HHS Public Access

Author manuscript

Vaccine. Author manuscript; available in PMC 2024 March 13.

Published in final edited form as:

Vaccine. 2021 October 08; 39(42): 6245–6249. doi:10.1016/j.vaccine.2021.09.023.

Viability evaluation of freeze dried and suspension anthrax spore vaccine formulations stored at different temperatures

T. Abayneh[a,*], **B. Getachew**[a], **E. Gelaye**[a], **R. Traxler**[b], **A.R. Vieira**[b]

[a]National Veterinary Institute, P.O.Box 19, Bishoftu/Debrezeit, Ethiopia

[b]Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

Anthrax is endemic in Ethiopia with sporadic outbreaks despite the regular vaccination of domestic livestock. This has raised concerns on the effectiveness of the vaccination strategy which may be associated with breaches in the vaccine cold chain maintenance. This study was aimed at demonstrating the tolerance of anthrax vaccine to cold chain breaches through evaluation of viable spore counts expressed as colony forming units per mL (CFU/mL) of freeze-dried and suspension anthrax vaccines stored at 5 °C, 20 °C and 37 °C for up to 6 months. Both vaccine formulations maintained above the recommended minimum required titre (2×10^6 culturable spores per dose for cattle, buffaloes and horses, and not $<1 \times 10^6$ for sheep and goats) for up to 6 months at 5 °C storage. In storage at 20 °C, the viability of freeze-dried anthrax vaccine maintained the minimum required titre up to 6 months while up to 90 days in case of the suspension formulation. Both types of vaccine formulations maintained the minimum titre per dose for up to 30 days at 37 °C storage. Generally, both vaccine formulations showed similar trends in titre fall in all of the three storage temperatures (5 °C, 20 °C and 37 °C) as observed in the almost linearly overlapping 95% confidence intervals (CI) up to day 90 at 5 °C and 20 °C storages while up to day 30 at 37 °C storage. However, a significant ($P < 0.05$) drop in titre was observed after day 90 for storages at 5 °C and 20 °C, and after day 30 for 37 °C storage as observed in the non overlapping 95% CI from the average titres of previous time points. This study showed that if temperature excursion occurs above the recommended temperature range (4–8 °C) during storage or transport, the vaccine should remain effective and can still be used in vaccination programs.

Keywords

Anthrax; Freeze dried vaccine; Viability evaluation

*Corresponding author at: National Veterinary Institute, P.O.Box 19, Bishoftu, Ethiopia. takeletefera99@gmail.com (T. Abayneh).

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.

1. Introduction

Anthrax, a zoonotic disease caused by *Bacillus anthracis*, causes mortality and morbidity in animals and humans. Owing to the spores of B. anthracis capable of spreading through aerosol, the organism can present a serious bio-threat [1]. Globally, an estimated 20,000 to 100,000 incidents of human anthrax occur per year [2] with a significant number of cases being in Chad, Ethiopia, Zambia, Zimbabwe and India. Anthrax ranks second next to rabies as the disease of greatest zoonotic concern in Ethiopia [3] with sporadic outbreaks occurring typically in May and June of the year [4]. In 2003, Ethiopia reported almost 2000 cases of human anthrax [5]. Anthrax outbreaks have been reported in Amhara, Oromia, Tigray and Southern regions of Ethiopia in domestic ruminants and humans. In Ethiopia, humans are at risk of infection mainly due to the tradition of consuming raw meat and sharing of home-butchered meat among the community, resulting in exposure of several people to contaminated meat from a single animal anthrax case.

In Ethiopia, vaccination of livestock has been the major strategy in the prevention and control of the disease in both animals and humans, a strategy considered to be the most effective and practically feasible. Currently, most countries, including Ethiopia, use vaccine prepared from live spores of a non-encapsulated but toxigenic variant of *Bacillus anthracis* 34F2 Sterne strain developed many decades ago [6,7]. The National Veterinary Institute (NVI) is the sole provider of anthrax vaccine in Ethiopia, which is a lyophilized spore vaccine. Vaccine distribution to the different regions is the responsibility of the Ministry of Agriculture. The NVI delivers lyophilized vaccine to regional vaccine storage hubs in case of bulk orders using cold trucks. Once the vaccine leaves the production facility, it is recommended to be maintained at 2–8 °C until reconstituted and used.

In Ethiopia, the regional states carry out the anthrax vaccination strategy. In most cases, districts have their own budget to order the vaccine, conduct both preventive, and reactive vaccination (ring vaccination following an outbreak). However, there may be insufficient incentive for the farmers to get their animals vaccinated routinely as they are expected to pay for the vaccine.

