3, 16 and 50 for clinical pathology (hematology and chemistry). Complete hematology and clinical chemistry assessment were performed using an Advia 120 (Bayer Corp., Tarrytown, NY) and a Synchron CX5 analyzer (Beckman Instruments, Inc., Brea, CA), respectively. Hematological parameters consisted of erythrocyte count and

morphology, platelet count, hematocrit, hemoglobin, mean cell volume, mean cellular hemoglobin, mean cellular hemoglobin concentration, mean platelet volume, reticulocyte count (absolute and relative) and differential white blood cell counts. Clinical chemistry parameters consisted of sodium, potassium, chloride, calcium, inorganic phosphorus, blood urea nitrogen, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, creatinine, creatine kinase, glucose, total protein, albumin, cholesterol and triglycerides; globulin levels and albumin/globulin ratios were calculated from the data.

2.7. Antigen preparation

Heat formalin-killed *F. tularensis* LVS and Schu-S4 antigens were used for ELISAs. These antigens were produced by Midwest Research Institute (Kansas City, MO) and obtained from ATCC, Manassas, Virginia (# NR-74 and NR-73, respectively) through the Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases (NIAID), NIH. The organisms used as starting material were grown from frozen WCB. The LVS strain was from the same vaccine lot used in the toxicology study (LVS #703–0603–017) and it was obtained as described above. The Schu-S4 strain was derived from Lot 623–42, originally prepared by The Salk Institute in Swiftwater, PA (1986); submaster vials were created by diluting the original Schu-S4 stock in gel saline containing 20% glycerol and were maintained at –80°C. The organisms were grown in peptone cysteine agar plates in the presence of modified casein partial hydrolysate. Bacterial pellets were resuspended in 3% formalin in PBS and heated at 60° C for 30 min. Sterility was confirmed by the absence of bacterial growth in glucose cysteine blood agar plates. The antigens were then washed with PBS, aliquoted and stored at –80° C.

2.8. ELISAs

Serum IgM, IgG and IgA antibodies specific for *F. tularensis* were measured by ELISA. Plates were incubated with LVS and Schu-S4 antigens (ATCC NR-74 and NR-73) diluted to a final concentration of 1×10^6 CFU/ml, for 3h at 37° C. Plates were then washed with PBS containing 0.05% Tween 20 (PBST) and blocked overnight at 4° C with 10% dried milk in PBS. Samples were tested in 2-fold serial dilutions in 10% dried milk in PBST (PBSTM), starting at 1:50. Specific antibodies were revealed with the horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (ZyMax Grade, Zymed Laboratories Inc. San Francisco, CA), anti-rabbit IgA and anti-rabbit IgM (Bethyl Laboratories) diluted 1:2,000, 1:250 and 1:500 in PBSTM, respectively. TMB Microwell Peroxidase (Kirkergaard & Perry Labs, Gaithersburg, MD) was used as substrate. The reaction was stopped with 100 µl of 1M phosphoric acid. Titers were calculated from linear regression curves as the inverse of the serum dilution that produces an absorbance value of 0.2 above the blank, and expressed in ELISA Units per ml (EU/ml).

2.9. Microagglutination

Rabbit sera diluted two-fold (starting 1:20) in 0.9% saline were incubated with stained *Pasteurella tularensis* antigen (National Drug Co. Code No: 101 AG, Lot 9, kindly provided by Diagnostic Systems Division, USAMRIID) in 96-well V bottom plates, overnight, at room temperature. A rabbit antiserum specific for *Pasteurella tularensis* (National Drug Co., Code No NDBR 101 AS, provided by Diagnostic Systems Division, USAMRIID) was used as positive control. Normal rabbit serum was used as negative control; a row containing antigen alone was also included as negative control. Titers were assigned as follows: 4+ positive (even lattice of agglutination with no stained cells or button on bottom wells); 3+ positive (even lattice of agglutination with a small transparent button of stained cells on bottom of well); 2+ negative (uneven lattice of agglutination with a moderate to large button of stained cells on bottom of well); 1+ negative (no agglutination visible- well clear with a large button on the bottom of the well). Criteria for acceptance of the assays were as follows: negative control <1:20; no

agglutination on negative control wells; end-point titers for the positive control within two-fold dilutions of the predetermined titer and no more than two fold difference in intra-assay duplicates.

