Indirect Enzyme-Linked Immunosorbent Assay Optimization for Vaccine Development and Verification of *Francisella tularensis*

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ABSTRACT

Tularemia is a highly infectious and readily aerosolized disease caused by the Gram-negative bacterium *Francisella tularensis*. There is currently no licensed vaccine, and therefore developing a vaccine that can effectively provide protection and prevent infection is vital. One method in determining vaccine efficacy is by animal survival after challenge with a virulent strain of *F. tularensis*. However, there is a clear need for a clinically relevant method of determining if a vaccine is working, which could potentially be correlated with antibody titer. This research aimed to develop and optimize a sensitive and reliable indirect ELISA assay to detect rabbit antibody response to SCHU S4, a virulent strain of *F. tularemia*. Primary (Ab1) and secondary (Ab2) antibody incubation temperature and background due to temperature and method of plate washing was investigated using 4 hyperimmune rabbit plasma samples 28 days post challenge with SCHU S4 that were vaccinated with attenuated recombinant mutants of SCHU S4. The IC50 and the Hill Coefficient values of four-parameter logistical regressions of ELISA assay data was analyzed to gain insight on the antibody binding curves. It was found that there is a significantly higher logIC50 values for 37°C than 4°C for Ab2 ($p = 0.006$, $n=3$) and for room temperature than 37°C for Ab1 ($p = 0.047$, $n=1$), however, the Hill Coefficients were not significantly varied among different temperatures for both experiments. The ELISA plate background was furthermore significantly greater when the plates were washed by hand with a pipette than with using a plate washer ($p = 0.023$). The optimization indirect ELISA assay parameters will then be applied to an experiment to examined rabbit antibody response 28 days post-vaccination against 5 strains of *F. tularensis*.

Francisella tularensis is a Gram-negative bacterium that causes the disease tularemia, or rabbit fever. There are two subspecies that are associated with human disease: the more virulent type A (*Francisella tularensis* subsp. *tularensis*) that is predominately found in North America, and type B (*Francisella tularensis* subsp. *holarctica)* (1,2). *F. tularensis* can be transmitted through direct contact with an infected human or animal tissue, but it can also become aerosolized. Even a low bacterium concentration (~10 bacterium) is extremely infectious, resulting in a 35 percent mortality rate when untreated, and furthermore antibiotic resistant and highly virulent strains were reported to be developed by both the United States and Soviet Union as a biological weapon (3). This resulted in *F. tularensis* to be listed as a tier 1 select agent by the Center for Disease Control (1,4). However, there is currently no licensed vaccine for *F. tularensis.* Previous research developed an ineffective killed vaccine, followed by trying an attenuated type B strain termed LVS, or live vaccine strain, which was effective against SCHU S4, a virulent type A strain (1,5). However, there are questions concerning LVS's ability to revert into a non-attenuated strain (1). Therefore, there is a demand for a *F. tularensis* vaccine that can stimulate an effective immune response and clear the bacterium to prevent infection.

Live attenuated vaccine strains developed from both type A and type B *F. tularensis* are currently being studied in animal models and in *in vitro* experiments. For example, *ΔclpB* is an attenuated SCHU S4 strain that is missing a key heat shock protein and was used in this study (6). The effectiveness of the vaccine can be measured by rabbit survival, but antibody titer can be used as a potential correlation to protection. There is a clear need to develop a sensitive and reliable method to quantitatively assess if the vaccines are

inducing an antibody response which can possibly become a prediction of animal protection. Furthermore, a clinically relevant way is needed to measure if the vaccine is working. Indirect enzyme linked immunosorbent assays (ELISA) is both a reliable and sensitive analysis that has been used in prior research for seroepidemiological investigations including *F. tularensis* (2). The overall purpose of this research is to optimize the indirect ELISA assay for detecting rabbit antibody response to the virulent type A strain SCHU S4 by investigating the significance of the secondary antibody incubation time and temperature, and plate washing methods. The informed results of this study were used to optimize the ELISA assay in looking at rabbit antibody binding to multiple type A and type B strains after vaccination with attenuated recombinant mutants of SCHU S4.