Failure to maintain the cold chain has been a major problem at district level due to frequent power outage, malfunctioning cold stores and lack of attention to abide by the manufacturer's instructions in vaccine storage and management. Thus, there is doubt as to whether the vaccines are transported, stored and handled appropriately at district level. . The availability of an effective vaccine and vaccination programme should have brought effective control of the disease. It has been suggested that anthrax outbreaks in Ethiopia may be partly due to the ineffectiveness of the vaccination programme. The situation on the ground suggested the need for an investigation into the possibility of vaccine failure due to lack of cold chain maintenance.

For anthrax vaccine to be effective, it should contain a minimum of 2×10^6 culturable spores per dose for cattle, buffaloes and horses, and not$<1 \times 10^6$ culturable spores per dose for sheep, goats and pigs [8]. The vaccine is expected to contain these spores in an appropriate volume, e.g. 2×10^6/ml [8]. This investigation was undertaken to examine vaccine stability

under controlled laboratory conditions at different temperatures and time points to determine the vaccine's tolerance to storage under adverse environmental conditions, and to evaluate potential differences between freeze-dried and suspension formulation vaccines.

2. Materials and methods

2.1. Anthrax vaccine samples

Samples from three different lots of freeze-dried anthrax spore vaccine produced and stored at NVI, and samples from two lots also produced by NVI but already distributed to two districts (field) were used for viabilty evaluation. Additionally, a suspension formulation of anthrax spore vaccine kindly provided by Colorado Serum Institute (CSI) (Colorado Serum Company, Denver, CO, USA) was also used for potency evaluation for comparison purposes with the freeze-dried anthrax spore vaccine. This suspension vaccine is also produced from the 34F2 Sterne strain of *B. anthracis* containing a suspension of viable *Bacillus anthracis* spores in Saponin.

The freeze dried anthrax spore vaccine is prepared from spores of *B. anthracis* freeze dried with saponin and skim milk used as an adjuvant and stabilizer, respectively.

2.2. Determination of viable spore count and vaccine storage conditions

Potency of anthrax vaccine can be determined either by determining the titre of the vaccine which refers the expected number of live organisms it contains per dose or mL of vaccine or in bioassay studies through determination of the protective efficacy [9,10,11]. In this study, determination of viable spore count of anthrax vaccines was used as a measure of potency and was done at the Research & Development Laboratory of the National Veterinary Institute, Ethiopia. Anthrax vaccine titre was determined by enumerating the number of culturable spores employing the plate colony count method according to standard plate count protocols described previously [12,13,14].

Viable spore count was done on two categories of vaccine samples which included: anthrax vaccine samples obtained from manufacturers (Freeze dried vaccine from NVI and suspension vaccine from CSI) and freeze-dried anthrax vaccine samples collected from field (selected districts in Ethiopia). In case of anthrax vaccine samples obtained directly from manufacturers, an accelerated stability test was conducted after keeping the vaccines under controlled storage conditions. In the accelerated stability test, vaccine samples were stored at two temperature points (20 °C and 37 °C) above the recommended storage temperature and at one temperature point (5 °C) within the recommended storage temperature range (2–8 °C). A total of nineteen time points at which viable spore count was determined, were considered throughout the selected temperature points (6 time points at 5 °C, 8 at 20 °C and 5 at 37 °C) (Table 1) [13,15]. Two calibrated incubators and a refrigerator were used for the experiment (in both freeze-dried and suspension Anthrax vaccines) and the temperature record was monitored twice a day until the end of the experiment. The timeframe of 6-month storage was set in this study due to the fact that customers unlikely store vaccines for >6 months after purchase.

Accordingly, nineteen randomly selected vaccine vials from each of the three most recently produced batches of freeze-dried anthrax vaccine from NVI cold storage and 19 vials from a single batch of the suspension anthrax spore vaccine from CSI were used for accelerated potency test after storage at 5 °C, 20 °C and 37 °C at the indicated time points shown in Table 1.

At each time point, triplicate plates were used for each serial dilution of the vaccine. The average CFU/ml of replicates from each batch and the average of the counts of the three batches of the freeze-dried vaccine as well as the suspension formulation were determined to estimate the viability of anthrax vaccines.

