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# Comparative immunogenicity and efficacy of thermostable (lyophilized) and liquid formulation of anthrax vaccine candidate AV7909

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ABSTRACT

The anthrax vaccine candidate AV7909 is being developed as a next-generation vaccine for a post-exposure prophylaxis (PEP) indication against anthrax. AV7909 consists of the anthrax vaccine adsorbed (AVA) (Emergent BioSolutions Inc., Lansing, MI) bulk drug substance adjuvanted with the immunostimulatory oligodeoxynucleotide (ODN) compound, CPG 7909. The addition of CPG 7909 to AVA enhances both the magnitude and the kinetics of antibody responses in animals and human subjects, making AV7909 a suitable next-generation vaccine for use in a PEP setting. Emergent has produced a thermostable (lyophilized) formulation of AV7909 vaccine utilizing drying technology. The purpose of the study described here was to assess the immunogenicity and efficacy of the lyophilized formulation of the AV7909 vaccine candidate as compared with the liquid formulation in the guinea pig general-use prophylaxis (GUP) model. The study also provides initial information on the relationship between the immune response induced by the thermostable formulation of the vaccine, as measured by the toxin neutralization assay (TNA), and animal survival following lethal anthrax aerosol challenge. Results demonstrated that there were no significant differences in the immunogenicity or efficacy of lyophilized AV7909 against lethal anthrax spore aerosol challenge in the guinea pig model as compared to liquid AV7909. For both vaccine formulations, logistic regression modeling showed that the probability of survival increased as the pre-challenge antibody levels increased.



## 1. Introduction

Anthrax is considered a serious biological threat due to the highly lethal effects of exposure via the inhalational route and the relative ease of weaponizing *Bacillus anthracis* spores. The virulence of *B. anthracis* is predicated upon two processes: encapsulation and the production of two interlinked toxins. The polyglutamate capsule prevents phagocytosis of the bacterium. Three polypeptides, protective antigen (PA), lethal factor (LF), and edema factor (EF), interact to form two interlinked toxins. PA and LF combine to produce anthrax lethal toxin (LT), and the PA and EF combine to produce edema toxin (ET). LT is the predominant cause of severe disease and death following inhalational spore exposure [1,2].

General-use prophylaxis (GUP) and post-exposure prophylaxis (PEP) are the two scenarios in which an anthrax vaccine is presently used. Since anthrax is considered a serious biological terrorism and military threat, a priority has been placed on PEP for emergency use in the civilian population. While antimicrobials administered post-exposure can reduce the incidence or progression of anthrax disease, they do not protect against subsequent disease resulting from germination of residual spores that may remain in the body after the cessation of the recommended 60-day antibiotic regimen [3,4]. Accordingly, confirmed or suspected exposure to *B. anthracis* spores would immediately be treated with an approved regimen of antibiotic therapy and anthrax vaccine. This combination therapy is based on the premise that germinated spores would be killed by antibiotic therapy while disease resulting from latent spore germination, which may occur after cessation of antibiotic therapy, would be prevented by a protective immune response generated by the vaccine. The Centers for Disease Control and Prevention (CDC) Advisory Committee on Immunization

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Practices (ACIP) currently recommends post-exposure vaccination with AVA in conjunction with a 60-day antibiotic regimen in the event of confirmed or suspected exposure to *B. anthracis* [5].

AVA consists of filtered *B. anthracis* culture supernatant adsorbed to Alhydrogel®. For GUP, AVA is administered by the intramuscular (IM) route as a three-dose primary series at 0, 1, and 6 months followed by boosters at 6 and 12 months after completion of the primary series and at 12-month intervals thereafter. For PEP, the vaccine is administered subcutaneously (SC) at 0, 2, and 4 weeks post-exposure combined with antimicrobial therapy. The predominant means of protection provided by this vaccine is thought to be mediated by antibodies generated against PA that neutralize the activities of LT and ET. AV7909, a next generation anthrax vaccine candidate, consists of AVA and the oligodeoxynucleotide CPG 7909. DNA sequences containing unmethylated cytosine and guanosine dinucleotide pairs (CpG) within the context of certain flanking sequences have proven to be immunostimulatory and may act as a potent vaccine adjuvant [6-9]. AV7909 was evaluated in Phase 1 and Phase 2 clinical trials that demonstrated the vaccine's safety and ability to elicit a high level of toxin neutralizing antibodies as measured by the toxin neutralization assay (TNA) [10].