RESULTS

Determining Positive Control Candidates in Hyper-Immune Rabbit Plasma Samples

New positive control candidates for future ELISA assays were identified after analyzing the antibody binding to SCHU S4 in 10 rabbit hyperimmune plasma samples from 2014. A four-parameter logistical regression in Graphpad Prism 7 was used as it was previously shown to best fit the binding response curve and furthermore provides insight into binding activity with the IC50 and slope values (Reed et al., 2002). Antibody binding saturation was also previously found to be ≥ 10 million Relative Light Units (RLU's), the read out from the illuminometer. HI Rabbit plasma samples 136-14 and 137-14 were both identified as new HI positive controls as the samples were saturated above 10 million RLU's for at least 3 half log dilutions (Figure 1). These identified samples were then used in addition to the positive control for the ELISA optimization experiments because they had reached antibody saturation using this protocol.

Optimization of Indirect ELISA's for *F. tularensis*

The optimization of antibody binding for a semi-quantitative ELISA assay analysis is important when evaluating vaccine efficacy. The Reed lab previously used standard dilutions for the amount of CFU/ml (antigen) concentration for coating the wells, and the concentration of the primary and secondary antibodies added. This research focused on the temperature of incubation for the primary and secondary antibody and the method of plate washing's effects on the background determined by the negative control. The logIC50, or how much antibody is needed to inhibit 50% of *F. tularensis*, is a semi-quantitative approach to assessing the antibody-antigen binding. A cutoff of log = 2.0 is the EC50 for mock-vaccinated rabbits and therefore the logIC50's must be above this threshold for the vaccine to provide protection. Another point of interest is the characteristic of the binding curves as a representation of the degree of interaction between the antigen and antibody, represented by

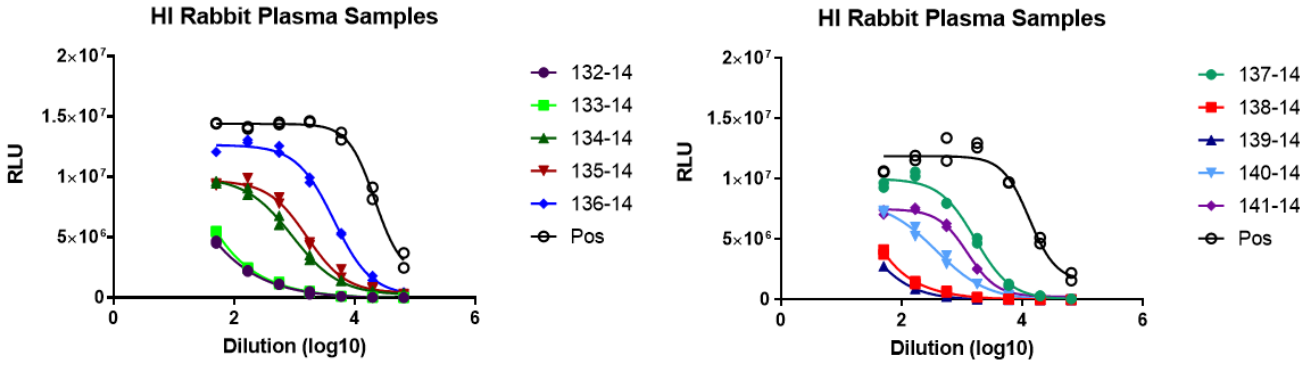

Figure 1. Antibody binding response curves of 10 hyperimmune rabbit plasma samples and a hyperimmune plasma positive control over 7 half log dilutions from ELISA assays. Fit of curves were determined by 4-parameter logistical regression. Binding is measured in Relative Light Units as read out by luminometer after adding POD. Each plasma sample was plated in duplicate.