Culturable spore counts of vaccine samples obtained from the field (two districts from Oromia region) were immediately analyzed for potency after retrieval from the field. In both districts visited, vaccine was purchased and transported from NVI in ice blocks although there is no means of verifying whether the recommended cold chain was maintained during transport. Although both districts use refrigerators and freezers to keep vaccines, they experience frequent power outages and technical malfunctioning of their equipment. In both cases, anthrax vaccines that were sampled for viability evaluation were retrieved from a storage room where they had been kept for eight (Dodota Sire district) and 11 (Zeway Dugda district) weeks at ambient temperature due to malfunctioning refrigerators. The average ambient temperature at the site of vaccine storage at Dodota Sire district during the time of the visit was 20 °C (range 13–24 °C) [16] while 18 °C (range 13–21 °C) at Zeway Dugda [17].

2.3. Data analysis

The colony forming unit (CFU) data was recorded in Microsoft excel and were log transformed. Descriptive statistics such as averages, standard deviations and 95% confidence intervals (CI) were used in summarizing and analyzing the data.

3. Results

3.1. Viable/culturable spore count of anthrax vaccines

The results of the laboratory analysis of anthrax vaccine samples are presented in Table 2.

Generally, both formulations of anthrax vaccine (freeze dried and suspension) maintained titre above the minimum recommended titre of 2×10^6 and 1×10^6 culturable spores per dose for cattle and small ruminants, respectively [8] all through the 180 days after storage at 5 °C, although a gradual decreasing trend in titre was observed with no significant difference ($P > 0.05$) among titres of the consecutive time points as observed in the almost linearly overlapping 95% CI except for the freeze dried vaccine formulation where the titre fall was significant ($P < 0.05$) after 90 days of storage as evidenced by the non-overlapping 95% CI (Fig. 1)

Storage at 20 °C did not cause a drop in titre below the recommended titre per dose up to day 90 for suspension vaccine and up to day 180 for lyophilized vaccine formulation. In both formulations although the titre did not drop below the minimum recommended dose at

storage at 37 °C up to day 30, a significant ($P < 0.05$) drop in titre was observed thenafter in both cases (Fig. 3).

Generally, both vaccine formulations showed similar trends in titre fall in all of the three storage temperatures (5 °C, 20 °C and 37 °C) as observed in the almost linearly overlapping 95% CI up to day 90 at 5 °C and 20 °C storages while up to day 30 at 37 °C storage and a significant ($P < 0.05$) drop after the above respective time points as seen in the non overlapping 95% CI suggesting similar temperature tolerance of the two vaccine formulations (Figs. 1–3). The lyophilized vaccine maintained the minimum recom mended titre per dose at 20 °C up to day 180 which may be due to its higher initial titre as both formulations showed similar trends in titre fall (Figs. 1–3).

The titre of anthrax vaccine collected from the two districts from Oromia region was 2×10^7 CFU/mL for Dodota Sire district while 3.5×10^7 for Zeway Dugda district; both with titers above the minimum recommended per dose. Records show that the vaccine batch tested (1/19) at Dodota Sire district was in their store for 8 weeks and that of Zeway Dugda district (batch 4/19) for 11 weeks both kept at ambient temperature averaging 18–20 °C [16,17].

In storage at 5 °C, the average plate count at day 30 (2.6×10^8) was higher than day 14 (2.4×10^8) in freeze dried vaccine formulation while in storage at 20 °C the average plate counts at day one was higher than the initial average counts in the suspension anthrax vaccine. In storage at 20 °C, the average plate count of the freeze-dried anthrax vaccine at day 3 (2.6×10^8) was higher than day one (2.4×10^8) and the initial count (2.5×10^8) while the average plate count at day seven (2.8×10^8) was higher than the count at initial, day one and three. At 37 °C, the average count at day three was higher than at day one in the freeze-dried anthrax vaccine (Table 2).

4. Discussion

Generally, being biological substances, all vaccines are sensitive to adverse environmental or storage conditions with a specific shelf life and gradually lose their potency. The loss of potency is much faster when the vaccine is exposed to temperatures outside the recommended storage ranges, indicating the strict requirement to abide by the manufacturer’s instruction in maintaining the recommended temperature conditions under which the vaccine is kept to the moment of administration in order that full vaccine potency is retained.. One of the methods used in potency evaluation of live vaccines is determining the titre of the vaccine, which refers the expected number of live organisms it contains per dose or an mL of vaccine suspension although measuring the biological activity/immunogenicity of the vaccine after administration into a living system (bioassay) as in determining protection against challenge is a more robust method of evaluating potency. Performing animal studies to evaluate potency, particularly in case of anthrax, is expensive, and requires specialized bio-containment facilities and experience which otherwise is hazardous due to the risks of environmental contamination and human health.