Lyophilized vaccine formulations can improve product stability, extend shelf life, and eliminates the need for cold chain transportation which can expand vaccine distribution and coverage. Developing a lyophilized formulation of an aluminum-adjuvanted vaccine would be particularly beneficial, given the ubiquity of aluminum-adjuvanted vaccines and the significant product losses due to unwanted freezing during cold chain distribution [11]. However, lyophilization (freeze drying) of aluminum-containing vaccines is challenging given that freezing aluminum-adjuvanted vaccines leads to a well-documented loss in potency [12,13] associated with a collapse of the aluminum gel and aggregation of aluminum particles [14,15]. Emergent has developed a formulation and lyophilization process that enables lyophilization of aluminum-adjuvanted vaccines while maintaining the integrity of the aluminum gel; this process was used to convert liquid AV7909 to a lyophilized formulation. This study provides a preliminary assessment of the immunogenicity and efficacy of the thermostable (lyophilized) formulation of the AV7909 vaccine candidate as compared with the liquid formulation in the guinea pig GUP model as measured by the TNA, and animal survival following lethal anthrax aerosol challenge.

## 2. Materials and methods

### 2.1. Experimental animals

Testing was performed at the Battelle Biomedical Research Center (West Jefferson, OH). All animal procedures were approved by Battelle's Institutional Animal Care and Use Committee (IACUC), followed the principles of the Guide for the Care and Use of Laboratory Animals from the National Research Council, and were conducted in compliance with the Animal Welfare Act. Animal room temperatures (68-79°F) and relative humidity (30–70%) were maintained and recorded twice daily. The light/dark cycle was approximately 12 h each daily.

An equal number of male and female Hartley guinea pigs (*Cavia porcellus*) weighing approximately 400–500 g, were purchased from Charles River Laboratories (Quebec, Canada). Guinea pigs were single-housed in polycarbonate cages on stainless steel racks equipped with water bottles. Animals were moved into the biosafety level 3 (BSL-3) laboratory approximately 7 days prior to challenge and housed there until the conclusion of the study.

### 2.2. Test and control articles

The liquid formulation of AV7909 final drug product was made by combining AVA bulk drug substance, prepared from the cell-free culture filtrate of an avirulent, non-encapsulated strain of *B. anthracis* adjuvanted with Alhydrogel adjuvant, with CPG 7909 to achieve a concentration of 0.5 mg CPG 7909 per mL.

The thermostable AV7909 was formulated applying novel drying technology to prevent aluminum particle aggregation and corresponding degradation of the antigen adsorbed on adjuvant resulting in potency loss [16]. The Alhydrogel adjuvant component of liquid AV7909 was allowed to settle in a custom-designed polycarbonate bioprocess vessel (Paw BioScience, Eatontown, NJ). Two thirds of the supernatant were removed and used to prepare a stabilizing excipient solution, which was then introduced back into the vessel containing AV7909 through a 0.2 μm filter, achieving a final total volume equivalent to the original AV7909 volume. Following mixing, 4.8 mL of the formulated bulk product was transferred into Type I borosilicate glass vials with lyophilization stoppers. Vials were then loaded into an SP Scientific 50L Ultra EL-85 lyophilizer (SP Scientific, Warminster, PA) with shelves pre-cooled to <−40 °C. The appearance of the lyophilized cake was observed by visual inspection, the residual moisture was measured by a Karl Fischer Titrator (Mettler Toledo, Columbus, OH), and the glass transition temperature was measured by a differential scanning calorimeter (TA Instruments, New Castle, DE).

To verify the stabilizing excipients protected the vaccine against freeze-thaw damage, the particle size distribution of reconstituted lyophilized AV7909 was tested using the Malvern MasterSizer 3000 (Malvern Instruments, Malvern, UK) and compared with the liquid AV7909 precursor and a sample without stabilizing excipients frozen at <−80 °C.