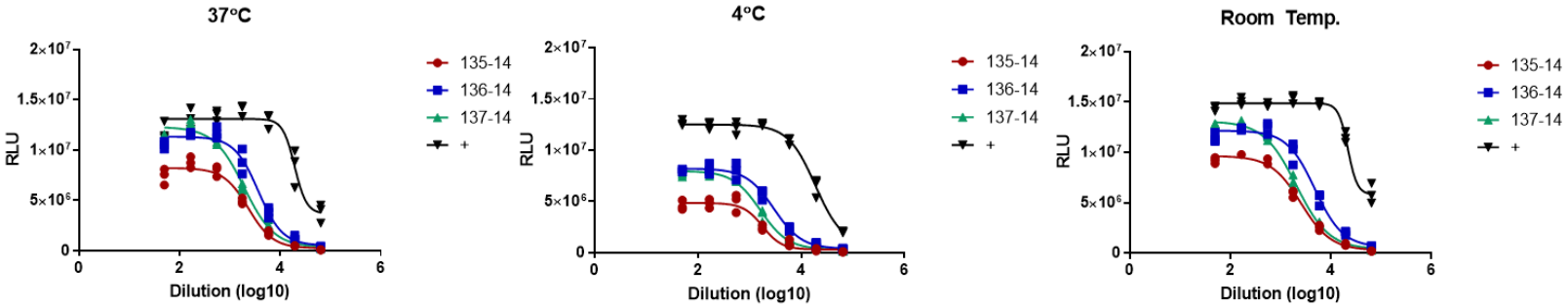

Figure 2. Example antibody response curves for 4 HI rabbit plasma samples for three different secondary antibody incubation temperatures – 37°C, 4°C, and room temperature. Fit of the binding curves over seven half log dilutions were obtained by using the four-parameter logistical regression analysis. Each plasma sample per plate was performed in triplicate. This experiment was repeated (n=3) times.

the Hill Coefficient. The Hill Coefficient is also representative of how steep the slope of the binding curve is, which can be an indication of antibody binding saturation.

Secondary Antibody Incubation Temperature

Secondary antibody incubation temperatures that were tested were 37°C, 4°C, and room temperature (20-25°C) for 1 hour (Figure 2). The ELISA protocol that had been previously used in the lab was 37°C, so therefore what was examined was the comparison between 37°C and room temperature and 37°C and 4°C. Illuminometer results were analyzed in Microsoft Excel and Graphpad Prism 7 where the data was fit by a four-parameter logistical regression which also gives logIC50 and Hill coefficient values. This study was conducted in triplicate and the mean logIC50 values were calculated for all rabbit HI plasma samples (Table 1).

Paired T-tests were conducted in Graphpad Prism 7 to determine if there was a statistically significant difference between the logIC50's which would indicate the optimal temperature for secondary antibody binding. It was found that there is no statistically significant difference between the logIC50 values for the 4 rabbit plasma samples between room temperature and 37°C ($p = 0.816$), but a significant difference between 4°C and 37°C ($p = 0.006$) with the 37°C having the higher logIC50 values. The higher the logIC50 value, the higher the antibody binding to the antigen and therefore optimizing the assay. These results indicate that 4°C is not optimal for antibody binding but that there is no significant difference between incubation temperature at 37°C or room temperature.

Paired T-tests between 37°C and room temperature and 37°C and 4°C of the mean Hill Coefficients for the rabbit HI plasma samples (Table 2). There was no statistically significant difference found between room temperature and 37°C ($p = 0.794$) and 4°C and 37°C ($p = 0.416$). Furthermore, a negative mean Hill Coefficient was found for all samples, meaning that there is negative cooperativity between the antibody and

Rabbit HI Plasma Sample	Mean logIC50 Values		
	37°C	Room Temp	4°C
135-14	3.2563	3.213	3.133
136-14	3.491	3.548	3.316
137-14	3.306	3.330	3.156
Positive Control	4.230	4.214	4.145

Table 1. Averaged logIC50 values from (n=3) experiments of different secondary antibody incubation temperatures obtained with four-parameter logistical regression analysis. 37°C had a statistically significantly higher logIC50 than 4°C.

