HUMORAL RESPONSE TO TYPE-A DERIVED GENETICALLY MODIFIED LIVE ATTENUATED *FRANCISELLA TULARENSIS* IN RABBITS

by

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ABSTRACT

Francisella tularensis is a gram negative, non-motile, coccobacillus that is the causative agent of tularemia. There is concern that *F. tularensis* could be used as a biological weapon and development of vaccines is a high priority to the military given the rise of terrorist attacks and significant impact a biological attack would have on public health. Type A strains are highly virulent but the current live vaccine strain (LVS) is based on a type B strain which is only partially protective against aerosol challenge with SCHU S4 (S4), a type A strain. Historically mice have been the most commonly used model to study vaccines and pathogenesis of *F. tularensis*. However, mice are acutely sensitive to tularemia such that strains that are attenuated or avirulent in other mammals cause lethal disease in mice. Therefore, the rabbit model has been used in these studies because New Zealand White rabbits have a disease course and susceptibility similar to humans.

The data presented here will demonstrate that the humoral immune response plays a role in the protection and survival from a *F. tularensis* infection. A novel ELISA assay has been used to examine antibody titers against *F. tularensis* in the plasma of rabbits post vaccination and analyzed to determine if there is a correlation to survival against challenge with SCHU S4. In addition, data was analyzed to compare various vaccines, vaccination routes, and vaccine doses to determine if one provided better protection and higher survival rate.

PREFACE

"The most exciting phrase to hear in science,

the one that heralds new discoveries,

is not 'Eureka!' but 'That's funny...'"

~Isaac Asimov

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I would like to acknowledge Le'Kneitah Smith, Nicolas Garcia, and Katherine Willet for their technical assistance and Dr. Eileen Barry for the development of the live attenuated vaccines. I would like to thank Dr. Doug Reed for guiding me through my Master's and being my mentor.

I would especially like to thank Amy Caroline for her help in developing the ELISA assay. You were more than a lab mentor, you became a lifelong friend.

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1.0 INTRODUCTION

Diseases have plagued mankind since the beginning. Military commanders saw how sickness could decimate an army. An enemy that came quickly, silently, was often deadly, and impossible to stop for centuries. It was only a matter of time before mankind began to use disease as a weapon of war.

"Bioterrorism- the deliberate release of viruses, bacteria, or other biological agents used to cause illness or death in people, animals, or plants."[1] As early as 600 BC, when the Athenian dictator Solon used helleborus roots to contaminate the water supply during the siege of Kirrha, acts of biological warfare have been used to weaken and kill the enemy[2]. In 2001, anthrax was sent in letters demonstrating the increasing knowledge of terrorists to produce and use biological weapons[1-4]. The anthrax attacks in 2001 solidified the warnings of a bioterrorism attack from a question of 'if' to a question of 'when'[4,5].

After World War I, the 1925 Geneva Protocol for preventing biological weapons proliferation attempted to control the development of biological weapons because of the use of chlorine gas by the Germans, but failed miserably[2,6]. During World War I and II, bioweapons were studied by both sides and during World War II the United States began its Biological Warfare program which continued into the Cold War[2,7]. However, in the midst of the Cold War, President Nixon took action to stop further offensive biological weapons development because it was believed biological weapons were of little use and the United States wanted to make progress in negotiations to ban biological and toxin weapons[7]. In 1975 the Biological Weapons Convention (BWC) became the first multilateral disarmament treaty banning the development, production and stockpiling of an entire category of weapons of mass destruction[8]. Agreements were made to

provide annual reports on data on research centers and laboratories, information on vaccine production facilities, information on national biological defense research and development programs, and other information that pertained to infectious disease outbreaks, past biological research, and regulations in the country pertaining to specific activities related to the BWC. Over the years the laws regarding potential bioweapons in the United States strengthened. In 1995, Congress passed the Antiterrorism and Effective Death Penalty Act of 1996 which required Health and Human Services to develop regulations for the transfer of select agents[1]. A select agent is defined as one that has the potential to pose a severe threat to human, animal, and/or plant health and safety[9]. Following the 2001 anthrax attacks the USA Patriot Act in 2001 and the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 required Health and Human Services and the United States Department of Agriculture to develop regulations for the possession, use, and transfer of select agents[1]. In 2010, an executive order directed the Department of Health and Human Services and Department of Agriculture to tier the select agents, with a Tier 1 designation presenting "the greatest risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence[1]."

F. tularensis causes a spectrum of disease that is mostly determined by the route of infection and is treatable with a wide range of antibiotics, but the antibiotics must be administered as quickly as possible to prevent relapses[10,11]. The most virulent form is pneumonic disease which has a 30-60% mortality rate if left untreated and can be lethal at extremely low doses, as low as 10 CFU[10-12]. The former Soviet Union and the United States (prior to 1969) developed *F. tularensis* as a biological weapon and due to the low infectious dose the Centers for Disease Control (CDC) have classified *F. tularensis* as a Tier 1 Select Agent[12]. In addition, the Soviet Union reportedly continued their biological weapons programs into the 1990s and

developed *F. tularensis* strains that were antibiotic resistant and could cause disease in LVS vaccinated individuals[13]. The potential of *F. tularensis* to be misused was recognized by the World Health Organization. The Word Health Organization modeled studies to predict the effects of an airborne release of 50 kg and calculated that in a population of 5 million people- 250,000 people would present with disease, one million people would require preventative antibiotics for at least 10 days, even if exposed individuals were treated with antibiotics within 48 hours 25,000 would require hospitalization and 2,500 would die[13,14]. Civilians and military personal massively exposed via aerosol or orally are likely to develop the most severe form of tularemia, resulting in high mortality rates; survivors would require hospitalization and have frequent relapses[15]. In addition, by releasing *F. tularensis* via aerosol it is likely that enzootic reservoirs would be established in wild animals resulting in subsequent outbreaks in humans[15]. These calculations were in 1970 and did not factor in an antibiotic resistant strain of *F. tularensis*[13,14]. *F. tularensis* is considered a biological agent that presents an abundant risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence, and pose a severe threat to public health and safety[9].

1.1 *Francisella tularensis*

Francisella tularensis is a gram-negative, intracellular bacterium that is the causative agent of tularemia, also known as rabbit fever. There are four subspecies of *F. tularensis,* two of which cause disease in humans, *Francisella tularensis* subsp. tularensis (a.k.a type A) and *Francisella tularensis* subsp. holarctica (a.k.a type B), of which type A is considered more virulent[16]. *F. tularensis* is a zoonotic disease and is most commonly found in rodents, hares, and rabbits; these

are not thought to be reservoirs because infection causes acute disease in these species[13]. Notably, human outbreaks often correlate with animal outbreaks of the disease[13,17]. In addition, arthropods including ticks, mosquitos, and biting flies have been shown to be primary vectors for tularemia[18-20]. Tularemia can also be transmitted to humans through direct contact with infected animals, transplanted organs, ingestion of contaminated food, water, or soil, and inhalation of infectious aerosols from dust from contamination hay or lawn mowing[21-25].

While the specific virulence factors of tularemia are not well understood, the pathogenesis of the bacterium is known. Once a human is infected *F. tularensis* enters a macrophage through phagocytosis and disrupts the phagosomal membrane to infiltrate the cytoplasm of the macrophage[26]. Once *F. tularensis* replicates inside the macrophage it is released through apoptosis and into the blood stream and plasma where it propagates a cycle of infection, escape, and reinfection[27]. The ability of *F. tularensis* to invade erythrocytes may contribute to relapses of tularemia after a short cycle of antibiotics because erthryocytes live for approximately four months[28].

Multiple antibiotics have been studied as post exposure prophylaxis treatments to *F. tularensis* exposure, with some antibiotics seeming to be more effective than others. Streptomycin and gentamicin have been shown to be effective, first line therapies because susceptibility testing shows all four subspecies of *F. tularensis* are vulnerable[29,30]. Ciprofloxacin and other fluoroquinolones have been used effectively, but the data on them is limited[31,32]. In addition, tetracycline and chloramphenicol can be used, but since they are bacteriostatic, relapses are more common than with other antibiotics[33,34]. Lastly, it has been suggested that doxycycline is a good first line therapy along with streptomycin and gentamicin because it has greater efficacy compared to ciprofloxacin, *F. tularensis* is less likely to develop resistance, and is less expensive; however,

relapses are possible[35,36]. Mice studies have suggested that doxycycline is most effective if administered within 24 hours and its effectiveness drops to 30% if not given until 48 hours post exposure, while ciprofloxacin continued to be 70% effective for up to 72 hours post exposure[37].