2.10. Statistical analysis

An automated data collection system (LABCAT, Innovative Programming Associates, Princeton, NJ) was used in both toxicology studies. Organ weight analysis was performed using SigmaStat (Systat Software, Inc. San Jose, CA). Toxicology data were analyzed by ANOVA with post-hoc comparisons using Dunnett's test. Immunological data were analyzed by multiple linear regression of log₁₀ antibody levels, with dose, necropsy day, and route of administration as covariates, and by correlation analysis. Associations between LVS and Shu-S4 titers and between ELISA and microagglutination titers were examined by Pearson's correlation on log-transformed data. Analysis was performed using SigmaStat and NCSS (Number Cruncher Statistical Systems, Kaysville, Utah). A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Acute toxicology

Although it is known that rabbits are naturally susceptible to tularemia, there is a dearth of information on their reaction to *F. tularensis* LVS. We therefore performed an initial acute toxicology study (outlined in Table 1) to establish the dosage range that would not produce overt pathology in this model in preparation for a definitive toxicity safety assessment. None of the rabbits died and no vaccine-related adverse clinical signs were observed during the 35-day monitoring period. No treatment-related effects on mean body weight, mean body weight gain, or mean food consumption were observed. Reactions at the inoculation site are shown in Figure 1. None of the rabbits showed ulceration at the injection site. Rabbits that received vehicle control by scarification had a very slight/mild erythema and/or edema on days 1–3 whereas rabbits that received vehicle control by SC or ID route did not show erythema/eschar or edema at any time point throughout the study. Reactogenicity scores appeared to be dose-related. Groups that received the higher doses exhibited the highest scores or the highest incidence of animals with a given score. Erythema/eschar was most severe and persisted longest in the animals vaccinated by the ID route, where scores up to 5 were observed in all rabbits that received the highest dose (three of the rabbits in this group did not return to score 0 for erythema/eschar before the end of the observation period). Erythema/eschar scores up to 2 were observed in the scarification route cohort; a maximum score of 1 for the erythema/eschar was observed in the SC route cohort. The observations of edema at the inoculation site were similar to those of the erythema/eschar. Edema scores up to 4 were recorded in rabbits that received the two highest doses of vaccine by the ID route. The edema persisted for the duration of the observation period in most of these animals, while scores no greater than 2 were observed in animals that received the same high doses in the SC and scarification cohorts (which returned to normal sooner). Gross necropsy findings were within normal limits in the tissues examined (liver, kidneys, spleen, stomach, small and large intestines).

Vaccine colonization was investigated in the spleen; both spleen and liver (examined in the definitive toxicology study) appear to be the main target organs of *F. tularensis* and bacterial dissemination and damage to these tissues have been related to the outcome of infection [14–17]. Vaccine organisms were not detected in the spleen in any of the animals by day 35, not even in the high dose groups.

3.2. Definitive toxicology

A definitive toxicology and safety study was conducted as outlined in Table 2. The in-life findings are summarized briefly in Table 4. No deaths and no adverse clinical signs were observed, except for reactogenicity (erythema and edema) at the inoculation site (as shown in Figure 1). The ID route was not included in the definitive toxicology study due to the significant reactogenicity observed using this route in the acute toxicology study, which would likely preclude its use in a clinical setting. No toxicologically significant effects on body weights, body weight gains, food consumption, ophthalmology, clinical chemistry or hematology were observed during the study (detailed clinical chemistry and hematology data are shown in Supplementary Tables 1–4 published online). Erythema and/or edema were observed in most of the animals that received the LVS vaccine in each cohort. The majority of these signs returned to normal (score of 0) by day 20; however, the reactogenicity persisted until day 49 in females that received the highest dose of LVS by the SC route. A statistically significant increase in the mean absolute spleen weight was observed on day 16 in males that received the highest dose by scarification although the corresponding mean relative spleen weight was not significantly increased. LVS organisms were not detected in the liver and spleen from any vaccinated animals in either cohort at day 16 or 50. Pigmentation change in the skeletal muscle was observed at the injection site at necropsy on day 16 in both male and female LVS recipients, likely as a result of administration of live bacteria. One female in the highest dose SC cohort had thick, yellow and red skin at the injection site at necropsy on day 50. This lesion correlated microscopically with necrosis surrounded by chronic inflammation within the subcutis, which was consistent with a foreign body reaction. All other gross lesions observed at necropsy (e.g. enlarged mediastinal lymph node or spleen) were interpreted as incidental findings or attributed to the dosing procedure. All microscopic findings were interpreted as consistent with a transient response associated with the administration of live bacteria, such as the inflammation observed in the lungs, or with incidental findings that are commonly present in rabbit toxicology studies.