Rabbit HI Plasma Sample	Mean Hill Coefficient		
	37°C	Room Temp.	4°C
135-14	-1.623	-1.301	-1.738
136-14	-1.641	-1.421	-1.482
137-14	-1.573	-1.461	-1.588
Positive Control	-2.672	-2.799	-1.846

Table 2. Averaged Hill Coefficient values from (n=3) experiments of different secondary antibody incubation temperatures obtained with four-parameter logistical regression analysis. There was no statistical difference between the mean Hill Coefficient for different secondary Ab temperatures.

antigen, which is likely because there is a set amount of antigen so no more antibody can bind than the amount of antigen present.

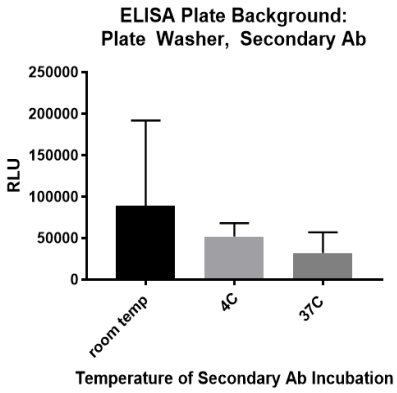

Figure 3. Plate background obtained from the negative control that had no primary antibody added for different secondary Ab temperatures. Room temperature had the overall highest background and higher variability, represented by the standard deviation bars

While higher logIC50 values are useful for ELISA assay optimization, it is also important to examine the plate background. A higher level of background and noise in the assay may result in misrepresentation of the data, offset logIC50 values, and overall decrease the sensitivity and reliability of the assay. Therefore, it is important to minimize the background. One row per plate was designated as the negative control that was still coated with antigen but Ab1 and Ab2 was not added to obtain the basal level of RLUs. It was found that there was no statistically significant difference in background noise amongst the different Ab2 incubation temperatures, although Ab2 at room temperature had the highest variability (SD) and background overall (Figure 3).

It was found that there was a statistically significantly higher logIC50 of 37°C than 4°C for Ab2 incubation temperature. However, even though there is no statistically significant difference in logIC50s between 37°C and room temperature, room temperature has a higher overall plate background and variability, although not statistically significant. Therefore, these results indicate that 37°C is the optimized Ab2 incubation temperature that has both a high logIC50 and low plate background.

Primary Antibody Temperature

Ab1 temperatures tested were the same as the Ab2 experiment at 37°C, room temperature, or 4°C for 1 hour. The four-parameter logistical regression was performed on the data to obtain the logIC50 and Hill coefficient values (Figure 4). This study however was only conducted once and will therefore have to be repeated. The results of changing Ab1 temperature was different than that found with changing Ab2 temperature. Paired T-tests found that there was a statistically significant higher logIC50 values for room temperature than 37°C ($p = 0.047$) and no statistical significance between 37°C and 4°C as found with Ab2. Like Ab2, there was no significant difference amongst the Hill Coefficients and were also displaying negative cooperativity, which is again expected.