1.2 Humoral Immune Response

While protective immunity to tularemia has been historically attributed to cell-mediated immunity generated by an effective T cell response due to replication in the cytoplasm, *F. tularensis* has a significant extracellular phase in the blood, which makes it vulnerable to the humoral immune response[27,38]. As early as the 1960s there was evidence to support that the adaptive immune system and antibody responses play a role in control of infection due to passive transfer studies carried out in animals[39-41].

It has been shown that in humans naturally infected with *F. tularensis* specific IgM, IgG, and IgA antibodies appear about two weeks post infection, peak 1-2 months post infection, and can be detected up to a year later[42]. In addition, a study in 1985 showed that IgG, IgM, and IgA antibodies to *F. tularensis* after a naturally acquired infections were still present retained the ability to agglutinate bacteria and fix complement up to 11 years post infection[43]. Likewise in humans vaccinated with LVS specific IgM, IgG, and IgA antibodies are present in the sera about two weeks post vaccination and can be detected at least a year and a half later[43-45].

Research regarding the role of the humoral immune response to an infection with *F. tularensis* is limited because of a long held belief that the cell-mediated immune response played more of a role in long lasting immunity. More research is required to further elucidate the importance and mechanisms from the humoral immune response that are important for long lasting immunity.

1.3Vaccines

As pneumonic tularemia poses a threat to the public as a potential bioweapon, it is essential to understand the protective mechanisms connected with a competent immune response so a safe, effective vaccine can be discovered. Killed bacterial vaccines were found to be ineffective. In the 1950s and 60s a type B strain was attenuated by passage in culture and was shown to provide good protection against an aerosol challenge with a virulent type a strain[12,39,46]. This strain was labeled the live vaccine strain (LVS), but is unlikely to be licensed by the FDA because the mechanism of attenuation is not known and there is potential for the virus to revert to a virulent strain[38,39,47]

There is no licensed vaccine that provides adequate protection against tularemia in the event of a biological attack. While LVS is given to at risk personnel, it not licensed by the FDA, primarily due to the inadequate data on safety and efficacy[12,15]. Therefore, scientists are investigating new vaccines that would provide better protection should there ever be a large outbreak. While mice are the dominant animal that is used to study new vaccines for tularemia, mice are acutely sensitive to strains that are attenuated in humans making them a less than ideal candidate to study efficacy and level of protection[12,15]. Therefore, the Reed lab has begun to use a rabbit model because the disease course, and likely the antibody response, is similar to humans[12,16].

2.0 SPECIFIC AIMS

The aim of this study is to examine the humoral immune response to vaccination with a live attenuated *F. tularensis* vaccine and whether that response correlates with protection against an aerosol challenge with virulent *F. tularensis*. This would contradict the long held dogma that cell mediated immunity plays a more important role in intracellular infections.

2.1AIM 1: Evaluate the humoral response in New Zealand white rabbits that were vaccinated or challenged with *F. Tularensis* to determine whether antibody responses contribute to protection

- Develop and use an ELISA assay to determine titers of igg in vaccinated rabbits before and after aerosol challenge with SCHU S4
- Determine whether a correlation exists between igg levels and survival of rabbits challenged with SCHU S4

2.2 AIM 2: Determine whether additional factors including hyper-immune sera or immunoglobulins, IgA and IgM, can influence rate of rabbits using the same novel ELISA assay

- Ascertain when IgM first appears in the plasma in response to the vaccination since it is the first antibody made in response to infection and reveal if there is a correlation to survival.
- Determine IgA levels from the last plasma sample taken after vaccination because IgA is involved in intracellular infections and analyze data to determine if there is a survival correlation

3.0 METHODS

3.1 Biosafety

All experiments using virulent *F. tularensis* SCHU S4 were performed at Biosafety Level 3 (BSL-3) in the Regional Biocontainment Laboratory (RBL) at the University of Pittsburgh. Powered air purifying respirators (PAPRs) were worn for respiratory protection, and all work was conducted in a class II biosafety cabinet using Vesphene IIse (diluted 1:128, Steris Corporation, cat. #646101) as a disinfectant.

3.2 Bacteria

For aerosol exposures, virulent *F. tularensis* strain SCHU S4 was grown in brain heart infusion (BHI) broth at BSL-3. Stocks of attenuated mutants of SCHU S4 and LVS were generated previously and stored at -80°C in BSL-3. For ELISAs SCHU S4 was grown overnight in BHI broth and heat killed by incubating for 3 hours at 60^{o}C in accordance with IBC-approved safety protocols. A small amount of material was plated to confirm that the bacteria were killed prior to removal from BSL-3.

Plasma for ELISA assays was heat inactivated at 56^{o}C for thirty five minutes in a water bath in accordance with IBC-approved safety protocols. The outside of the vials were then disinfected by briefly submerging them in Vesphene IIse. The vials were then dried, sealed in a small biohazard bag, that bag was then sprayed with Vesphene IIse and dried, sealed in another medium biohazard bag where the process for disinfection was repeated. The bag of vials was then taken out of BSL-3 and stored at -20^{o}C in BSL-2 until needed for an ELISA.

3.3 Rabbits

Young female New Zealand White (NZW) rabbits were housed in the University of Pittsburgh Regional Biocontainment Laboratory (RBL) at animal biosafety level 3+ (ABSL3+) for the duration of the study. All studies were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

3.4 Vaccines & Challenge

Dose: $1x10^9$ cfu

Route: Scarification, Oral, Aerosol

Attenuated derivatives of *F. tularensis* were produced by Dr. Eileen Barry at the University of Maryland; Table 1 indicates the attenuating mutation and the virulence/protection seen in mice from studies conducted by Dr. Barry.

Vaccinations and challenge were conducted by Dr. Doug Reed who was assisted by Le'Kneitah Smith (2010-2012), Amy Caroline (2014), Nicolas Garcia (2014), and Katherine Willett (2015).

Table 1: Vaccinations and Attenuating Mutation

Name	Attenuating Mutation	Mouse	
		Virulence	Protection
ΔaroC	Chorismate synthase; synthesize aromatic amino acids	Attenuated	No
ΔguaBA	Guanine biosynthesis	Attenuated	No
ΔaroD	3-hydroquinate dehydratase; synthesize aromatic amino acids	Attenuated	Yes
LVS	Unknown: likely multiple mutations	Reduced	Partial

In early experiments rabbits were vaccinated once and challenged thirty days later. In later experiments, prime & boost vaccinations were proposed and based on the timeline shown in Figure 1 challenge remained thirty days after the final vaccination. Challenge was by aerosol with virulent SCHU S4 grown in BHI at doses ranging from 280-10,000 cfu (12-430 LD_{50}). Rabbits were monitored for 30 days for clinical signs of disease and bled at regular intervals to assess changes in white blood cells, bacteremia, erythrocyte sedimentation rate, and antibody titer.

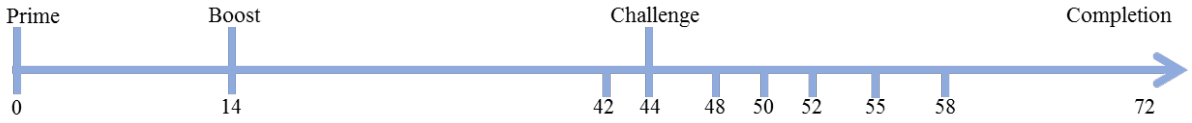

Figure 1: Timeline of Prime & Boost Vaccinations with Challenge

3.5 ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed using standard ELISA procedures. Plates were coated with heat-killed SCHU S4 and stored at 4°C until use. 1:50 dilutions of rabbit sera in phosphate-buffered saline (PBS)-Tween and 5% nonfat milk were plated in duplicate in half log dilutions and incubated for 1 hour at 37°C on 96 well plates coated with heat killed SCHU S4. After washing plates with PBS-Tween on Skan Washer 300 Version B plate washer (Skatron Instruments), secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) at 1:5000 dilution, secondary goat anti-rabbit IgM alpha chain HRP at 1:2500 dilution, or goat anti-rabbit IgM mu chain HRP at 1:2500 dilution was added to the plates at and incubated for 1 hour at 37°C. Antibodies were diluted in PBS-Tween with 5% nonfat milk. After incubation with secondary antibody, the plates were washed again with PBS-Tween. BM chemiluminescence

ELISA substrate (POD) (Roche 11582950001) was prepared mixing 100 parts of solution A with 1 part of solution B. The POD chemiluminescence solution was added to the plates and plates were immediately read on a SpectraMax L (Molecular Devices) or Orion Microplate Luminometer (Berthold Detection Systems) plate reader at 405 nm and absorbance was recorded in Microsoft Excel 2010.

