Evaluation of quantitative anti-F1 IgG and anti-V IgG ELISAs for use as an in vitro-based potency assay of plague vaccine in mice[☆]

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Abstract

Quantitative anti-F1 and anti-V IgG enzyme-linked immunosorbent assays (ELISAs) were developed to measure the serological response of female Swiss Webster mice after vaccination with the recombinant fusion protein, rF1-V, which is being developed as a plague vaccine. Several fundamental parameters of the ELISA were evaluated: specificity, precision, accuracy, and stability. Experimental results suggested that a potency assay based upon the serological response of female Swiss Webster mice, as measured by quantitative anti-F1 IgG and anti-V IgG ELISAs, might be used to evaluate the rF1-V fusion protein vaccine.
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1. Introduction

Two major proteins of *Yersinia pestis* are associated with protection against plague. The first is Fraction 1 (F1) capsular protein [1,2] which is encoded on the 100 kb pFra plasmid, has anti-phagocytic activity [3] but is not considered to be an essential virulence factor based upon studies in mice and non-human primates using non-encapsulated isogenic strains [4–7]. Anti-F1 antibody, however, is protective against encapsulated strains [8]. The second protective protein is virulence-associated V antigen (V or LcrV) [9,10] which is encoded on the LCR plasmid. V antigen is considered to be a virulence factor in that it plays a role in formation of the type III secretion system and translocation of *Yersinia* outer proteins (Yops) [11], inhibits chemotaxis [12], and modulates the cytokine response [13,14]. Two early plague vaccines that were developed were either a *Y. pestis* live attenuated strain EV76 or a formaldehyde-killed virulent strain 195/P [15]. The live, attenuated vaccine was never licensed in the U.S. because of the risk of severe side effects. Injection with the formaldehyde-killed vaccine, known as killed whole cell (KWC), Plague Vaccine USP, or Cutter USP [15], elicited an antibody response to F1 but not to V antigens [16]. One of the major drawbacks with KWC became evident when it was demonstrated that it did not provide protection in animal models against an aerosol challenge or challenged with F1$^-$ *Y. pestis* strains [2,17–19]. It was this inability of KWC to fully protect against infection that eventually led to removal of KWC from production and highlighted the need of an improved vaccine to protect against infection with *Y. pestis*. Two different approaches have been pursued in the development of newer vaccines which incorporate both the F1 capsular protein and the virulence-associated V antigen of *Y. pestis*. The first is based upon F1, purified from *Y. pestis*, and recombinant V antigen (rV), purified from *E. coli*, which are co-administered [16,17]. The second approach proposed the use of a vaccine

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14. ABSTRACT
Quantitative anti-F1 and anti-V IgG enzyme-linked immunosorbent assays (ELISAs) were developed to measure the serological response of female Swiss Webster mice after vaccination with the recombinant fusion protein, rF1-V, which is being developed as a plague vaccine. Several fundamental parameters of the ELISA were evaluated: specificity, precision, accuracy, and stability. Experimental results suggested that a potency assay based upon the serological response of female Swiss Webster mice, as measured by quantitative anti-F1 IgG and anti-V IgG ELISAs, might be used to evaluate the rF1-V fusion protein vaccine.

15. SUBJECT TERMS
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based upon a recombinant fusion protein between F1 and V (rF1-V) [18,19]. While both proposed vaccine preparations have been shown to be effective against aerosol challenge with fully virulent *Y. pestis* [16−19], the F1-V fusion vaccine demonstrated a higher relative potency against lethal exposure in the mouse model than the F1 + V cocktail or USP vaccine [20].

Serological evaluation of the antibody response of mice injected with F1 and V is currently conducted by ELISA end-point titers, which have been reported to correlate with protection [10,21]. Additionally, passive protection studies with anti-F1 and anti-V sera have demonstrated that antibodies to both antigens are protective against encapsulated *Y. pestis* infection [6,8,22,23]. Data are presented here evaluating various parameters of a quantitative anti-F1 IgG ELISA and a quantitative anti-V IgG ELISA as assays to measure the serological response of female Swiss Webster mice after vaccination with F1-V-based vaccine in support of its use as a possible in vitro serology-based potency assay to monitor consistency of the F1-V vaccine. An in vitro serology-based potency assay will not require stringent biosafety containment measures as necessitated by the current potency assays using efficacy testing in animal models [1,24−26].

2. Materials and methods

2.1. rF1, rV, and rF1-V fusion proteins

Recombinant F1 capsule protein (rF1; 15.563 kDa) [2], recombinant virulence-associated V antigen (rV; 37.240 kDa) (B. Powell, unpublished data), and recombinant fusion protein F1-V (rF1-V) [19] were prepared as described and stored at −70 °C. Two different lots of rF1-V were used through out these studies.

2.2. Animals

Female Swiss Webster mice (NCI-FCRC, Frederick, MD) and BALB/c mice (Charles River Laboratories, Wilmington, MA) were used when 6−8 weeks old at the start of each experiment. The animals received food and water ad libitum. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. ELISA

2.3.1. Preparation of reference standard

The reference standards for the ELISAs were prepared from ascitic fluids produced in female BALB/c mice that had been injected with either F1 or V adsorbed to aluminum hydroxide gel (Alhydrogel, 2% Al_2O_3; HCL Biosector, Frederikssund,

Denmark) [27]. Mice were injected intraperitoneally (i.p.) with 0.2 ml of 2,6,10,14-tetramethylpentadecane (pristine; Sigma Chemical Co., St. Louis, MO) on day 0. Ten days, 17 days, and 38 days later, mice were injected i.p. with 50 μg of either F1 or V adsorbed to Alhydrogel at 0.5 mg of aluminum per dose. On day 42, mice were injected i.p. with 1×10^6 of Sp2/0-Ag14 myeloma cells. Ascitic fluid was collected from the mice as it was produced. The pooled ascitic fluids were centrifuged at $25\,000 \times g$, passed through 0.2 μm filters, and aliquots were stored at −70 °C until purified. The ascitic fluids were diluted 1:1 with 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) before being chromatographed over a HiTrap Protein G column (AmershamPharmacia, Piscataway, NJ). The bound IgG was eluted with 0.1 M glycine/HCl, pH 2.7, and the eluted fractions were neutralized by adding 1 M Tris/HCl, pH 9.0, and dialyzed against PBS at 4−6 °C overnight. Antigen affinity columns were prepared by binding F1 or V to AminoLink resin (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's directions. Protein G-purified IgG was passed over the respective affinity column in 20 mM Tris/HCl, pH 7.0 and washed to the baseline absorbance value. Bound antibody was eluted from the affinity resin with 20 mM Tris/HCl, pH 3, and the fractions neutralized by adding 1 M Tris/HCl, pH 9.0. The affinity-purified IgG was dialyzed against PBS at 4−6 °C overnight, filtered through 0.22 μm filters, and aliquots were frozen at −70 °C. Protein concentration was determined by using the BioRad microplate protein assay (BioRad Laboratories, Hercules, CA). The IgG concentration was determined by using a mouse IgG ELISA (Roche Applied Science, Indianapolis, IN). For both the affinity-purified mouse anti-F1 and anti-V sera, seven dilutions of each were prepared as the reference standards and three dilutions (high (Pos1), mid (Pos2), and low (Pos3)) were prepared as positive controls. Normal mouse serum (NMS; Sigma Chemical Company, St. Louis, MO) was used as the negative control. The dilutions were prepared as twofold higher concentrations in PBS containing 0.5% Tween 20 and 5% non-fat dry milk and stored at −70 °C.