ELISA plate background was also analyzed as the optimized assay should have a

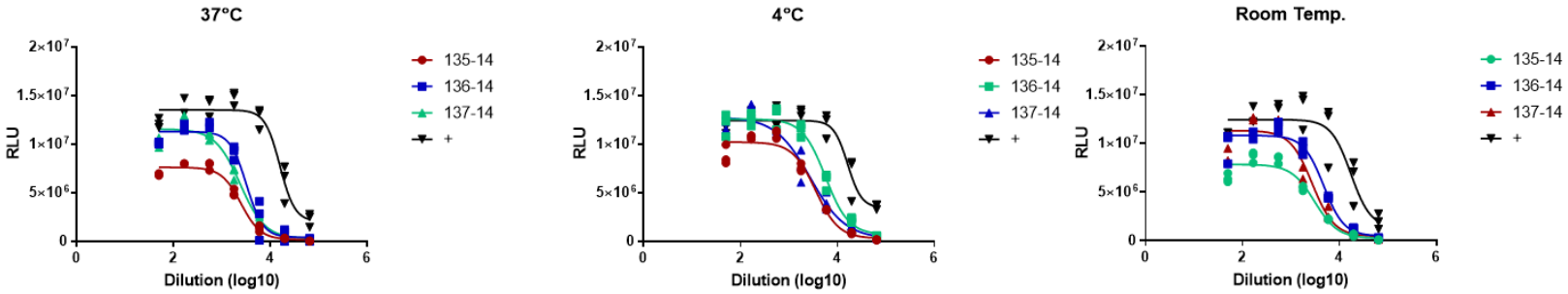

Figure 4. Example antibody response curves for 4 HI rabbit plasma samples for 3 primary antibody incubation temperatures – 37°C, 4°C, and room temperature. Fit of the binding curves over seven half log dilutions were obtained by using the four-parameter logistical regression analysis. Each plasma sample per plate was performed in triplicate. This experiment was repeated (n=1) times.

minimal and non-variable background noise. It was found that there was no statistical difference between the backgrounds at different Ab1 incubation temperatures. Overall 37°C had the highest plate background and variability measured in standard deviation (Figure 5).

Room temperature had a statistically higher logIC50 than 37°C and furthermore had a relatively low level of plate background noise. The trend of these results suggest that the Ab1 incubation should be at room temperature, however, this experiment needs to be repeated at least 2 more times to draw conclusive results to inform future ELISA assays.

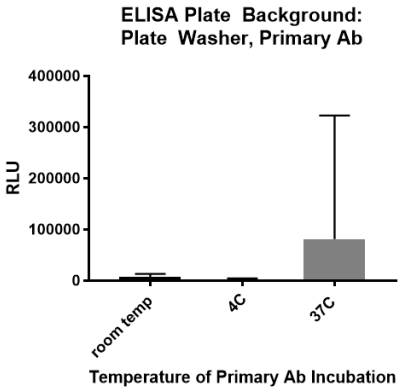

Figure 5. ELISA plate background determined by a negative control per plate that had no primary Ab. When primary Ab incubation was varied by temperature, it was found that the highest and most variable background was found at 37°C, with the greatest RLU count and standard deviation.

ELISA Assay Negative Control Plate Background

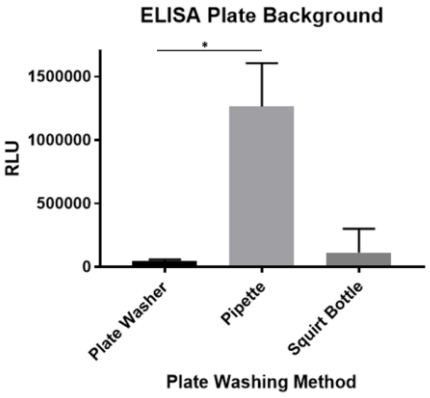

Figure 6. Plate background determined from different methods of plate washing. There was a statistically significant difference in background (RLUs) between using a plate washer and pipette, indicated by *.

The next part of the ELISA assay to be examined for optimization was the method of plate washing. Three different methods – plate washer, pipette, and squirt bottle – were used in another set of experiments that looked at Ab2 incubation temperature and averaged across all. Paired T-tests were performed comparing plate washer to either pipette or squirt bottle since the standard protocol previously used a plate washer. The overall goal was to find which technique minimized plate background for all three temperatures, and it was found that using a pipette to hand-wash plates had a statistically significant higher background than using a plate washer ($p = 0.023$). While there was no significant difference between using a squirt bottle or plate washer, the plate washer overall had the lowest background and variability (SD) (Figure 6). Therefore, using a plate washer is key to minimizing background which is important for assay optimization.