Liquid AV7909 and thermostable AV7909 reconstituted with sterile water were diluted from the human dose (0.5 mL) in sterile normal saline (Hospira, Lake Forest, IL) for use in the study. The control group was injected with sterile saline.

### 2.3. Study design

An equal number of male and female guinea pigs were randomized into each study group. Animals were immunized by IM injection on Study Days 0 and 14 with 0.5 mL of liquid AV7909 or thermostable AV7909 at various dilutions. The vaccine dilutions and total number of animals per Study Group are shown in Table 1. The control group (9) was injected with 0.5 mL sterile saline. On Study Day 70, animals were challenged via the inhalational route with aerosolized *B. anthracis* (Ames strain) spores. Animals were observed for clinical signs of disease and mortality for 21 days post-challenge.

### 2.4. Aerosol challenge

Aqueous suspensions of *B. anthracis* spores were aerosolized using a 6-jet Collision nebulizer with a precious fluid jar and delivered to the animals via a nose-only aerosol exposure system. A challenge dose of 200 $LD_{50}$ *B. anthracis* spores was targeted using an $LD_{50}$ value of $5.01 \times 10^4$ spores/animal [16]. The average (±standard deviation) aerosol exposure dose for all animals in the study was 313 ± 41 *B. anthracis* (Ames strain) $LD_{50}$ equivalents.

Real-time verification of the atmospheric concentrations of spores in the exposure system was not possible; instead the concentration of spores per volume air in the nebulizer was established by prior performance tests without animals. Respiratory rates, tidal volumes, and minute ventilation during aerosol challenge were estimated by the Guyton formula [18]. Exposure

**Table 1**
Survival of Animals Vaccinated with Liquid and Thermostable AV7909 Vaccine Formulation.

| Study Group | Number of Animals[a] | Vaccine | Vaccine Dilution | Mean $LD_{50}$ Challenge Dose | Survival[b] | Time to Death[c] |
|---|---|---|---|---|---|---|
| 1 | 27 | Thermostable AV7909 | 1:96 | 308 | 96 (26/27) | ∴ (∴, ∴) |
| 2 | 28 | | 1:128 | 305 | 71 (20/28) | ∴ (∴, ∴) |
| 3 | 29 | | 1:192 | 320 | 35 (10/29) | 4.91 (3.92, ∴) |
| 4 | 26 | | 1:256 | 313 | 42 (11/26) | 4.89 (2.94, ∴) |
| 5 | 30 | Liquid AV7909 | 1:96 | 314 | 83 (25/30 | ∴ (∴, ∴) |
| 6 | 28 | | 1:128 | 325 | 71 (20/28) | ∴ (∴, ∴) |
| 7 | 29 | | 1:192 | 306 | 59 (17/29) | ∴ (4.84, ∴) |
| 8 | 30 | | 1:256 | 303 | 40 (12/30) | 5.35 (3.95, ∴) |
| 9 | 12 | Saline | n/a | 332 | 0 (0/12) | 2.11 (1.94, 2.89) |

[a] Animals that survived to the end of in-life period of the study or the terminal (death prior to the end of in-life) blood collection.
[b] % Survival (Number Survived / Total Challenged). Equal numbers of males and females were vaccinated on Day 0. The initial group size was 30 for vaccinated animals and 12 for the control group. Several animals died prior to the challenge due to complications from vena cava blood collection and were not included any further analyses.
[c] Time to death = Days (95% Confidence Interval); ∴ = Not enough animals died in this group to calculate median or confidence intervals for time to death.

duration was based on the time required for each animal to achieve the designated volume of inspired air.

Test system atmosphere samples were collected from one of the ports on the exposure system into a glass impinger (Model 7541, Ace Glass Inc.) and colony-forming units (spores)/mL in the impinger samples were enumerated to determine the concentration of spores during aerosol exposure. The estimated inhaled dose was calculated using the atmospheric spore concentration multiplied by the cumulative tidal volume inhaled by animals during exposure. The mean mass median aerodynamic diameter of challenge aerosol particles was 1.42–1.43 μm as determined with an Aerodynamic Particle Sizer (APS Model 3321, TSI Inc., Shoreview, MN), indicating the correct particle size to reach the alveoli.