2.3.2. ELISA

The ELISA was designed to measure anti-F1 or anti-V-specific IgG by using F1 or V, respectively, as the solid-phase capture antigens. Each plate contained three positive controls, one negative control (NMS), one blank, seven dilutions of the reference standard, and five, fourfold serial dilutions of four test samples. All standards, controls, and test samples were tested in triplicate on each plate.

Wells of 96-well plates (Immulon II HB, ThermoLab Systems, Franklin, MA or ReactiBind, Pierce, Rockford, IL) were coated with 100 μl of F1 or V diluted to 1 μg/ml in borate buffer, pH 9.5, wrapped in plastic wrap, and incubated overnight at 4 °C. The plates were washed three times with PBS containing 0.1% Tween 20 (PBST) using a Dynex Ultrawash-Plus microplate washer (Dynex Technologies, Chantilly, VA) before adding 50 μl of reference standards and controls to an equal volume of PBST containing 5% non-fat dry milk (PBSTM). Samples were diluted in PBSTM then serially

diluted fourfold in PBSTM (100 µl per well). The plates were incubated for 1 h at 37 °C. The plates were washed three times in PBST and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ) (Kirkegaard & Perry, Gaithersburg, MD) diluted to 1:1000 in PBSTM was added to the wells and the plates were incubated for 1 h at 37 °C. The plates were washed three times with PBST, rotated 180°, and washed again three times and incubated with the two-component substrate (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ABTS; Kirkegaard & Perry) at 37 °C for 30 min. Stop solution (Kirkegaard and Perry) was added and absorbance readings at 405 nm were obtained using a BioTek ELx808 microplate reader (BioTek, Winooski, VT). The mean absorbance values and coefficient of variation (%CV) for each triplicate dilution of all reference standards, controls, and test samples were obtained using the KC4 software program (BioTek Instruments). The IgG concentration of each unknown sample and control was calculated from each corresponding reference standard curve using a 4-parameter logistic regression equation of the KC4 program. Data were reported as arithmetic mean ± the standard error of the arithmetic mean (SEM). Further data analysis was performed using XLfit4 (IDBS, Inc., Emeryville, CA), SigmaPlot (Systat Software, Inc. San Jose, CA), and GraphPad InStat and Prism (GraphPad Software, Inc., San Diego, CA) software.

2.4. Specificity

Specificity was measured by several different approaches. The first was by determining the binding of anti-F1 and anti-V affinity-purified antiserum to their respective (homologous) protein and a heterologous antigen in the ELISA above. Two analysts each performed the assay on three plates with triplicate samples.

To determine the concentration of soluble homologous antigen that would inhibit binding of the respective antiserum to the homologous antigen in the solid phase, a competitive binding ELISA was performed. Various concentrations of F1 were pre-incubated with a 1:5000 dilution of anti-F1 serum, or V were pre-incubated with a 1:3000 dilution of anti-V serum for 1 h at 37 °C before transferring 100 µl to triplicate wells of three ELSIA plates prepared with the homologous respective antigen and the ELISA was completed as above. Half-maximal binding to the plate was determined by XLfit4 software (IDBS, Inc.).

Specificity was further examined by incubating equal volumes of a 1:2500 dilution of mouse affinity purified anti-F1 serum or a 1:5000 dilution of mouse affinity-purified anti-V serum with F1 protein at 1 µg/ml or with V protein at 10 µg/ml for 1 h at 37 °C. After the incubation, 100 µl were transferred to each of three wells of three homologous antigen-coated ELISA plates in addition to the homologous reference standards for each respective plate. After incubation for 1 h at 37 °C, the ELISA was completed as above. Assay was performed by two analysts.

Finally, analyte specificity was further assayed by determining parallelism between the titration curves of the reference standards with two test samples, mouse anti-F1-V serum and either mouse anti-F1 or mouse anti-V serum. The sera were prepared in female Swiss Webster mice that had been injected subcutaneously with either 20 µg of F1, 40 µg of V, or 60 µg of F1-V, each adsorbed to 0.186 mg of aluminum in Alhydrogel. Mice were bled on day 36. The relationship between the absorbance value and the corresponding reciprocal of the dilution was made linear by the fully specified logit–log model using the formula; $\text{Logit}(A_{405})_{\text{fs}} = \log(A_{405} - A_{405\text{min}})/(A_{405\text{max}} - A_{405})$ [28]. In this formula, '$A_{405\text{min}}$' and '$A_{405\text{max}}$' are unknown values corresponding to the lower and upper asymptotes, respectively of the four-parameter logistic-log model [28]. For our calculations, these values were determined by the XLfit4 software (IDBS, Inc.). The plot between the $\text{Logit}(A_{405})_{\text{fs}}$ and the respective log of the reciprocal of the dilution was a straight line. Test sera were each tested on three plates in triplicate against the respective antigens. Six plates from each assay contained the respective calibration standards which were combined for each analysis.

2.5. Accuracy and linearity

An initial 1:100 dilution of the mouse anti-F1 IgG reference standard and the mouse anti-V IgG reference standard were prepared in NMS. A starting dilution of 1:5000 was prepared in PBSTM and serial twofold dilutions were then prepared in PBSTM. Additionally, a 1:15 000 starting test dilution was prepared from the 1:5000 dilution and serial twofold dilutions were subsequently prepared as above. Three wells for each dilution were tested on three plates each by two analysts using the same sample dilutions. Linearity was evaluated by plotting the $\log_{10}$ of the antibody concentration against the respective absorbance value for each dilution and determining the correlation coefficient (r^2) (XLfit 4 software). Accuracy was reported as the percent recovery from the average concentration of the samples from 18 wells on six plates.