3.3. Antibody responses

We optimized ELISAs to measure antibodies to *F. tularensis* using heat formalin-killed Schu-S4 and LVS strains as capture antigens. The assay was qualified and validated using a standard rabbit antiserum and in house positive and negative control samples along with experimental rabbit sera. Intra- and inter-assay coefficients of variation were 3 and 10%, respectively. Specificity was demonstrated by inhibition of antibody binding using samples absorbed with *F. tularensis* LVS and Schu-S4 antigens; antigenic preparations from other Gram negative bacteria (*Salmonella* and *Shigella*) were included as negative controls. Figures 2 and 3 show serum IgM, IgA and IgG titers against LVS and Schu-S4 antigens, respectively, in rabbits that received control vehicle or *F. tularensis* LVS by the scarification or SC route; titers were measured on sera collected prior to necropsy on days 16 and 50. Multiple linear regression modeling was performed to assess the effects of target dose, necropsy day, route and gender on the six outcome variables (IgM, IgG and IgA to LVS and to Schu-S4). Table 5 summarizes parameter estimates and p-values associated with dose, day, and route of administration. The regression models indicate significant differences (at the 0.05 level) in dose and time points for each antibody class and antigen, except for IgG to LVS. It also indicates a significant difference for IgA responses to either antigen with regards to the route of administration; scarification elicited higher antibody levels than SC (Table 5). The positive estimates for each antigen (i.e., LVS and Schu-S4) and antibody class indicate significantly higher responses in animals that received the higher dose of LVS (1×10^8 CFU). The significant negative parameter estimates for IgA and IgM responses indicate higher titers on day 16 than on day 50 (Table 5). In contrast, IgG responses to Schu-S4 on day 16 were significantly lower than those observed on day 50. When interactions with dose were included in the models along with day and route (i.e., dose and day, dose and route), the interaction terms were never significant at the 0.05 level. There was no evidence of association of any antibody level with sex ($P \geq 0.60$ for bivariate

association of sex with antibody level for all six antibodies). In order to examine the magnitude of increase in antibody levels post vaccination and to express the data in a manner that would allow for comparisons with other studies using different immunological readouts, we examined the fold-increases in antibody titers in individual animals for each group (Table 6). All animals that received *F. tularensis* LVS seroconverted (≥ 4 -fold rise above the mean titer in the control group) by day 16 for all three antibody classes. Seroconversion was maintained on day 50, albeit at lower levels for IgM and IgA and higher levels for IgG with the exception of LVS IgG in the high dose (1×10^8) scarification group that had a similar mean fold increase at both time points. No responses were measured in the control group.

We also investigated whether there was an association between antibody titers measured against LVS and Schu-S4. Scatter plots for IgM, IgA and IgG titers against LVS and Schu-S4 for all animals are shown in Figure 4. Taking into account all doses, days, and routes, the LVS and Schu-S4 antibody levels were highly correlated (Pearson correlations $P < 0.001$ for all antibodies). Of note, associations approximate straight lines with intercept 0 and slope 1.

Antibody responses to *F. tularensis* have been traditionally measured by agglutination techniques. To compare our serological data generated with optimized ELISAs with those reported in the literature, we also assessed the LVS-induced antibodies using a standard microagglutination assay (Table 7). The range of antibody titers measured by microagglutination was much lower (< 10 to 640) than the range obtained by ELISA (12.5 to $> 1.4 \times 10^5$ EU/ml). Among the vaccinated rabbits, 100% seroconverted according to the ELISA whereas 87% seroconverted as determined by microagglutination. These titers, however, were similar to those previously reported in LVS-immunized rabbits [18] and in humans that contracted the disease [19]. Furthermore, there was a significant correlation between all three IgM, IgA and IgG ELISA and microagglutination titers; the closest association was observed between agglutination and IgM levels (Figure 5), in agreement with the superior microagglutination titers observed on day 16.

4. Discussion

There is an urgent need for a safe and effective tularemia vaccine that can serve not only as a biodefense prophylactic tool but also to protect exposed individuals from endemic areas. Despite its many drawbacks, the LVS has been the only vaccine candidate that showed protective efficacy in humans and is one that can be readily accessible if urgently needed. An improved LVS formulation (Lot # 703–0603–017) for evaluation in Phase 1 clinical studies was recently manufactured under cGMP conditions, resulting in a more highly-defined product than previous LVS lots prepared using research-grade reagents and employing no purification. Herein we demonstrate that this new vaccine formulation was safe and highly immunogenic during preclinical studies in rabbits.