3.6 Statistical Methods

Data was transferred from Microsoft 2010 to GraphPad Prism version 6.00 for Windows for analysis. Graphing and four parameter logistical regression of the absorbances was performed in GraphPad Prism 6. Statistical analyses including calculation of EC-50, slope, one-way analysis of variance (ANOVA), unpaired t test, and Mantel-Cox rank test were all done on GraphPad. All tests were analyzed for significance.

4.0 RESULTS

4.1 IgG titers in vaccinated rabbits

Analyses were first performed to determine if the live attenuated vaccines developed by Dr. Barry protected or extended time to death against a virulent aerosol challenge with SCHU S4. Initial experiments delivered attenuated strains by scarification; one group was given LVS for comparison purposes while another was inoculated with PBS (mock-vaccinated controls). As shown in Figure 2, both ΔguaBA and ΔaroD significantly extended time to death (p=0.0002) with some rabbits surviving challenge while LVS only extended time to death.

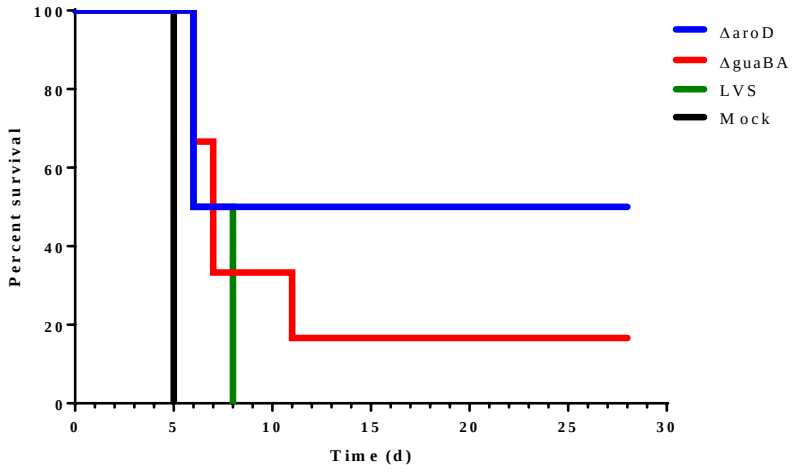

Figure 2: Live attenuated vaccines protect against an aerosol challenge with virulent SCHU S4.

To assess the humoral response, blood was drawn on days 7, 14, 21, and 28 post-vaccination. Figure 3 is a representative example of the change in IgG titers overtime as measured by ELISA. As time progressed, the antibody titers increased. The most prominent change was on day 28 between survivors, shown in green, and non survivors, shown in red. Rabbits that survived

subsequent aerosol exposure to SCHU S4 after vaccination had a notably increased antibody response on day 28.

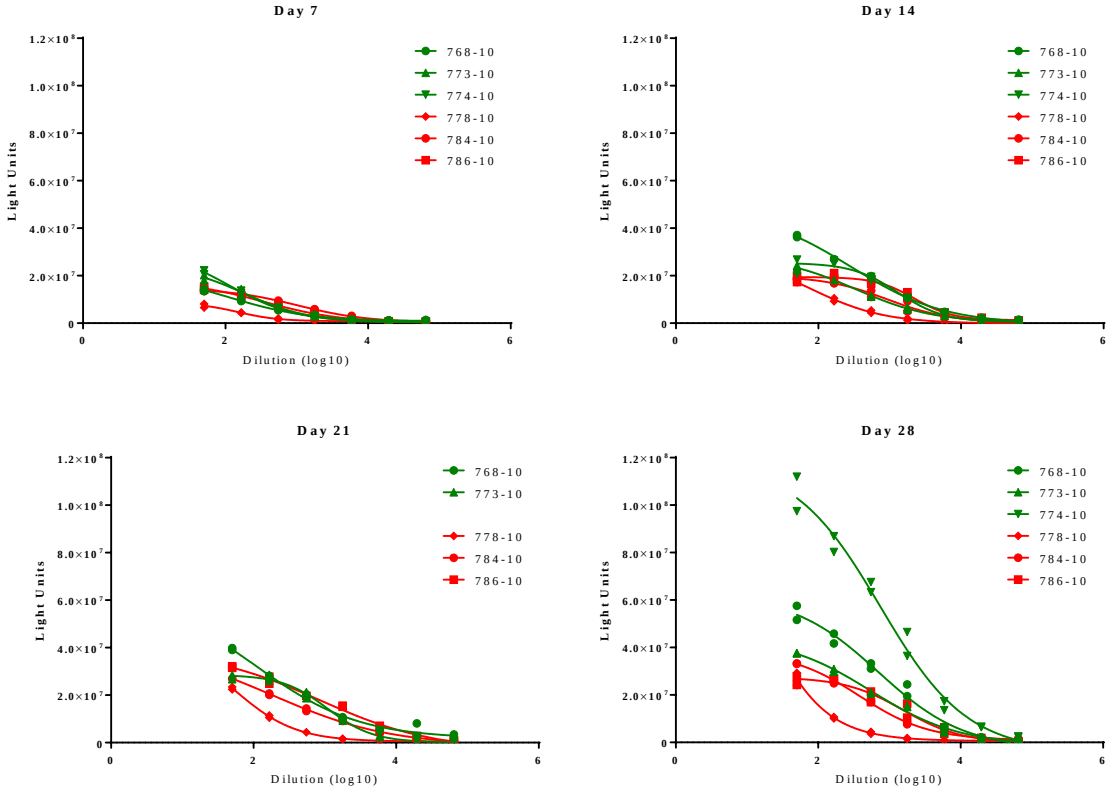

Figure 3: Measurement of IgG titers against heat killed SCHU S4 (hkSCHU S4) in individual rabbits after inoculation with ΔaroD by scarification.

Graphs show four-parameter logistical regression analysis.

Multiple experiments using different vaccines and different routes of inoculation were performed and IgG titers were measured for each animal using ELISA. The first set of experiments looked at ΔguaBA, ΔaroD, ΔaroC, and LVS via while a control group was inoculated with PBS (mock-vaccinated). Blood was drawn at days 7, 14, 21, and 28 post vaccination and using four parameter logistical regression of the absorbances the median effective concentration (EC_{50}) was calculated.