2.6. Precision

Assay repeatability and intermediate precision between days that assays were performed were estimated based upon the results from the three positive controls from three separate ELISAs for each antigen. Each assay consisted of three plates with samples run in triplicate on each plate. Precision was expressed as %CV for triplicate wells on each plate (intrawell), for a set of three plates (intra-plate), for the three assays (inter-assay), and for two analysts.

2.7. Potency assay

Polyclonal antisera, obtained at 2 and 4 weeks after intramuscular injection of female Swiss Webster mice with various concentrations of F1-V vaccine adsorbed to 0.20 g aluminum in Alhydrogel, were tested in the anti-F1 and anti-V IgG ELISAs. Sera were collected from six mice per dose of F1-V and from four separate experiments in which two different F1-V lots were used. Each data point represents 24 mice unless the ELISA titer data were identified as an outlier [29] or a sample

was unavailable for testing. The linear relationship between F1-V vaccine dose and week 2 and week 4 quantitative ELISA titers were plotted and the r^2 was calculated using SigmaPlot software (SPSS, Inc., Chicago, IL).

3. Results and discussion

3.1. Reference standards four-parameter logistic curve

In this study, we evaluated quantitative anti-F1 and anti-V IgG ELISAs to measure the IgG antibody response of female Swiss Webster mice that had been inoculated with F1-V fusion protein adsorbed to Alhydrogel. The ELISAs were developed to determine their possible use as potency assays for the F1-V fusion protein vaccine. The reference standards used for the quantitative ELISAs, mouse anti-F1 IgG and mouse anti-V IgG, were produced as polyclonal ascitic fluids [27] in female BALB/c mice by using Alhydrogel as the adjuvant (0.5 mg aluminum per injection). However, unlike previous experience with this method, we observed unexpected deaths of the mice 13 and 14 days after injection of the Sp2/O myeloma cells. For the F1-injected mice, on day 13, 17/30 (57.7%) had died and one of the remaining 13 mice was euthanized after removing the ascitic fluid and on day 14, 10/12 mice had died (83.3%) and the remaining two mice were euthanized after removing the ascetic fluids. For the V-injected mice, on day 13, 7/30 (23.3%) of the mice had died and six of the remaining 23 mice were euthanized after removal of the ascitic fluid and on day 14, 8/17 (47%) of the remaining mice had died and the remaining nine mice were euthanized after removal of the ascitic fluids. We collected 25 ml of anti-F1 ascitic fluid and 50 ml of anti-V ascitic fluid. The IgG was purified from the ascitic fluids by affinity chromatography over Protein G resin and the respective antigen affinity columns prepared by coupling F1 or V to AminoLink Plus gel (Pierce Biotechnology). The relationship between the absorbance values at $A_{405 \text{ nm}}$ and the known IgG concentrations of the antiserum prepared for use as reference standards were plotted by a sigmoid curve defined by a four-parameter logistic equation (KC4), $y = (a-d)/(1 + (x/c)^b) + d$, where '$d$' is the y-value corresponding to the upper asymptote, 'a' is the y-value corresponding to the lower asymptote, 'c' is the x-value corresponding to the y-midpoint between 'd' and 'a', and 'b' the slope factor of the curve. The anti-F1 reference standard curve consisted of seven dilutions of affinity-purified mouse anti-F1 IgG from 1279.6 ng/ml (S1, 1:500 dilution) to 0.064 ng/ml (S7, 1:10 000 000 dilution). Data from 11 assays consisting of 33 reference standard curves (three plates per assay of triplicate samples) for the F1 ELISA were calculated to be: $d = 3.287 \pm 0.0141$; $a = 0.003 \pm 0.0039$; $c = 30.2 \pm 1.40$; $b = 1.15 \pm 0.015$, and $r^2 = 0.9993 \pm 0.0001$ (Fig. 1). For non-immune mouse serum, the absorbance value was 0.301 ± 0.0219 at a 1:100 dilution during the assay. Likewise, the anti-V antigen reference standard curve consisted of seven dilutions of affinity-purified mouse anti-V antigen IgG from 594.2 ng/ml (S1, 1:500 dilution) to 0.06 ng/ml (S7, 1:5 000 000 dilution). Data from 11 assays consisting of 33
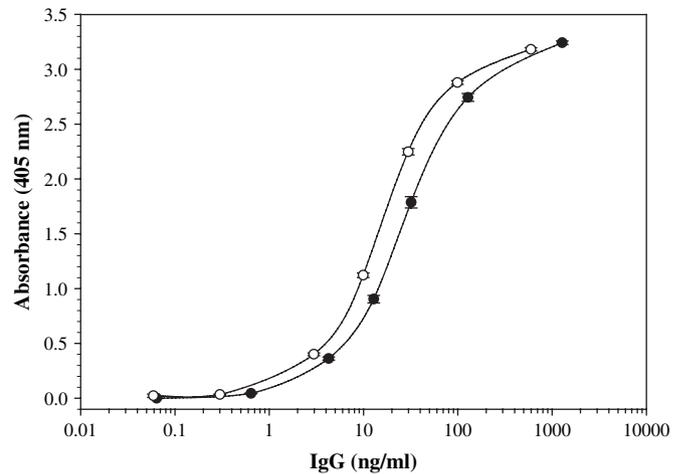

Fig. 1. Four-parameter logistic plot of the average absorbance value $\pm$ SEM for affinity-purified anti-F1 IgG (●) or affinity purified anti-V IgG (○) reference standards.

reference standard curves for the V ELISA were calculated to be: $d = 3.206 \pm 0.0143$; $a = 0.029 \pm 0.0102$; $c = 16.3 \pm 0.46$; $b = 1.27 \pm 0.019$, and $r^2 = 0.9990 \pm 0.0003$ (Fig. 1). For non-immune mouse serum, the absorbance value was 0.217 ± 0.0227 at a 1:100 dilution during the assay.