These studies are the first to provide a comprehensive toxicological and immunological analysis of *F. tularensis* LVS administered to rabbits in escalating doses and by different routes. There are only two reports in the literature, from studies conducted back in the 1960s, describing immune responses to *F. tularensis* LVS given to rabbits by different routes [18; 20]. One of them focused on cellular interactions between *F. tularensis* and macrophages [18] and the other one on procedures to enhance antibody production, such as combination of LVS with Freund adjuvant and use of accelerated immunization schedules, in proof of principle experiments [20]. These studies did not address vaccine safety and tolerability nor describe the pathology or microbiology related to LVS inoculations [17]. The measurement of antibody responses was limited to bacterial agglutinins with no further analysis of serological outcomes. Moreover, the LVS used in those studies was prepared for research purposes, without the rigorous quality control standards currently required for human testing.

To date, most of the research with *F. tularensis* has been carried out in mice [21]. However, since mice and humans respond very differently to this organism (e.g., LVS is lethal to mice when injected i.p. but it does not cause disease in humans [21]), it is unclear to what extent the information obtained in the mouse model translates to humans [17]. To more adequately understand the true immunological and pathological consequences of tularemia, it is important to use animal models that more accurately reflect infection in humans. The primate model is currently the model that best recapitulates the human disease and probably the best choice to assess vaccine efficacy [17]. Because of the difficulties associated with the use of non-human primates, however, a more practical yet reliable small animal model amenable for routine vaccine testing is still needed. For this reason the current study employed the rabbit, an accepted tularemia animal model for which there is nonetheless an overt lack of information on pathogenesis and immune responses.

Sensitive and specific ELISAs were used to measure serum antibodies to LVS and Schu-S4 and titers obtained by this method highly correlated with those measured by microagglutination. Antibodies to tularemia antigens have been traditionally measured by agglutination techniques [22;23] and although several articles describe the use of different ELISA permutations and other techniques to measure antibodies to *F. tularensis* [22;24–28], none of them were applied to evaluate responses to vaccine candidates in rabbits. To our knowledge, this is the first study reporting a close association between antibody titers to *F. tularensis* measured by ELISA and microagglutination in an experimental rabbit model for vaccine evaluation.

Cottontail rabbits from suburban regions that survived infection with *F. tularensis* had been found to develop IgM and IgG antibody titers which seemingly prevented chronic infection [28]. It was interesting to see in our studies that all three immunoglobulins evaluated (IgA, IgG and IgM) were elicited in response to LVS. Higher IgM and IgA levels were detected on day 16, whereas increased IgG titers were seen on day 50. A similar response profile characterized by simultaneous, but not parallel production of IgM, IgA and IgG was reported in humans exposed to *F. tularensis* [26;29;30], with IgM and IgA responses disappearing some months after exposure and IgG persisting longer [29]. Our data showed higher IgA responses to LVS when the vaccine was administered via scarification, suggesting that this route of immunization primes B cells with capacity for IgA synthesis that could be available in mucosal sites to prevent bacteria colonization in mucosal surfaces. High levels of IgA have been observed in humans that received LVS by scarification and a rise in specific IgA antibodies was suggested as an excellent predictor of vaccine effectiveness [30]. It is thus reasonable to speculate that vaccination with LVS using the scarification route might lead to enhanced protection against respiratory infection, particularly at early times after immunization.

Of importance, antibodies to LVS also recognized antigens from the virulent Schu-S4, most likely due to the antigenic similitude between these *F. tularensis* subspecies. This is a critical observation suggesting that antibodies generated by LVS have the potential to recognize and protect against the more virulent strain, Schu-S4 in humans.

Although our understanding of the immunological mechanisms of protective immunity against tularemia is far from complete, it is likely that in addition to antibody responses, T cell immunity also plays a major role in host defense [5;31]. Unfortunately, it was not possible to investigate cell-mediated immunity in the present studies. Similarly, since the animals were euthanized for safety/pathology assessment, challenge was not performed and no correlates of protection could be derived from the present data. We have nonetheless identified unique parameters in the response to LVS in rabbits, such as local but not systemic reactions, lack of pathological findings and *in vivo* recovery of live organisms, and a defined profile of antibodies to LVS and Schu-S4 measured by ELISA and microagglutination that can serve as guidance

in the evaluation of new vaccine candidates. In fact, these results paved the way for studies in humans. An NIAID-sponsored Phase I, double blind, placebo-controlled dose escalation to study the safety and reactogenicity of *F. tularensis* DBR101 Lot 4-derived LVS administered by scarification and SC routes is underway.