DISCUSSION

Current research aims to develop a live attenuated vaccine for *F. tularensis* that can induce an immune response to clear bacteria in the body and protect against infection. However, there is a need to develop a highly sensitive and reliable assay that can assess protection against virulent strains that is not just in the form of animal survival to have clinical relevance. ELISA assays previously have been used in diagnosing *F. tularensis* in clinical settings (8). The aim of this research was to optimize indirect ELISA assays for detecting rabbit antibody response 28 days post-challenge with SCHU S4 to the virulent type A strain SCHU S4. It was found that 37°C was the optimal incubation temperature for Ab2 that maximized logIC50 and minimized the plate background levels. Surprisingly, room temperature was found to be optimal for Ab1 with a statistically significant higher logIC50 than 37°C and less background levels than 37°C. However, the Ab1 experiment was only conducted 1 time and therefore will need to be repeated at least 2 more times to have conclusive results. Furthermore, the method of plate washing during the ELISA assays was also important for plate background levels, and there were significantly higher levels of background when the plates were washed by pipetting 200 µL of PBS+ 0.5% Tween

than with using a plate washer. One possible reason for this is that there is not enough force applied to the bottom of the well when using a pipette relative to a plate washer or squirt bottle and therefore the wash is not as effective.

This research also proposed the using of the Hill Coefficient produced by the four-parameter logistical regression equation, as a method of characterizing the slope of the binding curve that can allow insight into antigen-antibody binding. This equation is as follows,

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} \qquad (9)$$

where *y* is the value of the response at value *x* where *x* is the independent and *y* the dependent variable. The variable a is the minimum value of the binding response curve, *d* is the maximum value, and *c* is the point of inflection of the binding curve which is halfway between values a and *d*, also known as the IC50. *b* is Hill's slope of the curve, or in this case the Hill Coefficient reported, which represents the steepness of the curve at the inflection point *c* (9). Interestingly, there was no statistically significant difference amongst the Hill slope's when incubation temperature for Ab1 and Ab2. Therefore, further research is needed to determine if the Hill Coefficient provides insight vital to assess vaccine protection and antibody titer.

Areas of future research include optimizing the incubation time for both the Ab1 and Ab2 to not only optimize antibody binding but to potentially reduce the time to complete the assay for clinical application. These experiments furthermore only focused on antibody response against the virulent strain SCHU S4, and it would be insightful to investigate if these optimization parameters are specific for this antigen or are corroborated with using another *F. tularensis* strain as the antigen. This would also answer if this optimized ELISA assay is antigen dependent or independent. Possible limitations of this research include that the 405 LS Microplate Washer (Biotek) broke after the first Ab2 experiment, and we had to switch to the Skan Washer 300 Version B, which could be a potential source of variability. These experiments furthermore could be repeated more times to further verify the significant results. Furthermore, the ELISA optimization results will be used for a parallel vaccine testing of rabbit antibodies 28-day post-vaccine with an attenuated strain of SCHU S4 against a mix of 5 type A and type B *F. tularensis* strains to see if the vaccine provides protection against a range of strains.

EXPERIMENTAL PROCEDURES

F. tularensis Strains

SCHU S4 (hkSCHU S4) was previously cultured in Brain Heart Infusion broth (BHI) and was heat killed at 65°C for 3 hours. *ΔClpB*, a strain of SCHUS4 that is missing a heat shock protein, was provided by Wayne Conlan, National Research Council, Canada. *ΔClpB* was streaked on a cysteine heart agar plate (CHA) and incubated in 37°C for 48 hours and transferred into a BHI broth culture at a concentration of ~10^9 CFU's in a 37°C shaker overnight. 500 µL of BHI broth culture was used to created serial dilutions from 10^0 to 10^{-9} to determine the concentration (cfu/ml) of *ΔClpB* for the glycerol stocks and hk stock. Glycerol stocks were created for *ΔClpB* and stored at -80°C. A separate overnight broth culture was made for hk-ClpB, which was heat killed at 65°C for 3 hours. A plate of 20µL of hk-ClpB was then plated on CHA and was verified 10 days later of no *ΔClpB* growth. HkSCHU S4 and hkClpb stocks were stored at -20°C.