A logit–log plot [28] of S1–S6 anti-F1 (1279.6 ng/ml IgG to 0.64 ng/ml IgG) and of S1–S6 anti-V IgG (594.2 ng/ml IgG to 0.30 ng/ml IgG) reference standards showed a linear relationship between the $\log_{10}$ concentration of the reference standards and the logit$(A_{405})_{fs}$ from the 11 assays above (Fig. 2). The last standard (S7) for both the F1 and V ELISAs was omitted from the analysis as the logit$(A_{405})_{fs}$ could not be plotted; the average absorbance value of S7 (-0.001 and 0.023 for the F1 and V ELISAs, respectively) were less than the average minimum absorbance value from the four-parameter logistic equation ($A_{405} = 0.003$ for F1 ELISAs and $A_{405} = 0.029$ for V ELISAs; value 'a' above). The r^2 was 0.9996 and 0.9896
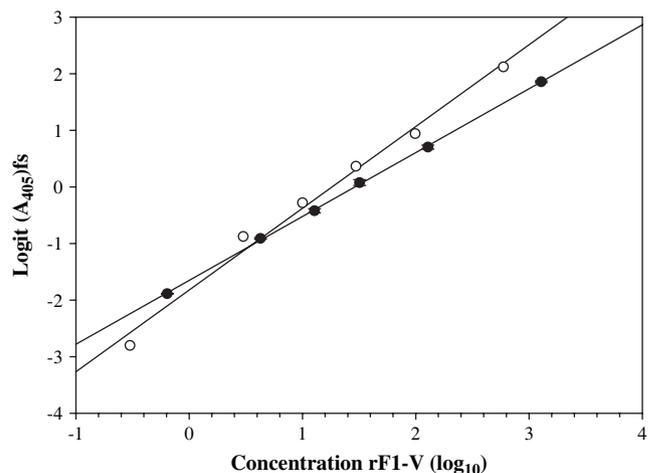

Fig. 2. Logit–log plot of S1–S6 anti-F1 IgG (●) and anti-V IgG (○) reference standards. Each point represents the average of 11 assays each performed with three plates.

for both the six data points plotted for anti-F1 IgG and for the six data points plotted for anti-V, respectively.

3.2. Specificity

Specificity was measured first by determining the binding of each reference standard antiserum to its respective (homologous) protein and a heterologous antigen (either F1 or V). We did not examine the cross-reactivity of our *Y. pestis* anti-V serum with that of LcrV from *Yersinia enterocolitica* or *Yersinia pseudotuberculosis* [30], PcrV from *Pseudomonas aeruginosa* [31,32], or AscV from *Aeromonas salmonicida* [33,34], integral components in type III secretion systems. However, Goure et al. [32] reported inhibition of assembly of the *Pseudomonas* and *Yersinia* translocation pore, PopB/PopD and YopB/YopD, respectively, by anti-V antibodies, suggesting cross reactivity. Results showed that both mouse anti-F1 and mouse anti-V bound to their respective antigens, but neither antisera bound to the heterologous antigen in the solid phase (Table 1). Additionally, as noted above, normal mouse serum did not show significant reactivity with either F1 ($A_{405\ nm} = 0.301$) or V ($A_{405\ nm} = 0.217$) in the ELISA at a 1:100 dilution. A competitive binding ELISA was performed to estimate the concentration of soluble antigen that would result in half-maximal binding of the respective homologous antiserum in the ELISA. Various concentrations of antigen were pre-incubated with a fixed concentration of its respective antiserum before transferring the mixture to its respective antigen-coated plate and completing the ELISA. Half-maximal binding of anti-F1 IgG to the F1-coated ELISA plate by soluble F1 was measured at 24.2 ng F1 per ml. Half-maximal binding of anti-V IgG to the V-coated ELISA plate by soluble V was measured at 3739 ng of V per ml. Pre-incubating F1 (0.5 μg per ml) with anti-F1 serum (1:5000 final dilution) reduced the absorbance value to background readings ($A_{405\ nm} = 0.097$), while pre-incubating with V (5 μg per ml) did not appear to influence binding to F1 in the solid phase ($A_{405\ nm} = 2.820$) (Table 1). Pre-incubating V (5 μg per ml) with anti-V serum (1:10 000 final dilution) reduced the absorbance value by only 72% ($A_{405\ nm} = 0.638$) of the absorbance

value obtained from the control ($A_{405\ nm} = 2.305$; Table 1). Pre-incubating F1 protein with anti-V serum did not appear to inhibit binding to V antigen-coated plates ($A_{405\ nm} = 2.049$) (Table 1). We chose to limit the concentration of V that was used to 5 μg per ml, which was just slightly more than the concentration that resulted in half-maximal binding of anti-V IgG to the V-coated ELISA plate by soluble V (3739 ng/ml), which may have accounted for the incomplete inhibition that was observed.

Finally, analyte specificity was further assayed by determining parallelism between the titration curves of the reference standards with two test samples, mouse anti-F1-V serum, and either mouse anti-F1 or mouse anti-V serum. Test sera were prepared in female Swiss Webster mice that had been injected subcutaneously with either 20 μg of F1, 40 μg of V, or 60 μg of F1-V adsorbed to 0.186 mg aluminum in Alhydrogel and bled on day 36. Test sera were each evaluated on three plates in triplicate against the respective antigens. Six plates from each assay contained the respective calibration standards which were combined for the analysis. The slopes and r^2 of the logit−log plot measured for the curves from F1 ELISA plates were; F1 reference standards, 1.1150, 0.9995; mouse anti-F1/V serum, 1.1331, 0.9989; and mouse anti-F1 serum, 1.0128, 0.9975. The differences between the slopes of the mouse anti-F1 IgG reference standards and mouse anti-F1-V serum and mouse anti-F1 serum were −0.0181 and 0.1022, respectively. The slopes and r^2 of the logit−log plot measured for the curves from V ELISA plates were; V reference standards, 1.2205, 0.9971; mouse anti-F1-V serum, 1.1869, 0.9945; and mouse anti-V serum, 0.9390, 0.9912. The differences between the slopes of the mouse anti-V IgG reference standards and mouse anti-F1-V serum and mouse anti-V serum were 0.0336 and 0.2813, respectively. The differences between the slopes for both assays appear to be negligible and suggest that the assays will provide a reliable estimate of the antibody concentrations.