The LVS vaccine is available and known to afford significant protection against respiratory tularemia and to mitigate the course of ulceroglandular disease [4;6;8]. In contrast, current prospects for the development of an effective subunit, well-defined tularemia vaccine are uncertain [4;5]. Ongoing efforts to revive the LVS vaccine include the definition of the attenuating mutations (now ever closer with the available genome sequences of LVS and Schu-S4), standardization of manufacturing practices and further clinical assessment of safety and immunogenicity in humans. Here we provide evidence that the rabbit model is adequate for preclinical safety and immunogenicity evaluation of new vaccine candidates in preparation for human studies, with clinical and immunological outcomes likely to reflect those observed in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Dennis DT, Inglesby TV, Henderson DA, et al. Tularemia as a biological weapon: medical and public health management. *JAMA* 2001 Jun 6;285(21):2763–73. [PubMed: 11386933]
2. Sjostedt A. Virulence determinants and protective antigens of *Francisella tularensis*. *Curr Opin Microbiol* 2003 Feb;6(1):66–71. [PubMed: 12615222]
3. Oyston PC, Sjostedt A, Titball RW. Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev Microbiol* 2004 Dec;2(12):967–78. [PubMed: 15550942]
4. Conlan JW, Oyston PC. Vaccines against *Francisella tularensis*. *Ann N Y Acad Sci* 2007 Jun; 1105:325–50. [PubMed: 17395730]
5. Isherwood KE, Titball RW, Davies DH, Felgner PL, Morrow WJ. Vaccination strategies for *Francisella tularensis*. *Adv Drug Deliv Rev* 2005 Jun 17;57(9):1403–14. [PubMed: 15919131]
6. Cross AS, Calia FM, Edelman R. From rabbits to humans: the contributions of Dr. Theodore E. Woodward to tularemia research. *Clin Infect Dis* 2007 Jul 15;45(Suppl 1):S61–S67. [PubMed: 17582573]
7. Sjostedt A, Tarnvik A, Sandstrom G. *Francisella tularensis*: host-parasite interaction. *FEMS Immunol Med Microbiol* 1996 Mar;13(3):181–4. [PubMed: 8861025]
8. Burke DS. Immunization against tularemia: analysis of the effectiveness of live *Francisella tularensis* vaccine in prevention of laboratory-acquired tularemia. *J Infect Dis* 1977 Jan;135(1):55–60. [PubMed: 833449]
9. Sandstrom G. The tularemia vaccine. *J Chem Technol Biotechnol* 1994 Apr;59(4):315–20. [PubMed: 7764815]
10. Tarnvik A, Berglund L. Tularemia. *Eur Respir J* 2003 Feb;21(2):361–73. [PubMed: 12608453]