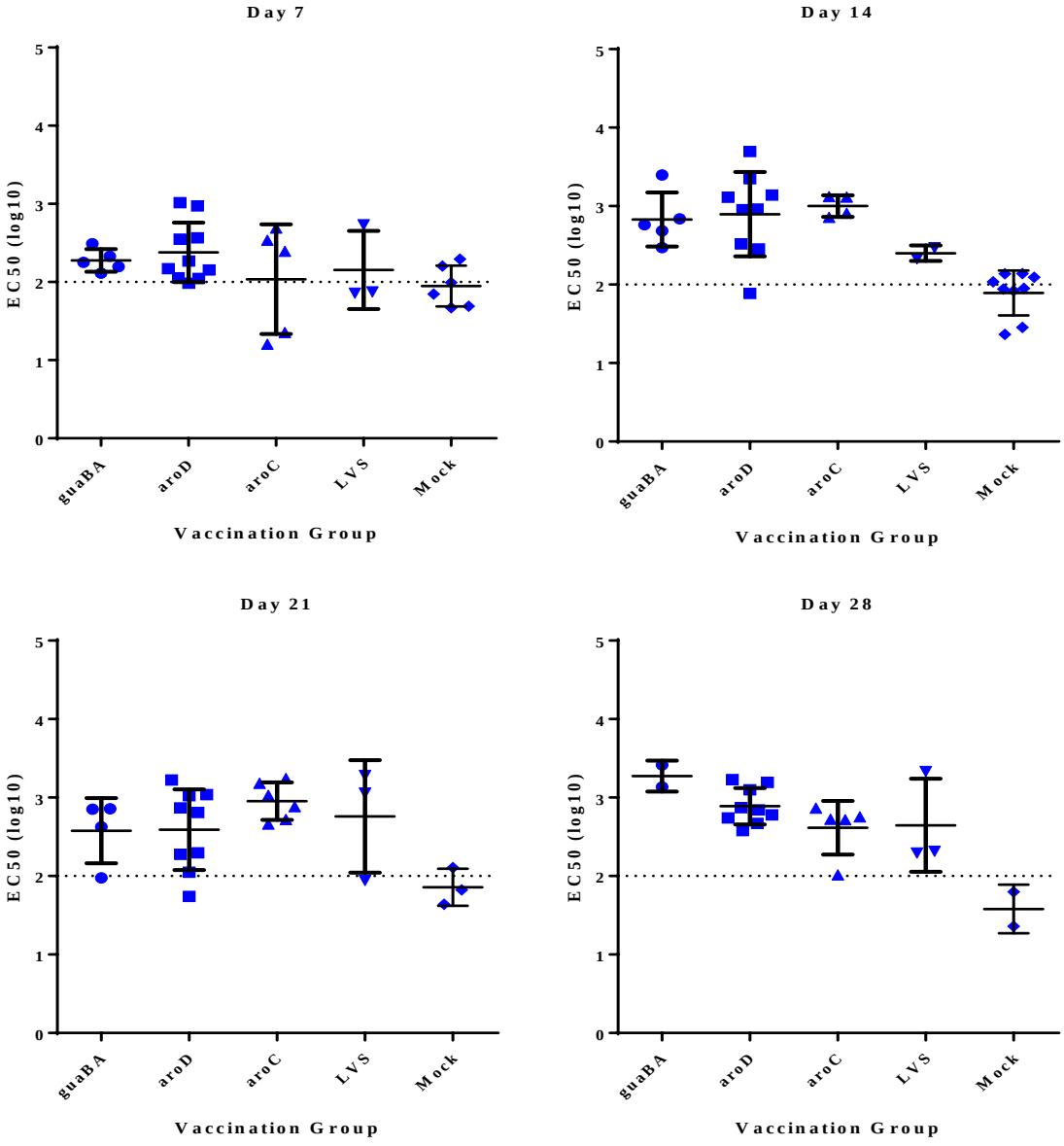

Figure 4: EC_{50} of serum IgG titers elicited by ΔguaBA, ΔaroD, ΔaroC, and LVS after scarification.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

Figure 4 shows the individual and averaged EC_{50} values for rabbits inoculated with ΔguaBA, ΔaroD, ΔaroC, and LVS by scarification compared against the mock at four time points.

While day 7 showed no significant difference between vaccine recipients or controls, days 14, 21, and 28 did show significant comparisons. Day 14 had three significant points, ΔguaBA vs mock, ΔaroD vs mock, and ΔaroC vs mock, with $p=0.0020$, $p<0.0001$, and $p=0.0007$, respectively. Day 21 only had one slightly significant comparison with ΔaroC vs mock with $p=0.0254$. Day 28, however, had the most four significant comparisons with ΔguaBA vs mock $p=0.0010$, ΔaroD vs mock $p=0.0011$, ΔaroC vs mock $p=0.0174$, and LVS vs the mock $p=0.0271$.

The next set of experiments examined oral inoculation with vaccines ΔguaBA, ΔaroD, and LVS. A fourth group was given a prime and a boost of ΔaroD orally (2x ΔaroD). Blood was drawn on days 7, 14, 21, and 28 post-inoculation to measure serum IgG titers. Results are shown in Figure 5. IgG titers were elevated in the LVS and 2x ΔaroD group on both day 14 and day 21 and on day 28 for 2x ΔaroD. However, one-ways ANOVAS performed for each time point found that none of the vaccine groups were found to be statistically significant from mock-vaccinated controls.

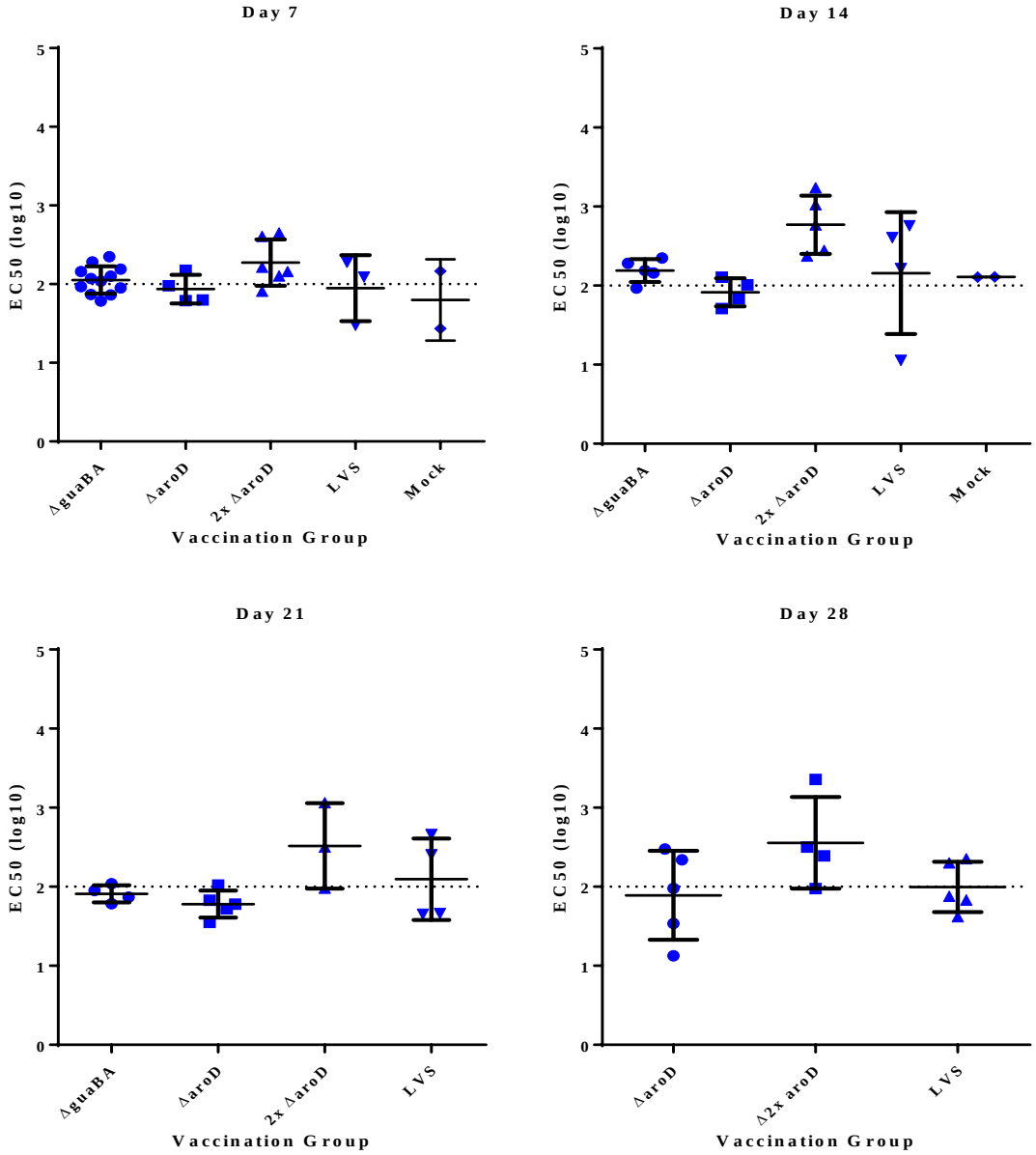

Figure 5: EC_{50} of serum IgG titers elicited by ΔguaBA, ΔaroD, 2 times the dose of ΔaroD (2x ΔaroD), and LVS given orally.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

Based on the protection see in murine and rabbit studies, ΔaroD was chosen to be the main focus of future experiments. Rabbits were vaccinated by aerosol exposure to ΔaroD or LVS. The IgG titers of LVS and ΔaroD were then compared with mock-vaccinated controls using one-way ANOVA to calculate significance.