3.3. Accuracy

Normal mouse serum was spiked with an initial 1:100 dilution of affinity-purified mouse anti-F1 IgG or anti-V IgG from which an initial starting dilution of 1:5000 in PBSTM was prepared. A 1:15 000 dilution in PBSTM was also prepared from the 1:5000 dilution. From both the 1:5000 and 1:15 000 dilutions, twofold serial dilutions were made and 100 μl added to the respective ELISA plate in triplicate. For the six F1-coated ELISA plates, a plot between the $\log_{10}$ of the antibody concentration against the respective absorbance value for each dilution had a correlation coefficient of $r^2 = 0.9944$ and the percent recovery was between 139% and 100% (Table 2). For the six V-coated ELISA plates, a plot between the $\log_{10}$ of the antibody concentration against the respective absorbance value for each dilution had a correlation coefficient of $r^2 = 0.9803$ and the percent recovery was between 103% and 73% (Table 2). For the two antisera, the observed percent recovery measurements, except for three test dilutions, was within 25% of the expected concentrations.

Table 1

Specificity. Absorbance values of either mouse anti-F1 IgG or mouse anti-V IgG bound to their homologous or heterologous antigen in the solid phase in the presence of homologous or heterologous soluble antigen

Sample	Pre-incubation	Dilution in well	Absorbance value	
			F1 plate	V plate
Anti-F1 IgG	None	1:5000	2.816	0.021
	F1	1:5000[a]	0.097	nt[b]
	V	1:5000[a]	2.820	nt
Anti-V IgG	None	1:10 000	−0.019	2.305
	F1	1:10 000[a]	nt	2.049
	V	1:10 000[a]	nt	0.638

Pre-incubation of antisera at a dilution similar to a reference standard dilution with either the homologous or heterologous antigen.

[a] Mean absorbance values of triplicate wells from duplicate assays of three plates each.

[b] nt, not tested.

Table 2
Accuracy. Percent recovery of test sample dilutions

Antiserum	Percent recovery for each test dilution[a]							
	1:5 k	1:10 k	1:15 k	1:20 k	1:30 k	1:40 k	1:60 k	1:80 k
Anti-F1	139%	126%	120%	120%	116%	100%	109%	104%
Anti-V	103%	88%	76%	80%	73%	84%	80%	94%

[a] Values represent the percent recovery for each test dilution from triplicate wells on six plates.

3.4. Precision

Assay repeatability and intermediate precision were examined by determining the %CV for the concentration of the three positive control samples from three separate assays, the two specificity assays and the accuracy and linearity assay, performed by two analysts. Repeatability was determined from the triplicate estimates on each of three plates (intra-plate). Intermediate precision was examined by determining the %CV for each set of three plates for each assay (inter-plate), day-to-day variation (inter-day) for three separate assays, and between analysts (inter-analyst) on three assays. Repeatability and intermediate precision for the anti-F1 ELISAs are shown in Table 3. For both analysts, intra-plate precision for Pos1, Pos2, and Pos3 samples ranged between 5.1% and 21.5%, inter-plate %CVs ranged from 0.4% to 10.0%, the %CV for all plates (inter-day) was between 6.2% and 14.1%, and the %CV for the inter-analyst determination ranged from 6.6% to 11.8%. Repeatability and intermediate precision for the anti-V ELISAs are shown in Table 4. For both analysts, intra-plate precision for Pos1, Pos2, and Pos3 samples ranged between 4.6% and 26.5%, inter-plate %CVs ranged from 0.8% to 8.2%, the %CV for all plates (inter-day) was between 4.4% and 8.7%, and the %CV for the inter-analyst determination ranged from 5.1% to 7.7%.

3.5. Stability

To evaluate the short-term stability of F1 or V as capture antigens on microtiter plates, the ELISA was conducted with plates that had been coated either 3 days or 10 days in advance

Table 3
Precision. Repeatability and intermediate precision measured by the percent coefficient of variation for the three anti-F1 positive control sera

Sample (ng IgG per ml)	%CV						Inter-analyst
	Analyst 1			Analyst 2			
	Intra-plate	Inter-plate	Inter-day	Intra-assay	Inter-assay	Inter-day	
Pos1 (53.32)	17.3			**21.5**			
	10.1			12.4			
	14.6	7.9		12.3	5.5		
	9.9			15.1			
	13.2			18.9			
	15.3	6.9		12.4	10.0		
	9.5			16.5			
	20.8			**21.0**			
	12.8	7.4	6.9	13.2	4.5	14.1	11.8
Pos2 (19.95)	17.4			8.9			
	10.8			11.1			
	9.3	9.4		11.4	2.6		
	14.0			15.0			
	12.2			17.4			
	12.2	6.3		5.7	6.0		
	6.3			5.1			
	18.6			6.1			
	19.8	4.2	6.4	11.0	6.5	8.5	7.4
Pos3 (6.4)	11.6			10.0			
	7.1			12.6			
	12.1	9.0		6.7	3.1		
	7.5			**20.5**			
	7.7			15.0			
	14.3	3.6		6.7	2.0		
	13.4			18.1			
	10.1			12.8			
	9.3	5.1	6.2	7.5	0.4	7.2	6.6

Results are from three separate assays (specificity and accuracy and linearity) showing well-to-well variation within a plate (intra-plate), variation between three plates per assay (inter-plate), day-to-day variation for each analyst (inter-day), and analyst-to-analyst variation (inter-analyst). Numbers in bold identify %CV values >20%.

Table 4
Precision. Repeatability and intermediate precision measured by the percent coefficient of variation for the three anti-V positive control sera

Sample (ng IgG per ml)	%CV						Inter-analyst
	Analyst 1			Analyst 2			
	Intra-plate	Inter-plate	Inter-day	Intra-assay	Inter-assay	Inter-day	
Pos1 (42.52)	8.6			12.8			
	18.8			19.3			
	13.7	3.6		6.0	8.2		
	13.3			8.6			
	18.5			16.4			
	7.6	2.4		9.2	4.3		
	6.1			15.5			
	24.8			**26.5**			
	23.2	2.8	6.0	15.4	4.1	8.7	7.7
Pos2 (20.01)	9.2			7.6			
	12.2			4.6			
	8.3	2.6		11.2	3.6		
	5.1			9.5			
	12.2			16.5			
	11.0	3.7		12.8	0.8		
	10.3			14.5			
	11.9			**21.4**			
	9.6	1.6	4.4	19.5	4.3	4.4	5.1
Pos3 (5.0)	9.5			7.4			
	9.4			7.8			
	9.5	2.5		9.1	2.7		
	8.4			6.9			
	8.1			9.6			
	9.7	7.0		19.4	7.1		
	15.9			14.3			
	8.7			5.0			
	9.6	2.2	4.4	14.2	2.5	7.3	5.8

Results are from three separate assays (specificity and accuracy and linearity) showing well-to-well variation within a plate (intra-plate), variation between three plates per assay (inter-plate), day-to-day variation for each analyst (inter-day), and analyst-to-analyst variation (inter-analyst). Numbers in bold identify %CV values >20%.