### 2.5. Clinical observations

Animals were observed twice daily for clinical signs of anthrax infection, which may have included but was not limited to respiratory distress, rough hair coat, abnormal posture, changes in appetite, and changes in activity. Any animal found moribund, unresponsive, recumbent, or in respiratory distress and all animals that survived the post-challenge observation period were euthanized.

#### 2.5.1. Blood collection

Pre-challenge blood was collected, processed to serum, and stored at <−70 °C until immune assessment by TNA assay. Bacteremia assessment was performed on post-challenge blood samples to confirm anthrax infection. Prior to blood collection, animals were sedated with a combination of ketamine (40–70 mg/kg) and xylazine (5–10 mg/kg) via the intraperitoneal (IP) route. Quantitative bacteremia assessment was performed on all terminal blood samples (animals that were found dead or euthanized after aerosol challenge) and survivors (Study Day 91). One hundred microliters of whole blood sample were spread onto trypsin soy agar plates and incubated at 37 °C for 16–96 h. A plate containing at least one colony with morphology consistent with *B. anthracis* was considered positive.

#### 2.5.2. Toxin neutralization assay (TNA)

Anthrax toxin-neutralizing antibody levels in guinea pig serum samples were measured using the TNA. The TNA is a cell-based cytotoxicity method developed to detect and quantify the functional ability of serum to neutralize *B. anthracis* LT activity [19]. Serum-mediated neutralization of anthrax LT manifests as a suppression of cytotoxicity, and hence preservation of cell viability. The limit of quantitation (LOQ) for the guinea pig TNA 50% neutralization factor ($NF_{50}$) is 0.062 [19].

On Day 1, the cell plates were seeded with J774A.1 cells. On either Day 1 or Day 2, the serum samples [test samples (TS), positive quality control (QC), negative control (NC), and reference standard (RS)] were diluted to their appropriate starting dilution. If prepared on Day 1, diluted serum samples were stored at 2–8 °C until utilized in the subsequent step.

On Day 2, the TS, RS, QC, and NC serum were transferred from the dilution tubes to a 96-well preparation plate (which does not contain cells), further serially diluted down the plate (except the NC, which is not serially diluted), and incubated with freshly prepared LT (neutralization step; 30 min ± 5 min, at 37 °C ± 2 °C, 5% ± 2% $CO_2$). After the neutralization incubation, spent media was removed from the cell plate that was seeded on Day 1 and the serum/LT cocktail was then transferred to the cell plate and incubated (intoxication step; 4 h ± 5 min at 37 °C ± 2 °C, 5% ± 2% $CO_2$). Following intoxication, MTT {3-[4,5-dimethylthiazol-2-yl]-2,5-dip henyltetrazolium bromide} was then added (to be reduced by viable cells) and plates incubated for 2 h ± 5 min at 37 °C ± 2 °C, 5% ± 2% $CO_2$. Solubilization buffer was added (which lyses the cells and solubilizes the reduced MTT) and plates were incubated for 16–20 h, at 37 °C ± 2 °C, 5% ± 2% $CO_2$.

On Day 3, the colorimetric reaction was read using a microplate reader measuring a wavelength of 570 nm (using 690 nm as a reference filter) to determine the cell viability/serum neutralizing capability. The reference standard and test sample OD values are fitted to a 4PL curve used to calculate the reportable values, the $ED_{50}$ and $NF_{50}$.

### 2.6. Pathology

Complete gross necropsies were performed on all guinea pigs found dead or euthanized, including animals that died prior to challenge and survivors euthanized at the end of study. Sections of target tissues including brain, lungs, spleen, liver, kidney, mediastinal lymph nodes, and all gross lesions were preserved in 10% neutral buffered formalin. Histopathology was performed as deemed necessary by a board-certified pathologist to confirm death due to anthrax.