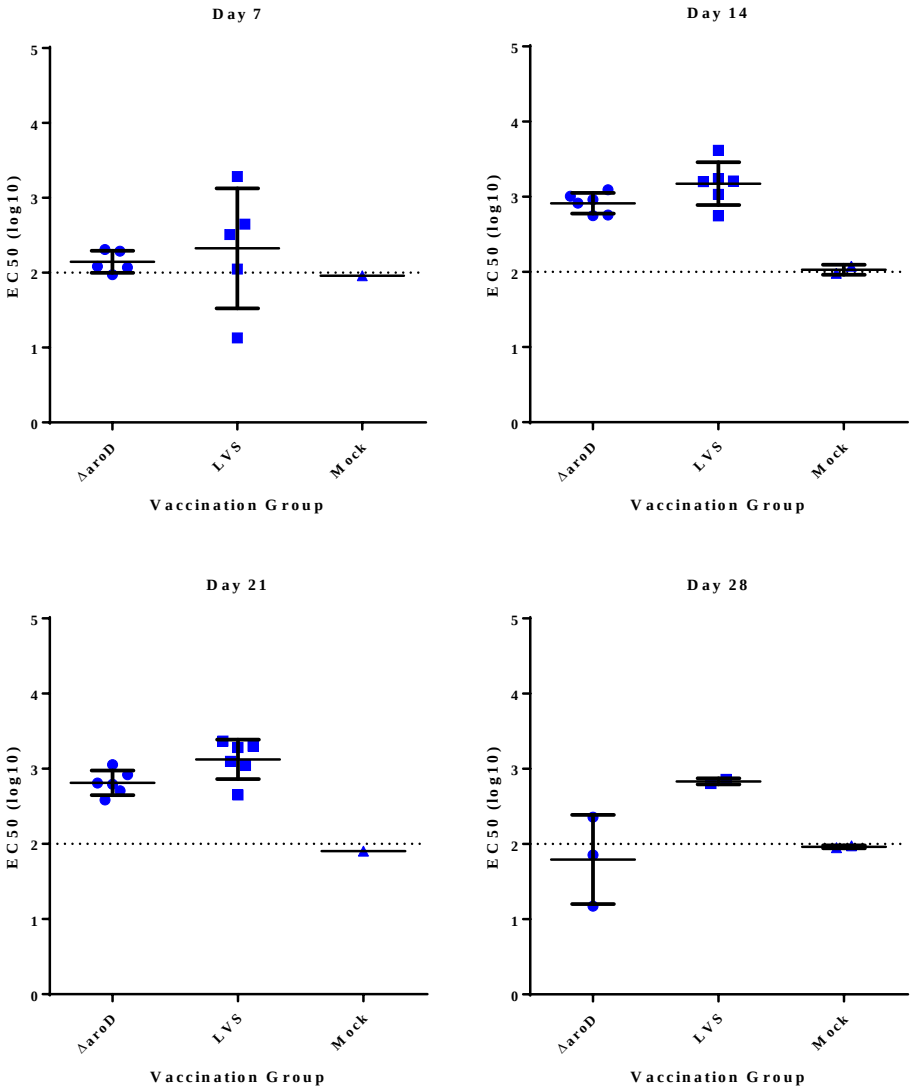

Figure 6: EC_{50} of serum IgG titers elicited by ΔaroD and LVS given by aerosol. Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

As shown in Figure 6, both ΔaroD and LVS had higher IgG levels compared to the control at days 14 and 21, and the higher levels on those days were significant. On day 14 ΔaroD vs mock p=0.0011 and LVS vs mock p=0.0001 as calculated by one-way ANOVA. An unpaired t-test was used for day 21 to compare LVS and ΔaroD; the difference was significant (p=0.0331). An unpaired t-test was chosen as opposed to a one-way ANOVA because there were not enough data points in the control group. When days 7 and 28 were analyzed, no significance was found between vaccination groups and mock-vaccinated controls. When the routes of vaccination- scarification, aerosol, or oral, with ΔaroD were analyzed in relation of time to death, scarification and aerosol had the best results, although differences with oral ΔaroD were not significant when analyzed using a Mantel-Cox test.

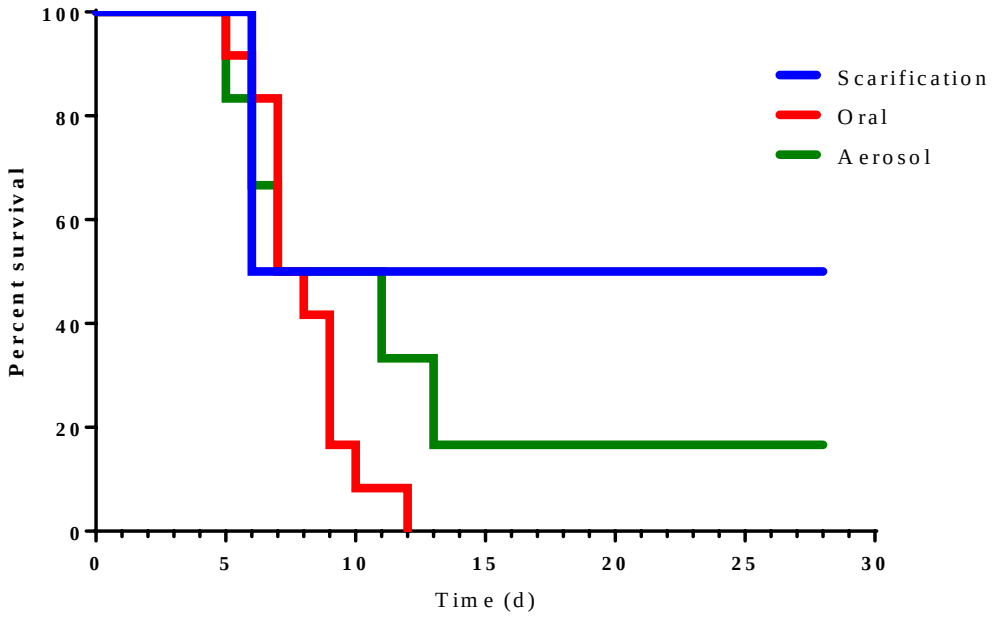

Figure 7: Impact of vaccination route on survival conferred by ΔaroD.

A prime/boost vaccination study with ΔaroD was carried out to evaluate whether this would boost the level of protection. Rabbits were vaccinated with an aerosol prime/scarification boost (AP/SB), scarification prime and aerosol boost (SP/AB), or an aerosol prime and aerosol boost (AP/AB). When determining the route of vaccination for the prime and boost experiments, scarification was chosen under the belief it would provide systemic immunity while aerosol would provide respiratory immunity. Based on the superior survival seen with the aerosol prime/boost of aroD (83% survival; see Figure 8), a subsequent experiment examined ΔguaBA and ΔaroD via aerosol in order to measure the IgG antibody response.

4.2 IgG titers imply protection

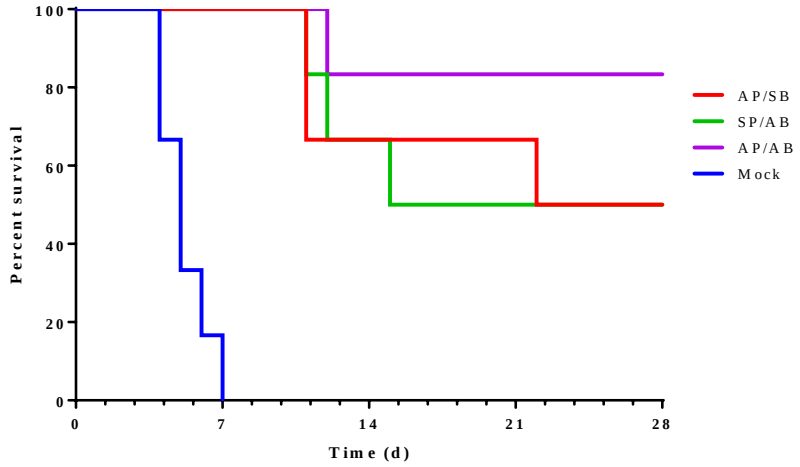

Figure 8: Impact of prime/boost vaccination on the level of protection.

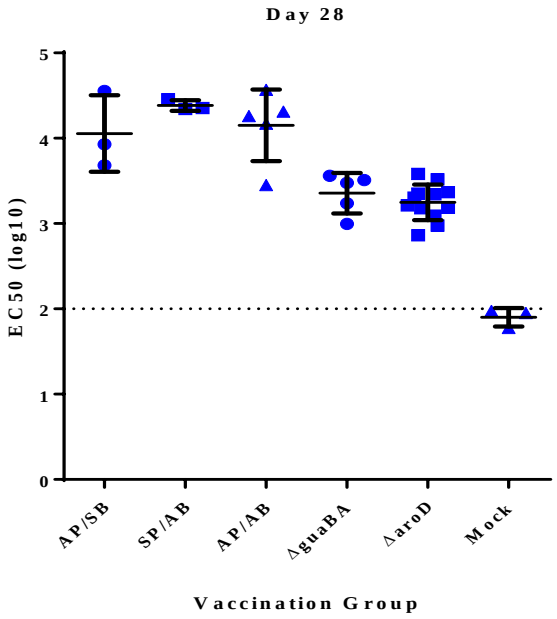

Figure 9: EC_{50} of serum IgG titers elicited from a prime boost study.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

When using one-way ANOVA to examine significance between groups in the prime boost study, multiple points of significance were calculated as shown in Table 2.

Table 2: Significance calculated by one-way ANOVA of groups in prime/boost study

Groups Compared	p value
AP/SB vs ΔguaBA	0.0247
AP/SB vs ΔaroD	0.0015
AP/SB vs mock	<0.0001
SP/AB vs ΔguaBA	0.0003
SP/AB vs ΔaroD	<0.0001
SP/AB vs mock	<0.0001
AP/AB vs ΔguaBA	0.0014
AP/AB vs ΔaroD	<0.0001
AP/AB vs mock	<0.0001
ΔguaBA vs mock	<0.0001
ΔaroD vs mock	<0.0001

Time to death was compared to day 28 post-vaccination EC_{50} of the IgG antibody titers (for most samples, the peak of the response) to determine if a possible correlation could be drawn.