Hyperimmune Rabbit Plasma

Hyper-immune (HI) rabbit plasma was previously isolated from 10 rabbits that survived for 28 days after challenge with SCHU S4 in a *F. tularensis* vaccination experiment in 2014 that was conducted in a Biosafety Level 3 (BSL-3) facility at the University of Pittsburgh Regional Biocontainment Center. These samples were cleared under biosafety protocol for usage in Biosafety Level 2 (BSL-2) laboratory settings and stored at -20°C. The HI plasma samples used were from rabbits 132-14 to 141-14. The positive control HI plasma sample 39-12 was obtained from a prior experiment in 2012 and was furthermore verified multiple times of high antibody binding and binding saturation to SCHU S4, of which the threshold is ≥10 million relative light units (RLUs).

ELISA plates

White high-binding 96 well plates were coated with $1.0x10^5$ cfu/well of hk-SCHUS4. 0.795g of Na_2CO_3 and 1.465g $NaHCO_3$ was added to 500 ml diH_2O to make the carbonate-bicarbonate buffer (pH =9.6). Hk-SHU S4 was diluted in carbonate-

bicarbonate buffer to achieve $1.0x10^5$ cfu/well and added to plates. The plates were sealed and incubated overnight at 20-26°C (room temperature). Blocking buffer was prepared using 1.05g of nonfat dry milk per 20 ml of PBS + 0.5%Tween. The coating antigen was then removed but not washed and 200 µL of blocking buffer was added per well. The plates were then sealed and incubated at 37°C for 3 hours. The blocking buffer was then removed and plates were sealed and stored at 4°C. This same process was then repeated using the *F. tularensis* strains ClpB, WY96, OR96, KY99, and MA00 (refer to future research section of discussion).

Indirect ELISA of F. tularensis

1:50 dilutions of plasma samples were prepared of 5 µL serum into 245 µL of blocking buffer. Plates were washed with PBS + 0.5% Tween using a spray bottle, pipette, or the plate washers Skan Washer 300 Version B or 405 LS Microplate Washer Biotek (see *Optimization Assays* below). Six half log dilutions of the HI serum down the plate was created by adding 50 µL of blocking buffer to all rows except for row A and 73µL of 1:50 dilution serum was added to row A. A series of dilutions was performed by mixing and removing 23 µL starting from row A downward, and the last row had no serum as a negative control. The plates were then sealed and incubated for 1 hour at 37°C. Secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) was diluted 1:2500 in blocking buffer which was previously verified by the Reed lab. Plates were removed from 37°C and washed 5X at room temperature with PBS + 0.5% Tween. 100µL of secondary Ab dilution was added to each well and the plates were sealed and incubated at 37°C for 1 hour (this differed in ELISA optimization assays, see *Optimization Assays* below). During this incubation period, POD chemiluminescence ELISA substrate was prepared by adding 100 parts of solution A, luminol/4-iodophenol, to 1 part of solution B, a stabilized form of H_2O_2 (Sigma-Aldrich). Plates were then washed 5X at room temperature with PBS + 0.5% and 100 µL of POD substrate was added per well and incubated for 3 minutes at room temperature. The plates were then read using an Orion Microplate Luminometer within 30 minutes of adding the POD substrate. Data and statistical analyses were conducted using Microsoft EXCEL and Graphpad PRISM.

Determining New Positive Control Candidates

Ten HI rabbit plasma samples (132-14 through 141-14 and positive control per plate) were used and performed in duplicate for indirect ELISA's. Plates were washed using a plate washer and followed ELISA protocol explained above.

Optimization Assays

Four HI rabbit plasma samples were used in ELISA optimization assays: 135-14, 136-14, 137-14, and the positive control, and were performed in triplicate per plate. The optimization assays specifically focused on the temperature of incubation of the primary and secondary antibody, as well as the plate washing method. For the temperature experiments (n=3), the plates were incubated at 37°C, 4°C, or at room temperature (20-25°C) during the secondary antibody incubation step. A separate experiment kept the temperature at 37°C but varied the incubated length period to 30, 60, or 90 minutes (n=1). Plate washing methods were also compared between using a plate washer, pipette, or squirt bottle all using PBS + 0.5% Tween, which was kept constant throughout the ELISA assay per plate.

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