11. Eigelsbach HT, Downs CM. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J Immunol* 1961 Oct;87:415–25. [PubMed: 13889609]
12. Guide for the Care and Use of Laboratory Animals. National Academy Press; Washington, DC: 1996.
13. Draize, JH. Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. Austin, TX: The Association of Food and Drug Officials of the United States, Bureau of Food and Drugs; 1959. Dermal Toxicity; p. 46-59.
14. Morner T, Sandstrom G, Mattsson R, Nilsson PO. Infections with *Francisella tularensis* biovar palaeartica in hares (*Lepus timidus*, *Lepus europaeus*) from Sweden. *J Wildl Dis* 1988 Jul;24(3): 422–33. [PubMed: 2900904]
15. Wayne CJ, Shen H, Kuolee R, Zhao X, Chen W. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an alphabeta T cell- and interferon gamma-dependent mechanism. *Vaccine* 2005 Mar 31;23(19):2477–85. [PubMed: 15752834]
16. Baron SD, Singh R, Metzger DW. Inactivated *Francisella tularensis* live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. *Infect Immun* 2007 May;75(5):2152–62. [PubMed: 17296747]
17. Lyons CR, Wu TH. Animal models of *Francisella tularensis* infection. *Ann N Y Acad Sci* 2007 Jun; 1105:238–65. [PubMed: 17395735]
18. Nutter JE, Myrvik QN. In vitro interactions between rabbit alveolar macrophages and *Pasteurella tularensis*. *J Bacteriol* 1966 Sep;92(3):645–51. [PubMed: 5922539]
19. Sato T, Fujita H, Ohara Y, Homma M. Microagglutination test for early and specific serodiagnosis of tularemia. *J Clin Microbiol* 1990 Oct;28(10):2372–4. [PubMed: 2229367]
20. Nutter JE. Effect of vaccine, route, and schedule on antibody response of rabbits to *Pasteurella tularensis*. *Appl Microbiol* 1969 Mar;17(3):355–9. [PubMed: 4976322]
21. Ellis J, Oyston PC, Green M, Titball RW. Tularemia. *Clin Microbiol Rev* 2002 Oct;15(4):631–46. [PubMed: 12364373]
22. Bevanger L, Maeland JA, Naess AI. Agglutinins and antibodies to *Francisella tularensis* outer membrane antigens in the early diagnosis of disease during an outbreak of tularemia. *J Clin Microbiol* 1988 Mar;26(3):433–7. [PubMed: 3356786]
23. Massey ED, Mangiafico JA. Microagglutination test for detecting and measuring serum agglutinins of *Francisella tularensis*. *Appl Microbiol* 1974 Jan;27(1):25–7. [PubMed: 4129476]
24. Porsch-Ozcurumez M, Kischel N, Priebe H, Splettstosser W, Finke EJ, Grunow R. Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clin Diagn Lab Immunol* 2004 Nov;11(6):1008–15. [PubMed: 15539498]
25. Koskela P. Humoral immunity induced by a live *Francisella tularensis* vaccine. Complement fixing antibodies determined by an enzyme-linked immunosorbent assay (CF-ELISA). *Vaccine* 1985 Dec; 3(5):389–91. [PubMed: 4082752]
26. Carlsson HE, Lindberg AA, Lindberg G, Hederstedt B, Karlsson KA, Agell BO. Enzyme-linked immunosorbent assay for immunological diagnosis of human tularemia. *J Clin Microbiol* 1979 Nov; 10(5):615–21. [PubMed: 120873]
27. Schmitt P, Splettstosser W, Porsch-Ozcurumez M, Finke EJ, Grunow R. A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. *Epidemiol Infect* 2005 Aug;133(4):759–66. [PubMed: 16050523]
28. Shoemaker D, Woolf A, Kirkpatrick R, Cooper M. Humoral immune response of cottontail rabbits naturally infected with *Francisella tularensis* in southern Illinois. *J Wildl Dis* 1997 Oct;33(4):733–7. [PubMed: 9391956]
29. Koskela P, Herva E. Cell-mediated and humoral immunity induced by a live *Francisella tularensis* vaccine. *Infect Immun* 1982 Jun;36(3):983–9. [PubMed: 7095859]
30. Waag DM, Galloway A, Sandstrom G, et al. Cell-mediated and humoral immune responses induced by scarification vaccination of human volunteers with a new lot of the live vaccine strain of *Francisella tularensis*. *J Clin Microbiol* 1992 Sep;30(9):2256–64. [PubMed: 1400988]

31. Metzger DW, Bakshi CS, Kirimanjeswara G. Mucosal Immunopathogenesis of *Francisella tularensis*. Ann N Y Acad Sci 2007 Mar 29;1105:266–83. [PubMed: 17395728]

FIGURE 1.

Reactogenicity at inoculation sites in the acute toxicology study. Graphic shows median reactogenicity scores at the injection site measured daily until day 35 in cohorts of rabbits that received escalating doses of LVS by the scarification, intradermal or subcutaneous route as outlined in Table 1. Pictures depict representative local injection sites following administration of PBS (control) by scarification or LVS by scarification, intradermal or subcutaneous injection; images correspond to day 7 post-injection.

FIGURE 3.

Antibody responses to *F. tularensis* Schu-S4. *F. tularensis* LVS was administered to rabbits as described in Figure 2 and Table 2. Antibody levels were measured by ELISA. Individual titers from 6 rabbits in each group and GMT are indicated. Statistical comparisons are summarized in Table 5.

FIGURE 4.

Scatter plot of antibody titers elicited by *F. tularensis* LVS measured by ELISA against LVS and Schu-S4 antigens. Rabbits were administered *F. tularensis* LVS as described in Figure 2 and Table 2. Data represent individual titers from vaccinated rabbits. Correlation coefficients (r) and P values for Pearson's correlation analysis are indicated.

FIGURE 5.

Scatter plots of antibody titers elicited by *F. tularensis* LVS measured by ELISA and microagglutination. Rabbits were administered *F. tularensis* LVS as described in Figure 2 and Table 2. Data represent individual titers from vaccinated rabbits. Correlation coefficients (r) and P values for Pearson's correlation analysis are indicated.