### 2.7. Statistical analysis

Pairwise log rank tests were performed to compare all pairs of groups when time to death was considered in addition to overall survival. Furthermore, Kaplan-Meier curves associated with time to death were plotted for each group. Noninferiority tests were performed to compare the survival proportions between each pair of groups immunized with a specific dilution.

Logistic regression analysis was performed to identify the relationship between immune response and survival for the AV7909 vaccinated animals. Individual TNA $NF_{50}$ values less than the LOQ replaced with the respective LOQ. A similar logistic regression

analysis was performed including all AV7909-vaccinated groups that included effects for vaccine type (thermostable or liquid) and the interaction between vaccine type and immune response. Quantitative bacteremia with values recorded as positive ("+") and those less than the LOQ replaced with LOQ. Quantitative bacteremia values reported as zero were replaced with the limit of detection (LOD).

## 3. Results

### 3.1. Lyophilization of AV7909

Lyophilized samples of AV7909 exhibited a well-shaped round cake with minimal evidence of reduction or collapse. The residual moisture was <2% and the glass transition temperature was >80 °C, indicating that the lyophilized product exhibited a high degree of physical thermostability.

The particle size distribution was measured for the reconstituted lyophilized AV7909, the liquid AV7909 precursor, and an AV7909 sample frozen at <−80 °C without stabilization and then thawed (Fig. 1). Freeze/thaw of AV7909 led to an increase in the aluminum hydroxide particle size, with the peak of the distribution shifting from 2.1 to 24.1 μm. Lyophilization protected the vaccine against this particle size increase, with the peak of the distribution remaining at 2.1 μm. These results demonstrate that the stabilization and lyophilization procedure employed protected AV7909 against the freeze/thaw damage typically observed for aluminum-adjuvanted vaccines, including an increase in aluminum particle size and a collapse of the aluminum gel.

Integrity of the key antigenic component of the vaccine, PA, was assessed by Western blot. No change in the molecular weight of protein was observed in the lyophilized material compared to the liquid formulation (data not shown).

### 3.2. Efficacy of liquid AV7909 and thermostable AV7909

Survival of guinea pigs following inhalational exposure to a lethal dose of *B. anthracis* Ames spores is shown in Table 1. All untreated control animals died confirming the lethality of the exposure doses. The most common clinical signs observed prior to death were consistent with inhalational anthrax in a guinea pig model described previously [17]. These signs included lethargy, lacrimation, respiratory abnormalities (e.g. labored breathing, increased respirations, respiratory distress), nasal discharge, and lack of appetite. Death due to anthrax was confirmed by bacteremia and/or histopathology. Both liquid AV7909 and thermostable AV7909 provided protection in a dose-dependent manner, with higher vaccine dose levels affording a statistically significant increase in survival compared to the control group. No statistically significant differences were observed between liquid AV7909 and thermostable AV7909 vaccines at the same dilution.

#### 3.2.1. Immunogenicity and relationship between immune response and survival

The TNA response induced by liquid AV7909 and thermostable AV7909 vaccination of guinea pigs are shown in Fig. 2. All animals had TNA $NF_{50}$ levels below the LOQ prior to vaccination. The peak

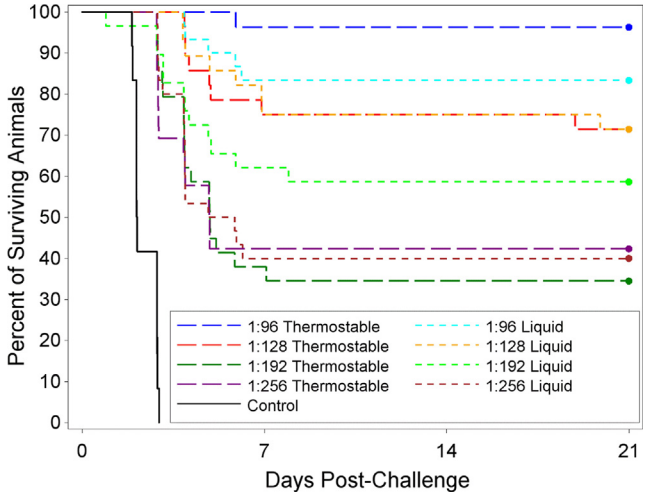


**Fig. 2.** Kaplan-Meier Curves Representing Time-to-Death and Survival for Each Group. Guinea pigs vaccinated with the indicated vaccine formulation and dilution were challenged with aerosolized *B. anthracis* (Ames strain) spores via inhalation and observed for clinical signs of inhalational anthrax disease and mortality for 21 days post-challenge. Kaplan-Meier curves associated with time to death were plotted for each group. Noninferiority tests were performed to compare the survival proportions between each pair of groups immunized with a specific dilution. Pairwise log rank tests were performed to compare all pairs of groups when time to death was considered in addition to the overall survival.