Table 5
Stability. Concentration of the anti-F1 Pos1, Pos2, and Pos3 control sera in the quantitative mouse anti-F1 IgG ELISA using plates coated with F1 3 days and 10 days beforehand

Sample	1 day old plates		3 day old plates		10 day old plates	
	IgG[a] (ng/ml)	%CV	IgG (ng/ml)	%CV	IgG (ng/ml)	%CV
Pos1 (53.3 ng/ml)	53.1 ± 3.11	14.3	53.9 ± 1.47	6.7		
Pos2 (20.0 ng/ml)	17.7 ± 0.70	9.7	20.0 ± 0.91	11.1		
Pos3 (6.4 ng/ml)	6.9 ± 0.33	11.7	7.5 ± 0.18	5.3		
Pos1 (53.3 ng/ml)	52.3 ± 4.96	23.2			56.6 ± 2.39	10.3
Pos2 (20.0 ng/ml)	20.4 ± 1.56	18.7			19.7 ± 0.84	10.5
Pos3 (6.4 ng/ml)	8.4 ± 0.77	22.3			7.8 ± 0.30	9.6

Three plates were run in duplicate assays.
[a] Arithmetic mean and SEM.

and held at 4–6 °C and compared with plates that had been coated 18–20 h ahead of time under standard assay conditions. Samples, which consisted of Pos1, Pos2, and Pos3 controls, were run in triplicate on each of three plates in duplicate assays conducted on different days. Comparison between the average concentrations of Pos1, Pos2, or Pos3 from each plate from two assays of F1 coated ELISA plates (Table 5) or the V antigen-coated ELISA plates (Table 6) by one-way analysis of variance (ANOVA) showed no significant differences between results.

3.6. Working range

Two methods were evaluated to determine the working range of the assays. In the first method, the four-parameter logistic plots from Fig. 1, which represented 33 separate assays for each standard antiserum, were converted to logit–log plots [28] (Fig. 2). We reasoned that the maximum concentration that could be determined was limited to the absorbance reading that could be accurately obtained and its value interpolated from the standard curve. The maximum value was defined as the concentration corresponding to the absorbance value of the upper asymptote for each standard reference curve which was about A_{405} of 3.20 for both anti-F1 and anti-V reference standard S1. The lower limit of measurement was reasoned to be the reference standard that would not cause the plot to veer from a straight line and would be greater than the normal

Table 6
Stability. Concentration of the anti-V Pos1, Pos2, and Pos3 control sera in the quantitative mouse anti-V IgG ELISA using plates coated with V 3 days and 10 days beforehand

Sample	1 day old plates		3 day old plates		10 day old plates	
	IgG (ng/ml)[a]	%CV	IgG (ng/ml)	%CV	IgG (ng/ml)	%CV
Pos1 (49.5 ng/ml)	49.3 ± 1.09	5.4	47.3 ± 1.82	9.4		
Pos2 (19.8 ng/ml)	19.9 ± 0.39	4.8	19.7 ± 0.27	3.3		
Pos3 (5.0 ng/ml)	5.3 ± 0.22	10.5	4.8 ± 0.06	3.2		
Pos1 (49.5 ng/ml)	48.6 ± 1.71	8.6			51.2 ± 2.23	10.7
Pos2 (19.8 ng/ml)	20.2 ± 0.28	3.3			20.1 ± 0.32	3.9
Pos3 (5.0 ng/ml)	5.0 ± 0.04	1.7			4.7 ± 0.16	8.1

Three plates were run in duplicate assays.
[a] Arithmetic mean and SEM.

mouse serum background absorbance values. This value corresponded to the concentration of reference standard S6 for both standard curves. For both anti-F1 IgG and anti-V IgG reference standards, a straight line was plotted by standards S1–S6. In these assays, mouse anti-F1 IgG reference standards S1–S6 gave a linear plot ($r^2 = 0.9996$, slope = 1.120) with a working range of 1279.6–0.64 ng IgG per ml. Mouse anti-V IgG reference standards S1–S6 gave a linear plot ($r^2 = 0.9896$, slope = 1.297) with a working range of 594.2–0.30 ng IgG per ml.

An alternative method examined the percent variation between the observed concentration and the expected concentration for each reference standard for each of 33 plates from 11 separate assays;

$$\%\text{variation} = (((\text{observed}/\text{expected}) - 1)) * 100.$$

Each data point plotted represented the percent variation of the average concentration of three wells for each plate. The working range of the assay was defined as the highest and lowest concentration for which the percent variation was within
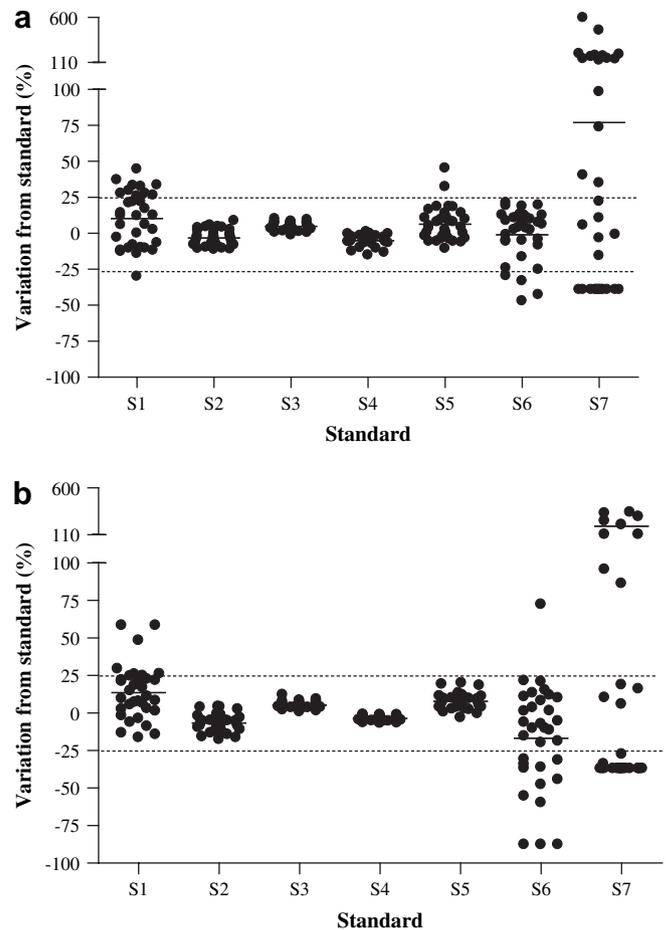

Fig. 3. a. Percent variation between the observed and the expected concentration of the mouse anti-F1 reference standards. Each data point represents the average of triplicate wells from each plate. b. Percent variation between the observed and the expected concentration of the mouse anti-V reference standards. Each data point represents the average of triplicate wells from each plate.