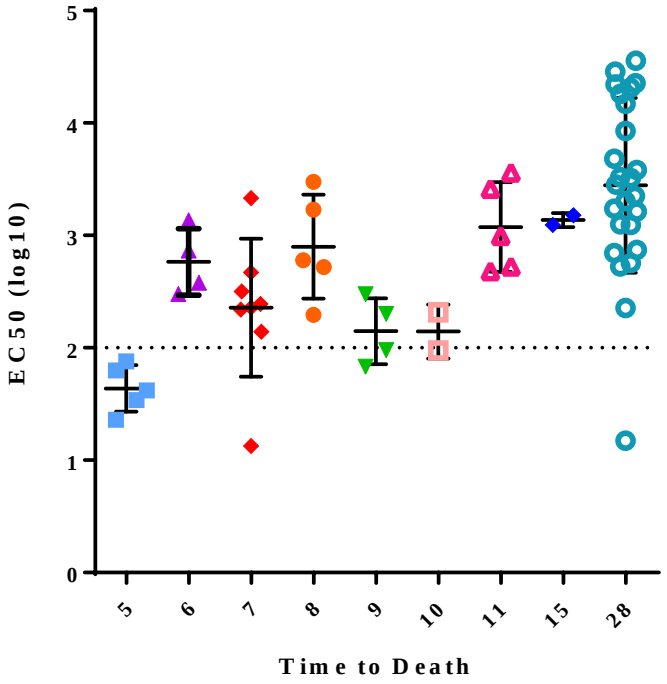

Figure 10: Serum IgG antibody titers correspond with survival against aerosol challenge with SCHU S4.

The antibody titers in Figure 10 are from 28 days post vaccination, or two days prior to challenge with SCHU S4. The data shown is across all vaccine groups and routes. Using a one-way ANOVA it was found that $r^2=0.5250$ and $p<0.0001$, implying that antibody titers can predict protection.

4.3 IgM and IgA titers in vaccinated rabbits

After examining IgG titers, antibodies IgM and IgA were studied because IgM is the typically the first antibody made in response to an infection and IgA is produced at mucosal

surfaces. IgA was chosen because pneumonic tularemia is the most fatal version of the disease and the respiratory tract and lungs are part of the mucosal immune system. First IgM and IgA levels were determined for rabbits inoculated with ΔguaBA, ΔaroD, ΔaroC, or LVS by scarification were measured. IgM titers were measured on days 7 and 14, while IgA was measured on day 28 (Figure 11).

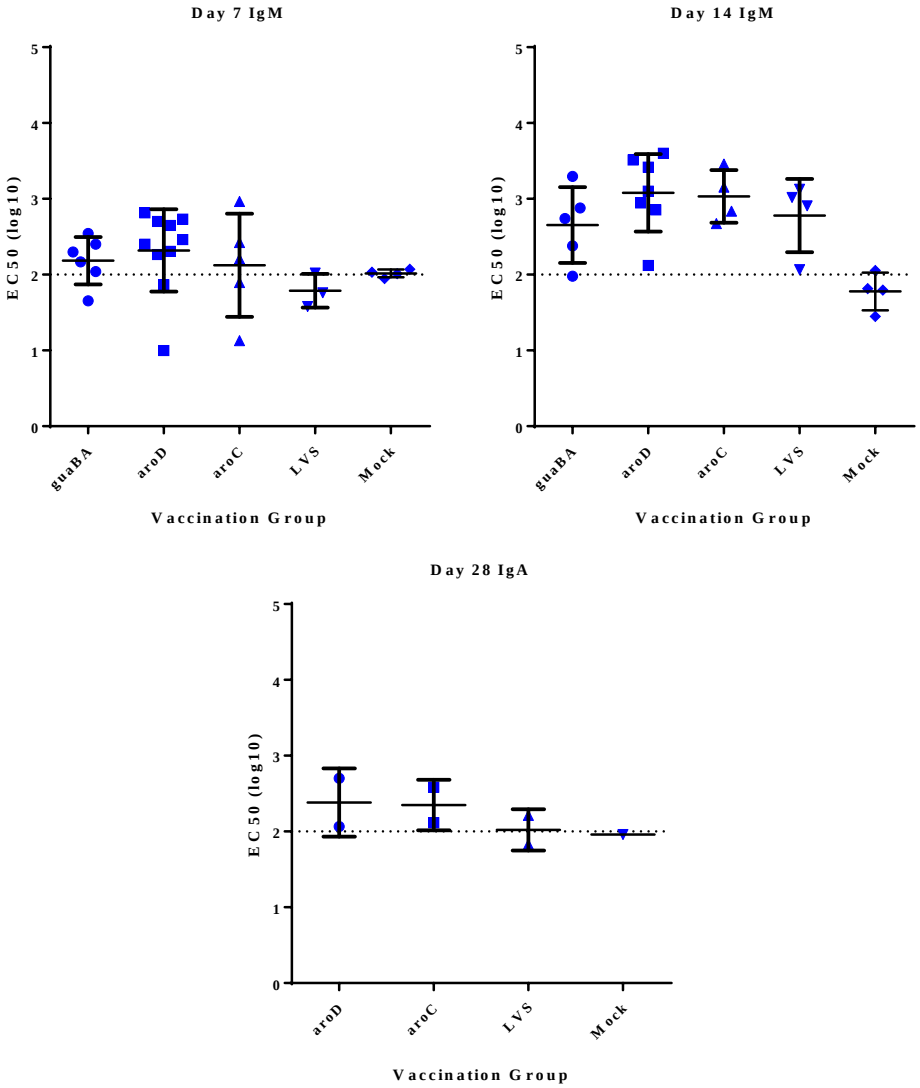

Figure 11: EC_{50} of serums IgM (days 7 and 14) and IgA (day 28) titers elicited by ΔguaBA, ΔaroD, ΔaroC, and LVS after scarification.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

Using one-way ANOVA in GraphPad it was determined that only IgM levels on day 14 were significant; where ΔaroD vs mock p=0.0018 and ΔaroC vs mock p=0.0085. No comparisons from day 7 or IgA on day 28 were found to be significant. Next, IgM and IgA levels were determined in rabbits vaccinated with ΔguaBA, ΔaroD, 2x ΔaroD, or LVS given orally (Figure 12).

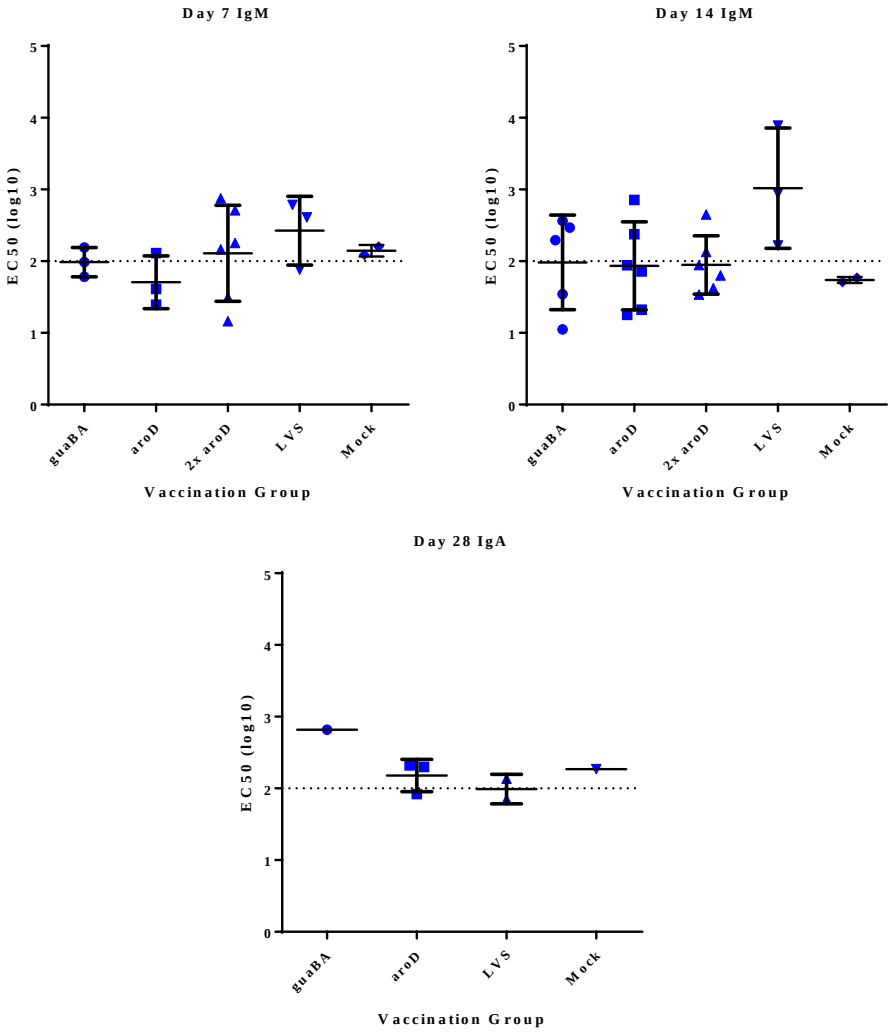

Figure 12: EC_{50} of serums IgM (days 7 and 14) and IgA (day 28) titers elicited by ΔguaBA, ΔaroD, 2 times the dose of ΔaroD (2x ΔaroD), and LVS given orally.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

No significance was found in the IgM levels for days 7 and 14 or in IgA levels. The next experiment looked at ΔaroD and LVS administered by aerosol (Figure 13).