TABLE 1

Acute toxicology study design

Cohort	Route	Treatment	Dose (CFU)	Animals*	
				per dose	total
1	PC	Control	0	5	5
		LVS	10 ⁵ , 10 ⁶ , 10 ⁷ , 10 ⁸ , 10 ⁹	5	25
2	ID	Control	0	5	5
		LVS	10 ⁵ , 10 ⁶ , 10 ⁷ , 10 ⁸ , 10 ⁹	5	25
3	SC	Control	0	5	5
		LVS	10 ⁵ , 10 ⁶ , 10 ⁷ , 10 ⁸ , 10 ⁹	5	25

PC: percutaneous (scarification), ID: intradermal, SC: subcutaneous administration; LVS: *F. tularensis* Live Vaccine Strain Lot # 703–1202–014. Control animals received phosphate buffer saline (PBS). Dose indicates CFU/animal. Animals were monitored for 34 days and necropsy was performed on day 35.

* all males.

TABLE 2

Definitive toxicology study design

Cohort	Group	Route	Treatment	Dose (CFU)	Necropsy day	Animals*
1	1A	SC	Control		16	6
	1B				50	6
	2A	SC	LVS	1x10 ⁶	16	6
	2B				50	6
	3A	SC	LVS	1x10 ⁸	16	6
	3B				50	6
2	1A	PC	Control		16	6
	1B				50	6
	2A	PC	LVS	1x10 ⁶	16	6
	2B				50	6
	3A	PC	LVS	1x10 ⁸	16	6
	3B				50	6

SC: subcutaneous, PC: percutaneous (scarification) administration; LVS: *F. tularensis* Live Vaccine Strain Lot # 703–0603–017. Control animals received Sterile Saline for Injection. Dose indicates CFU/animal.

* 3 males and 3 females per group.

TABLE 3

Parameters used to evaluate reactogenicity at the injection site.

Parameter	Score	Description
Erythema and eschar	0	No erythema
	1	Very slight erythema
	2	Well-defined erythema
	3	Moderate erythema
	4	Moderate-to-severe erythema
	5	Severe erythema to slight eschar
Edema	0	No edema
	1	Very slight edema
	2	Slight edema
	3	Slight-to-moderate edema
	4	Moderate edema
	5	Severe edema
Ulceration	0	No ulceration
	1	Very slight ulceration
	2	Slight ulceration
	3	Moderate ulceration
	4	Moderate-to-severe ulceration
	5	Severe ulceration

TABLE 4
Summary of in-life findings for the definitive toxicology study.

In-life Parameter	Scarification / dose (CFU)					Subcutaneous / dose (CFU)				
	Control	1x10 ⁶	1x10 ⁸	M	F	Control	1x10 ⁶	1x10 ⁸	M	F
Mortalities	0	0	0			0	0	0		
Adverse clinical signs noted	0	0	0			0	0	0		
Reactogenicity frequency	M	F	M	F	M	M	F	M	F	M
Erythema/eschar score										
0	6	6	6	3	3	6	6	6	6	6
1	6	6	6	6	6	0	0	1	3	5
2	0	0	1	3	6	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	4	0	0	0	0	2
5	0	0	0	0	0	0	0	0	0	0
Edema score										
0	6	6	6	6	4	6	6	6	6	4
1	6	6	6	6	6	0	0	4	6	6
2	0	0	4	4	5	0	0	2	0	2
3	0	0	0	0	0	0	0	0	0	0
4	0	0	1	0	1	0	0	1	0	3
5	0	0	0	0	0	0	0	0	0	1
Ulceration score										
0	6	6	6	6	6	6	6	6	6	6
1	0	0	0	0	5	0	0	0	0	2
2	0	0	0	0	0	0	0	0	0	1
3	0	0	0	0	0	0	0	0	0	0

In-life Parameter	Scarification / dose (CFU)			Subcutaneous / dose (CFU)					
	Control	1x10 ⁶	1x10 ⁸	Control	1x10 ⁶	1x10 ⁸	M	F	F
Mortalities	0	0	0	0	0	0			
Adverse clinical signs noted	0	0	0	0	0	0			
Reactogenicity frequency	M	F	M	F	M	F	M	F	F
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
Body weight	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE
Body weight gain	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE
Food consumption	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE
Ophthalmology	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE
Clinical chemistry	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE
Hematology	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE	NOE

F. tularensis Live Vaccine Strain Lot # 703-0603-017 was administered as percutaneous (scarification) or subcutaneous inoculations in doses of 1x10⁶ and 1x10⁸ CFU/animal.