±25% of the expected concentration. Fig. 3a shows the percent variation plotted against the expected concentration of the seven anti-F1 reference standards. Using these criteria, the working range for the mouse anti-F1 IgG reference standards was between standards S2 and S5 (127.96 ng IgG per ml and 4.26 ng IgG per ml) with the exception for reference standard S5 on two plates. The percent variation of S5 on these two plates was 32.4% (observed concentration of 5.65 ng IgG per ml) and 45.3% (observed concentration of 6.20 ng IgG per ml). Fig. 3b shows the percent variation plotted against the expected concentration of the seven anti-V reference standards. The working range for the mouse anti-V IgG reference

standards was between standards S2 and S5 (99.04 ng IgG per ml and 3.0 ng IgG per ml). In general, the working range for the assay was between 100 ng IgG per ml and 1 ng IgG per ml for both ELISAs.

3.7. Potency assay

Monitoring the lot-to-lot consistency of an immunobiological can be performed by a potency assay. Potency assays for bacterial vaccines, which are described under four federal regulations, include animal protection studies, serological determinations, characterization of immunogenic epitopes, and biochemical analysis of the antigen [35]. As part of pre-clinical experiments for a plague vaccine based upon F1-V fusion protein, we evaluated the serological response of mice in quantitative anti-F1 IgG and anti-V IgG ELISAs for use in in vitro serology-based potency assays using two different F1-V fusion protein lots in four different experiments with six mice per experimental group. Potency assays for plague vaccines have relied upon survival assays using mice, guinea pigs, rats, or langurs [1,24–26,36]. We currently use the Swiss Webster mouse survival model to evaluate new lots of rF1-V [20,37]. Week 2 and 4 quantitative anti-F1 and anti-V IgG ELISA titers of serum samples from Swiss Webster mice injected i.m. with F1-V vaccine preparations from four different experiments are plotted in Fig. 4a,b, respectively. The linear relationship between F1-V vaccine dose and week 2 and week 4 anti-F1 IgG titer was $r^2 = 0.9875$ and $r^2 = 0.8407$, respectively. Similarly, the linear relationship between F1-V vaccine dose and week 2 and week 4 anti-V IgG titer was $r^2 = 0.8230$ and $r^2 = 0.9231$, respectively. It thus appears that the ELISAs may be useful as in vitro serological assays to measure antibody levels in female Swiss Webster mice for use as serology-based potency assays for F1-V plague vaccine.
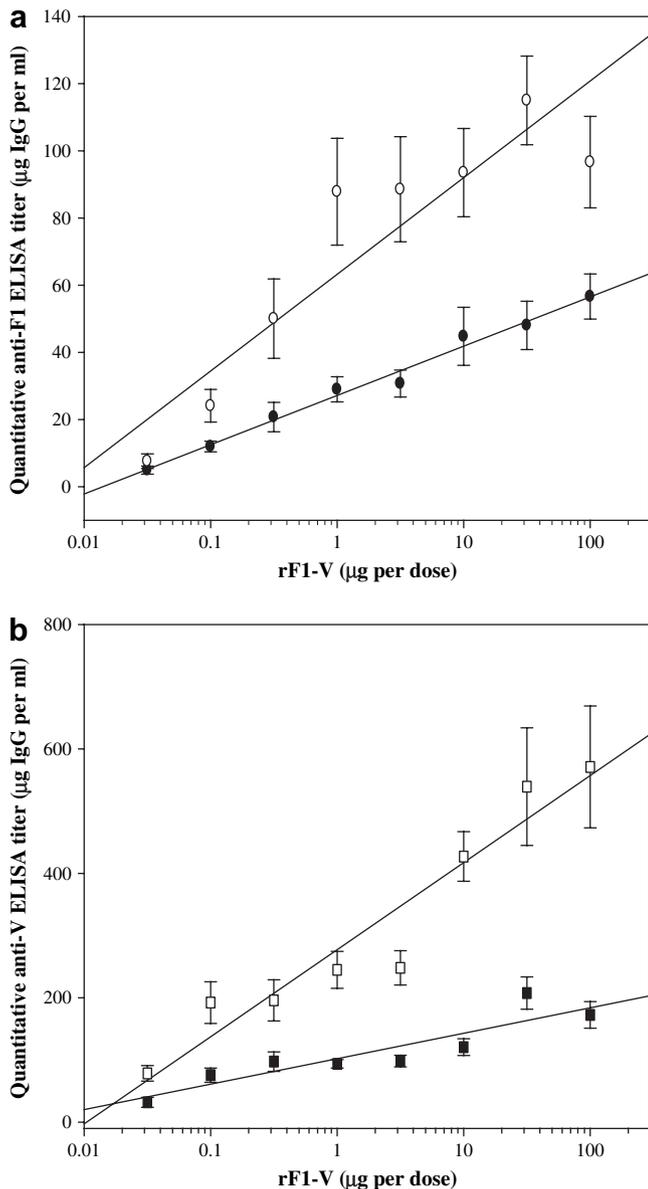
Acknowledgements

Fig. 4. a. Linear regression between F1-V vaccine dose (µg protein) and arithmetic mean and SEM quantitative anti-F1 IgG ELISA titers of female Swiss Webster mice at week 2 (●) and week 4 (○). b. Linear regression between F1-V vaccine dose (µg protein) and arithmetic mean and SEM quantitative anti-V IgG ELISA titers of female Swiss Webster mice at week 2 (■) and week 4 (□).

References

[1] Baker EE, Sommer H, Foster LE, Meyer E, Meyer KF. Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J Immunol 1952;68:131–45.

[2] Andrews GP, Heath DG, Anderson Jr GW, Welkos SL, Friedlander AM. Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and from an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge. Infect Immun 1996;64:2180–7.

[3] Williams Jr RC, Gewurz H, Quie PG. Effects of Fraction I from *Yersinia pestis* on phagocytosis in vitro. Infect Dis 2007;126:235–41.