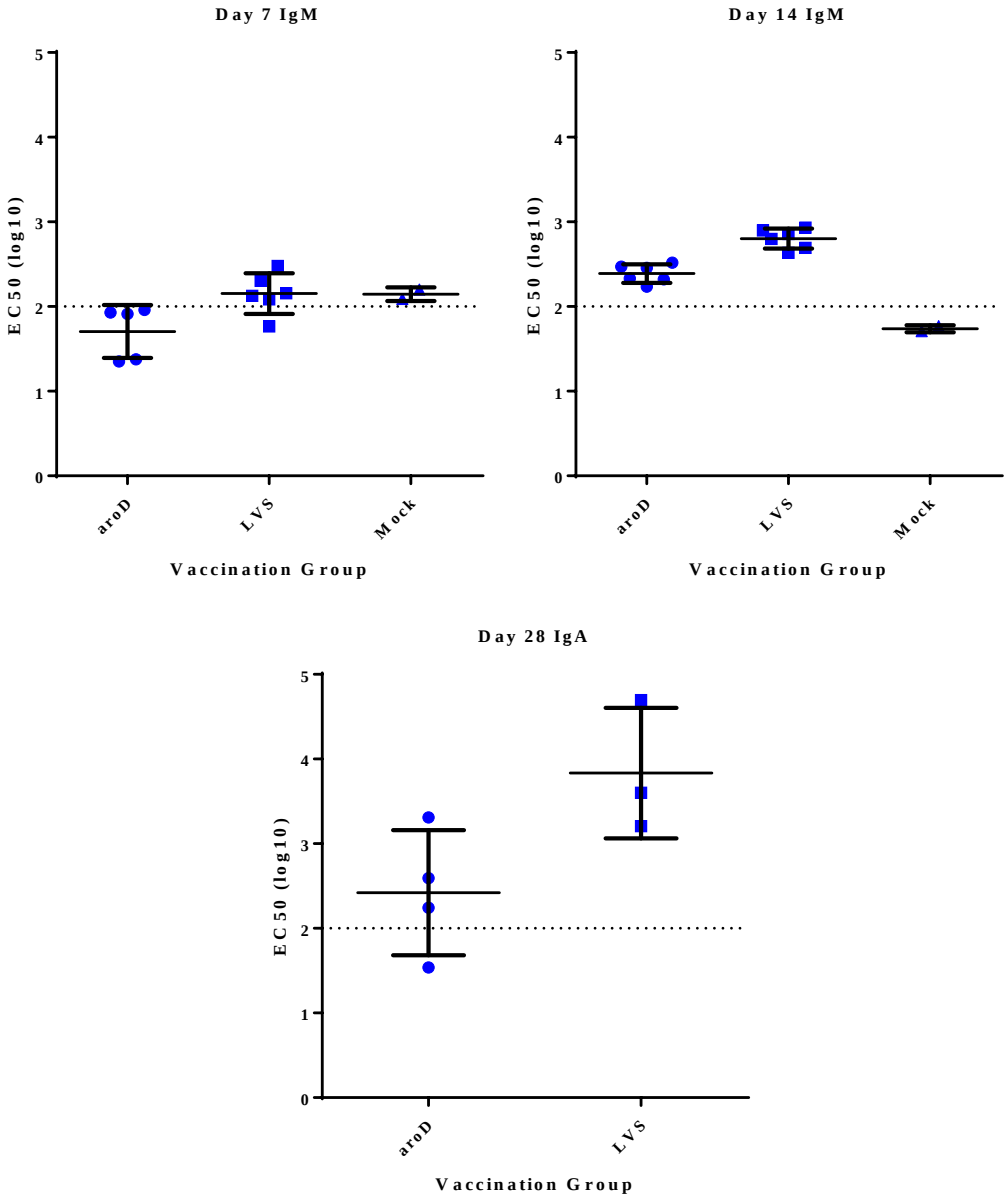

Figure 13: EC_{50} of serums IgM (days 7 and 14) and IgA (day 28) titers elicited by ΔaroD and LVS given by aerosol.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

In Figure 13, one way ANOVA tests were performed for IgM and an unpaired t test was performed for IgA because there were only two groups to compare and an ANOVA needs three or more. There was no significance in the IgA graph or IgM at day 7, but at day 14 all comparisons were highly significant with ΔaroD vs LVS $p=0.0001$, ΔaroD vs mock $p<0.0001$, and LVS vs mock $p<0.0001$. Following the layout of the IgG titers, IgM and IgA levels were next calculated for ΔguaBA, ΔaroD, and ΔaroD given as a prime/boost (Figure 14).

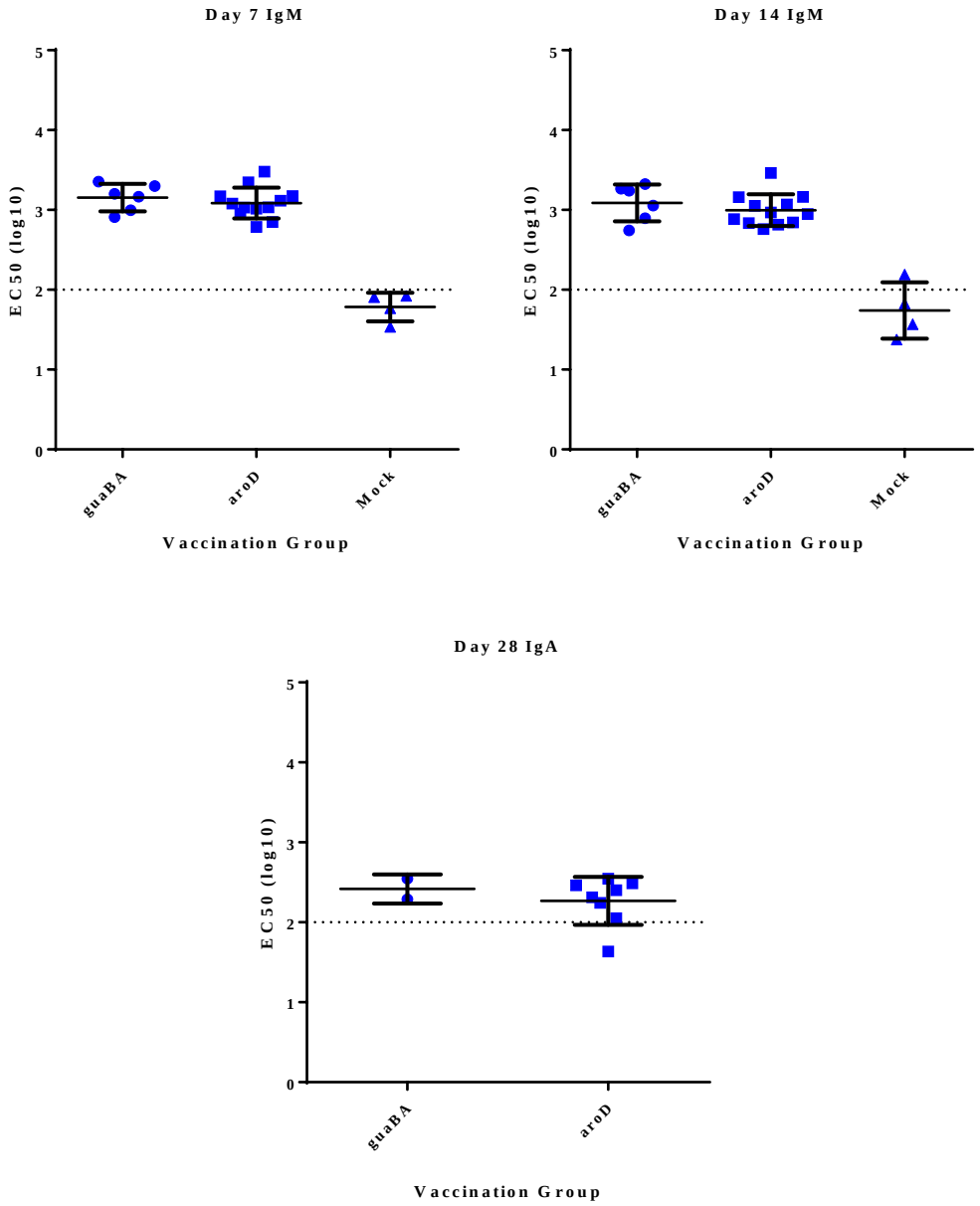

Figure 14: EC_{50} of serums IgM (days 7 and 14) and IgA (day 28) titers elicited from a prime boost study.