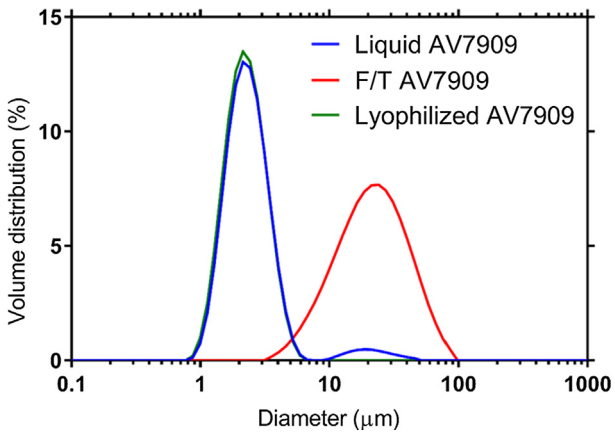


**Fig. 1.** Particle size distribution of aluminum hydroxide particles in liquid AV7909 (blue), liquid AV7909 subjected to freeze/thaw (red), and lyophilized AV7909 (green). The thermostable AV7909 was formulated as described in Materials and Methods. Particle size distribution of the reconstituted lyophilized AV7909 was tested using the Malvern MasterSizer 3000 (Malvern Instruments, Malvern, UK) and compared with the liquid AV7909 precursor as well as with liquid AV7909 frozen at <−80 °C.

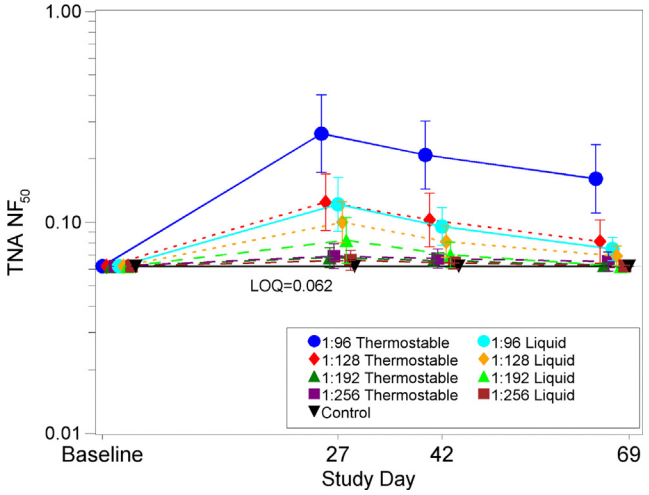


**Fig. 3.** Group Geometric Means and 95 Percent Confidence Intervals for TNA $NF_{50}$ by Study Day for All Vaccinated Groups and Saline Control. Guinea pigs were vaccinated by intramuscular (IM) injection on Study Days 0 and 14 with 0.5 mL of saline (control), liquid AV7909, or thermostable AV7909 at various dilutions. TNA was performed and $NF_{50}$ calculated as described in Materials and Methods.

**Table 2**
Survival Probabilities for the Liquid AV7909 and Thermostable AV7909 Vaccinated Groups at Study Day 69.

| Probability of Survival | TNA $NF_{50}$ | |
|---|---|---|
| | Liquid AV7909[a] | Thermostable AV7909 |
| 95% | 0.095 | 0.096 |
| 90% | 0.084 | 0.086 |
| 85% | 0.078 | 0.081 |
| 80% | 0.073 | 0.077 |
| 75% | 0.07 | 0.073 |
| 70% | 0.067 | 0.071 |
| 50% | <LOQ | 0.063 |

The table contains estimates for the TNA $NF_{50}$ associated with survival probabilities of 50, 70, 75, 80, 85, 90, and 95% on those study days on which the survival curve slope parameter for animals immunized with liquid and thermostable vaccine was significant.

immune response, following immunization at 0 and 14 days, occurred at Day 27 (see Fig. 3).