Reactogenicity frequency = number of animals exhibiting the score at some point during the study; M = Male; F = Female; NOE = no (toxicologically-significant or treatment-related) observed effects in either sex.

TABLE 5

Parameter estimates and p-values from multiple linear regression modeling of log₁₀ antibody levels on dose, necropsy day, and route of administration *

Treatment	Antibody	Dose 1x10 ⁶ vs. 1x10 ⁸ (CFU)	Necropsy day 16 vs. 50	Route Subcutaneous vs. Scarification
LVS	IgM	1.06 (<0.001)	-0.61 (<0.001)	0.07 (0.45)
	IgA	0.72 (<0.001)	-0.46 (<0.001)	0.24 (0.006)
	IgG	0.64 (<0.001)	0.21 (0.10)	0.14 (0.27)
Schu-S4	IgM	1.08 (<0.001)	-0.63 (<0.001)	-0.10 (0.30)
	IgA	0.73 (<0.001)	-0.45 (<0.001)	0.15 (0.037)
	IgG	0.76 (<0.001)	0.28 (0.017)	0.20 (0.076)

* Data indicate regression coefficients and p-values. The sample size for each model was 48 animals; control animals were not included because they had no detectable responses. Antibody data were log (base 10) transformed to more closely approximate a normal distribution. A positive parameter estimate indicates higher antibody titers for the higher dose, day 50 and scarification routes. Negative values indicate higher antibody titers with the lower dose, day 16 and SC route. Significant differences are bolded.

TABLE 6
Fold-increases in antibody titers in rabbits immunized with *F. tularensis* LVS.

Antibody	Antigen	Dose (CFU)	Scarification		Subcutaneous	
			Day 16	Day 50	Day 16	Day 50
IgM	LVS	1x10 ⁶	422 (223–774)	51 (27–139)	343 (98–3,522)	94 (36–422)
		1x10 ⁸	4,902 (2,510–8,609)	1,203 (662–1,992)	2,198 (1,726–2,889)	938 (499–1,881)
		1x10 ⁶	229 (130–467)	26 (14–65)	350 (103–3,972)	79 (36–448)
IgA	LVS	1x10 ⁸	3,056 (1,520–5,959)	604 (317–939)	2,290 (1,906–2,654)	789 (381–1,584)
		1x10 ⁶	114 (72–176)	74 (45–153)	96 (24–846)	27 (13–46)
		1x10 ⁸	813 (566–1,428)	253 (64–922)	550 (391–906)	139 (69–201)
IgG	LVS	1x10 ⁶	125 (71–191)	49 (31–87)	64 (20–596)	21 (10–29)
		1x10 ⁸	780 (567–1,049)	297 (119–657)	309 (206–589)	101 (46–157)
		1x10 ⁶	394 (163–1,232)	653 (316–2,381)	501 (55–5,738)	675 (49–4,795)
Schu-S4	Schu-S4	1x10 ⁸	3,555 (2,322–5,488)	3,474 (1,016–11,021)	896 (487–1,498)	2,876 (1,219–11,315)
		1x10 ⁶	215 (100–630)	396 (243–745)	167 (25–2,981)	460 (60–1,151)
		1x10 ⁸	2,232 (1,464–3,306)	2,391 (1,132–5,143)	740 (474–1,265)	1,861 (744–6,577)

F. tularensis LVS was administered to rabbits as described in Table 2. Data represent Geometric Mean fold increases in antibody titers and ranges (in parenthesis) for vaccinated groups compared with placebo.

TABLE 7
Microagglutination titers in rabbits immunized with *F. tularensis* LVS.

Group	Dose (CFU)	Scarification		Subcutaneous	
		Day 16	Day 50	Day 16	Day 50
LVS	1x10 ⁶	80* (40–160)	13 (10–40)	57 (20–320)	25 (5–40)
	1x10 ⁸	359* (320–640)	113 (40–320)	403* (160–640)	180 (80–320)
Placebo		5	5	5	5

LVS was administered to rabbits as described in Table 2. Data represent GMT and range of titers. Antibody responses in vaccinated groups were significantly higher than those observed in placebo groups ($P<0.001$) except for rabbits that received 1×10^6 by scarification; titers were higher in rabbits that received 1×10^8 CFU both on days 16 and 50 ($P<0.01$).

* $P<0.05$ titers were higher on day 16 than on day 50 except for rabbits that received 1×10^6 by SC route.