Data from mock-vaccinated rabbits are also shown. Graphs show results for individual rabbits (blue circles) at each time point; black lines are the mean for each group with the error bars indicating the standard deviation.

Data for mock vaccinated rabbits was not available for IgA analysis. Using one-way ANOVA calculations it was determined that IgM levels on day 7 and 14 were highly significant with $p<0.0001$, for both ΔguaBA and ΔaroD compared to mock vaccinated rabbits. The EC_{50} of IgM antibody titers measured at days 7 and 14 were then graphed with respect to time to death to ascertain whether there was a correlation with survival similar to what was seen with IgG (Figure 15).

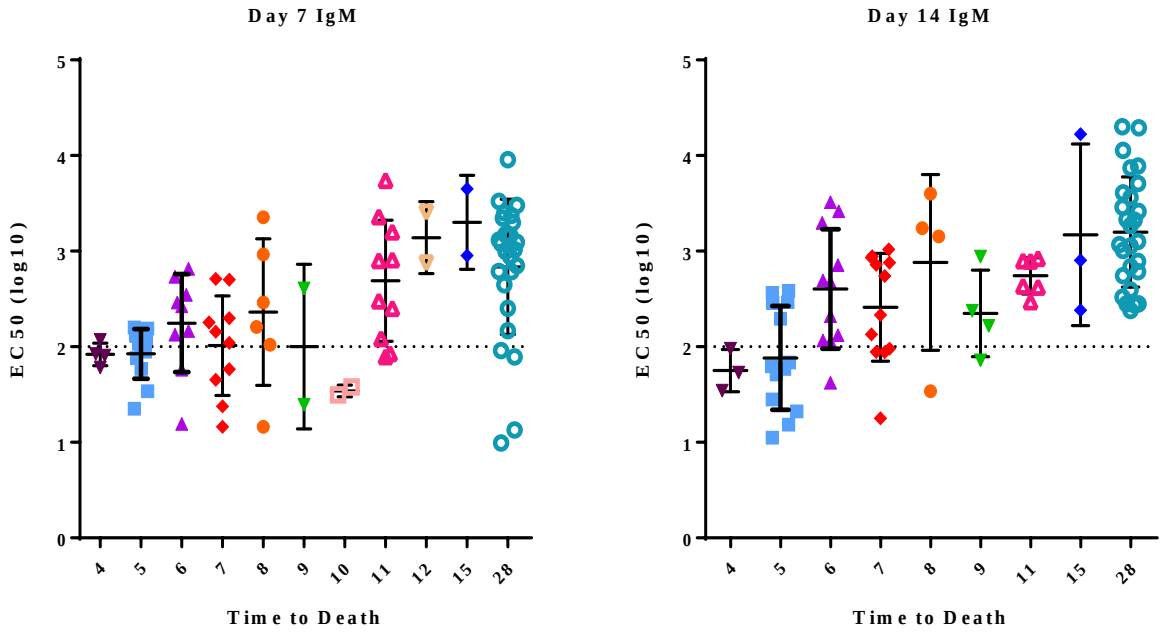

Figure 15: IgM antibody titers at days 7 and 14 correlate with survival

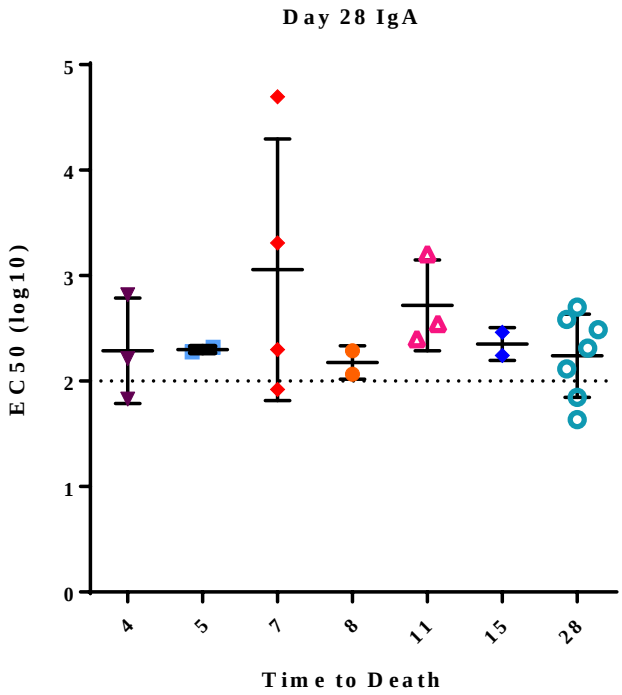

Figure 16: IgA levels do not seem to correlate with survival

Both day 7 and day 14 IgM titers were highly significant. Day 7 was $p<0.0001$ with $r^2=0.3799$ and day 14 was calculated to be $p<0.0001$ and $r^2=0.4505$ (Figure 15). However, IgA levels on day 28 did not correlate with survival, $p=0.4926$ and $r^2=0.2611$ (Figure 16).

5.0 DISCUSSION

Francisella tularensis is a potential bioweapon that has the ability to cripple the American society and leave us vulnerable to a follow up terrorist attack. The need for a safe and effective vaccine as well as a better understanding of how the immune system responds to a *F. tularensis* infection is essential. While LVS is currently given to at risk personnel as an investigational new drug, it is not currently licensed by the FDA and is unlikely to be due to the possibility of the virus mutating back to a virulent state. Due to these facts the Reed lab and collaborators have been experimenting with live attenuated strains of *F. tularensis* with the goal of developing one as a vaccine that would provide better protection that LVS and to gain a better understanding of the components of the immune system that contribute to survival.

In comparing the live attenuated vaccines developed by Dr. Barry with LVS it was found that rabbits vaccinated with ΔguaBA and ΔaroD survived challenge while LVS only extended time to death. IgG titers determined by ELISA for days 7, 14, 21, and 28 post-vaccination demonstrated that ΔaroD vaccinated rabbits that survived subsequent SCHU S4 challenge had higher antibody titers on day 28 than non-survivors. These results implied that antibodies play a role in survival against pneumonic tularemia. Various vaccines were then tested via scarification, oral inoculation and aerosol delivery. It was not surprising that day 7 IgG titers were not significant because the immune response is in the beginning stages. Serum IgG titers were elevated on days 14, 21, and 28 after scarification for aerosol vaccination (Figures 4 & 6). IgG titers were not significantly increased after oral vaccination with any of the vaccines tested. Across all vaccination studies, serum IgG titers on day 28 post-vaccination were found to predict survival after challenge.

IgM and IgA antibodies were also studied to see if they too could predict survival. IgA titers on day 28 post-vaccination were not found to be significant with any route of vaccination and did not correlate with protection. It is possible that IgA levels at earlier time points post-vaccination could predict protection but these were not evaluated. IgM levels were found to be significant with both scarification and aerosol vaccinations across all vaccine groups. As with IgG, the strongest IgM titers were seen in the prime/boost groups. Somewhat surprisingly, both day 7 and day 14 of the IgM titers correlated with survival although not quite as strongly as IgG titers.

Francisella tularensis is a facultative intracellular bacterial pathogen. Protection has been thought to be a result of cell-mediated immune responses. The data presented here, however, demonstrate that serum IgG and IgM titers elicited in rabbits after inoculation with live attenuated strains of *F. tularensis* correlate with survival after aerosol challenge with the virulent SCHU S4 of *F. tularensis*. Since the route of entry is the lung, it was surprising that IgA titers did not correlate with protection. Nevertheless, the data suggests that antibody may play a role in the protection although further experimentation will be needed to determine the mechanisms and antigens involved in the humoral response.

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