The relationship between TNA $NF_{50}$ levels and the survival of liquid AV7909- and thermostable AV7909-vaccinated animals following challenge was evaluated using logistic regression analysis. A strong correlation was observed between TNA $NF_{50}$ levels just prior to challenge and survival following challenge. The logistic regression model allowed estimation of pre-challenge TNA $NF_{50}$ titers associated with various probabilities of survival (Table 2). A 70% probability of survival was associated with TNA $NF_{50}$ titers of 0.067 and 0.071 and 0.067 for liquid AV7909 and thermostable AV7909, respectively.

## 4. Discussion

Freezing of vaccine compositions containing Alhydrogel adjuvant during the lyophilization process or to produce a frozen vaccine generally induces aggregation of the aluminum particles, causes reduction of the height of the settled aluminum gel (commonly referred to as gel collapse), and leads to degradation of the antigen adsorbed onto adjuvant resulting in potency loss [22–25]. A lyophilized formulation of AV7909 was developed to enhance the stability of the vaccine including its thermostability. The stabilization approach employed enabled conversion of liquid AV7909 to a lyophilized form without the freeze/thaw damage typically observed for aluminum-adjuvanted vaccines.

The primary objective of this study was to evaluate the protective efficacy of the thermostable AV7909 formulation in comparison with the liquid formulation in Hartley guinea pigs following lethal anthrax aerosol challenge and to compare the two formulations regarding immune response, as measured by the TNA. In the current study, animals were immunized on Study Days 0 and 14, whereas in previous studies vaccinations were administered on Study Days 0 and 28 [25] followed by challenge on Study Day 70. The changes in vaccination schedule were made to reflect the schedule proposed for use of AV7909 for PEP in humans.

The dilution factor had a significant effect on survival for both liquid and thermostable AV7909, with decreased probability of survival associated with an increased dilution factor. The interaction terms in these models were not significant, indicating that the relationships between dilution factor and survival were not significantly different for the two vaccine formulations.

In logistic regression models correlating survival to antibody levels, the slope parameters were significantly greater than zero on Study Days 27, 42, and 69 for TNA $NF_{50}$ for animals vaccinated with both liquid and thermostable AV7909, indicating that the probability of survival increased as the TNA levels increased on those study days. The interaction terms in these models were not significant, indicating that the relationships between TNA $NF_{50}$ levels and survival were not significantly different for the two vaccine formulations.

There was a strong correlation between pre-challenge TNA $NF_{50}$ titers and survival for both vaccine formulations. The TNA $NF_{50}$ thresholds associated with a 70% probability of survival following both liquid and thermostable AV7909 vaccination were comparable to those reported previously [25] and were lower than the TNA $NF_{50}$ 70% protective threshold values of 0.56 and 0.29 published for AVA-immunized rabbits and NHP, respectively [26]. The pre-challenge TNA $NF_{50}$ titers for all liquid and thermostable AV7909 groups (1:96, 1:128, 1:192, and 1:256 dilutions) were slightly lower than those induced by AVA (1:16 and 1:96 dilutions) in previous studies [25]. This finding is likely related to the differences in the maturity of the immune response resulting from the changes in the immunization schedule.

Overall, this study demonstrated there was no significant difference in the immunogenicity or efficacy of thermostable AV7909 against lethal anthrax spore aerosol challenge in the guinea pig model as compared to liquid AV7909. Taken together, these findings suggest that lyophilization of AV7909 improves stability of the vaccine, including resistance to freeze/thaw damage, without diminishing its ability to induce a robust immune response and confer protection against lethal anthrax spore challenge. Future work is warranted to evaluate efficacy of the AV7909 vaccine formulations in a PEP model, in which the vaccine is administered post-challenge in conjunction with antimicrobial treatment as described previously [26].

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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