[4] Worsham PL, Stein M-P, Welkos SL. Construction of defined F1 negative mutants of virulent *Yersinia pestis*. Contrib Microbiol Immunol 1995;13:325–8.

[5] Welkos SL, Davis KM, Pitt LM, Worsham PL, Friedlander AM. Studies on the contribution of the F1 capsule-associated plasmid pFra to the virulence of *Yersinia pestis*. Contrib Microbiol Immunol 1995;13:299–305.

[6] Friedlander AM, Welkos SL, Worsham PL, Andrews GP, Heath DG, Anderson Jr GW, et al. Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. Clin Infect Dis 1995;21:S178–81.

[7] Davis KJ, Fritz DL, Pitt ML, Welkos SL, Worsham PL, Friedlander AM. Pathology of experimental pneumonic plague produced by Fraction 1-positive and Fraction 1-negative *Yersinia pestis* in African green monkeys (*Cercopithecus aethiops*). Arch Pathol Lab Med 1996;120:156–63.

[8] Anderson Jr GW, Worsham PL, Bolt CR, Andrews GP, Welkos SL, Friedlander AM, et al. Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of *Yersinia pestis*. Am J Trop Med Hyg 1997;56:471–3.

[9] Burrows TW, Bacon GA. The basis of virulence in *Pasteurella pestis*: an antigen determining virulence. Br J Exp Pathol 1956;37:481–93.

[10] Leary SE, Williamson ED, Griffin KF, Russell P, Eley SM, Titball RW. Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. Infect Immun 1995;63:2854–8.

[11] Nilles ML, Fields KA, Straley SC. The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. J Bacteriol 1998;180:3410–20.

[12] Welkos S, Friedlander A, McDowell D, Weeks J, Tobery S. V antigen of *Yersinia pestis* inhibits neutrophil chemotaxis. Microb Pathog 1998;24: 185–96.

[13] Nakajima R, Motin VL, Brubaker RR. Suppression of cytokines in mice by Protein A-V antigen fusion peptide and restoration of synthesis by active immunization. Infect Immun 1995;63:3021–9.

[14] Sing A, Rost D, Tvardovskaia N, Roggenkamp A, Wiedemann A, Kirschning CJ, et al. Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. J Exp Med 2002;196:1017–24.

[15] Meyer KF. Effectiveness of live or killed plague vaccines in man. Bull WHO 1970;42:635–66.

[16] Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SEC, et al. A new improved sub-unit vaccine for plague: the basis of protection. FEMS Immunol Med Microbiol 1995;12:223–30.

[17] Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW. A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague. Vaccine 1997;15:1079–84.

[18] Anderson Jr GW, Heath DG, Bolt CR, Welkos SL, Friedlander AM. Short- and long-term efficacy of single-dose subunit vaccines against *Yersinia pestis* in mice. Am J Trop Med Hyg 1998;58:793–9.

[19] Heath DG, Anderson Jr GW, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, et al. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. Vaccine 1998;16:1131–7.

[20] Powell BS, Andrews GP, Enama JT, Jendrek S, Bolt C, Worsham P, et al. Design and testing for a non-tagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. Biotechnol Prog 2005; 21:1490–510.

[21] Simpson WJ, Thomas RE, Schwan TG. Recombinant capsular antigen (Fraction 1) from Yersinia pestis induces a protective antibody response in BLAB/c mice. Am J Trop Med Hyg 1990;43:389–96.

[22] Motin VL, Nakajima R, Smirnov GB, Brubaker RR. Passive immunity to Yersiniae mediated by anti-recombinant V antigen and Protein A-V antigen fusion peptide. Infect Immun 1994;62:4192–201.

[23] Hill J, Leary SEC, Griffin KF, Williamson ED, Titball RW. Regions of *Yersinia pestis* V antigen that contribute to protection against plague identified by passive and active immunization. Infect Immun 1997;65: 4476–82.

[24] Chen TH, Foster LE, Meyer KF. Experimental comparison of the immunogenicity of antigens in the residue of ultrasonated avirulent *Pasteurella pestis* with a vaccine prepared with killed virulent whole organisms. J Immunol 1961;87:64–71.

[25] von Metz E, Eisler DM, Hottle GA. Immunogenicity of plague vaccines in mice and guinea pigs. Appl Microbiol 1971;22:84–8.

[26] Chen TH, Foster LE, Meyer KF. Comparison of the immune response to three different *Yersinia pestis* vaccines in guinea pigs and langurs. J Infect Dis 1974;129:S53–61.

[27] Lacy MJ, Voss Jr EW. A modified method to induce immune polyclonal ascites fluid in BALB/c mice using Sp2/0-Ag14 cells. J Immunol Methods 1986;87:169–77.

[28] Plikaytis BD, Holder PF, Pais LB, Maslanka SE, Gheesling LL, Carlone GM. Determination of parallelism and nonparallelism in bioassay dilution curves. J Clin Microbiol 1994;32:2441–7.

[29] Bliss CI. Statistics in biology: statistical methods for research in the natural sciences, Linear regression. New York: McGraw-Hill; 1967. p. 152-185.

[30] Une T, Brubaker RR. Roles of V antigen in promoting virulence and immunity in yersiniae. J Immunol 1984;133:2226–30.

[31] Yahr TL, Mende-Mueller LM, Friese MB, Frank DW. Identification of Type III secreted products of the *Pseudomonas aeruginosa* exoenzyme S regulon. J Bacteriol 1997;179:7165–8.

[32] Goure J, Broz P, Attree O, Cornelis GR, Attree I. Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. J Infect Dis 2005;192:218–25.

[33] Burr SE, Stuber K, Wahli T, Frey J. Evidence for a Type III secretion system in *Aeromonas salmonicida* subst. *salmonicida*. J Bacteriol 2002; 184:5966–70.

[34] Burr SE, Stuber K, Frey J. The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida* subsp. *salmonicida* is translocated via a Type III secretion pathway. J Bacteriol 2003;185:6583–91.

[35] Habig WH. Potency testing of bacterial vaccines for human use. Vet Microbiol 1993;37:343–51.

[36] Smith H, Packman LP. A filtered non-toxic plague vaccine which protects guinea-pigs and mice. Br J Exp Pathol 1966;47:25–34.

[37] Goodin JL, Nellis DF, Powell BS, Vyas VV, Enama JT, Wang LC, et al. Purification and protective efficacy of monomeric and modified *Yersinia pestis* capsular F1-V antigen fusion proteins for vaccination against plague. Prot Exp Purif 2007;53:63–79.