Thus, it was beyond the scope of this work. Because the anthrax vaccine is a live vaccine, a decrease in viability would affect the dose given and potentially affect potency. Anthrax

vaccine is a spore that can tolerate extremes in environmental conditions. However, it is not known whether the vaccine is susceptible or tolerant to conditions outside manufacturers' recommended storage conditions warranting an investigation.

The results of this study revealed that both freeze-dried and suspension formulations of anthrax vaccine are highly tolerant to storage above the recommended temperatures (4–8 °C) showing similar trends in titre fall. Freeze-dried anthrax vaccine maintained at least the recommended minimum titre for up to six months at storage at 20 °C (room temperature) while the suspension formulation was up to 90 days. Both types of vaccine formulations maintained the minimum titre per dose for a month at storage at 37 °C. These findings are corroborated by previous work where it has been demonstrated that storage at moderate temperature that does not exceed 20 °C with minimum temperature variation and vaccine protected from direct sunlight was stable and effective up to two years [13].

The disparities observed in the current study in the plate count data could be due to the compound effect of the inherent limitation associated with the plate count method and subjective factors related to the laboratory technician. Since CFU shows only an estimate of the number of cells present, it is a skewed estimate based on the only cells that are able to form colonies and that can grow under the conditions of the test which include media, temperature, time, and oxygen conditions, which may be difficult to control. This can contribute to the variations in plate counts between cultures. Another limitation of the plate count or CFU method is that clumps of bacteria cells can be miscounted as single colonies [18] since results are reported as CFU/mL rather than bacteria/ml. Moreover, the plate count method has the relatively narrow countable range, generally considered to be 25–250 CFU bacteria on a standard petri dish, which lowers its precision.

In addition, the recorded plate count data is the interpretation of the technician which is known to vary among technicians even on the same sample. Generally, although plate count data are the best data that we can have, they are an interpretation of the number of colonies on the plate that can be influenced by the colony morphology, colony density, and the temperament of the counter [18]. There are also a number of variables contributing for poor reproducibility inherent with the method which may include errors associated with plating, dilution, sampling, counting and recording. Such variability can only be minimized by increasing the number of replicate platings [18]. The current study employed the standard triplicate plates per dilution, and thus increasing the replicates was expected to increase the accuracy of the method.

It is known that vaccine storage conditions at the district level can vary greatly and are very often suboptimal. At many of the districts where vaccine is stored until delivery, there are inconsistencies with availability of cold storage, with potential freeze–thaw cycles due to frequent power outages, and lack of equipment that refrigerates reliably. However, in this study, in working with vaccines retrieved from storage at the district level there was more than sufficient titre to supply protection after storage at ambient temperatures for eight to 11 weeks. Combining the results from the laboratory study as well as the study of vaccine retrieved from the field, we conclude that potentially adverse vaccine storage conditions are not likely to be responsible for anthrax vaccination failures. This suggests that the sporadic

occurrence of anthrax outbreaks observed in Ethiopia doesn't appear to be associated with vaccine failure, but rather related to timing of vaccination and coverage or some other causes which may need further investigation.

In conclusion, the results of this study revealed that both freeze dried and suspension formulations of anthrax vaccine were highly tolerant to storage above the recommended temperatures (4–8 °C) showing similar trend in titre fall. Freeze dried anthrax vaccine maintained the recommended minimum titre for up to six months storage at 20 °C (room temperature) while the suspension formulation for up to 90 days. Both types of vaccine formulations maintained the minimum titre per dose for one-month storage at 37 °C.

This study shows that if temperature excursion occurs above the recommended temperature range (4–8 °C) during storage or transport, the vaccine should remain effective and can still be used in vaccination programs. The high tolerance of anthrax vaccine to storage above the recommended temperature range observed in this study suggests that temperature excursions might not require canceling a vaccination campaign, if replacement vaccine is not available.

Acknowledgements

The authors would like to thank the National Veterinary Institute and laboratory staffs at the bacteriology laboratory for their administrative and technical support, respectively. The kind cooperation of the Colorado Serum Company in provision of the suspension formulation of Anthrax vaccine is highly appreciated.

Funding

This publication was supported by Cooperative Agreement 6 NU2GGH001807-03-05 11 awarded to the American Society for Microbiology by the Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention or the Department of Health and Human Services.

References

[1]. Verma A, Burns DL. Improving the stability of recombinant anthrax protective antigen vaccine. Vaccine 2018;36(43):6379–82. 10.1016/j.vaccine.2018.09.012. [PubMed: 30228030]

[2]. Dutta T, Sujatha S, Sahoo R. Anthrax-update on diagnosis and management. J Assoc Physicians India 2011;59:573–8. [PubMed: 22334971]

[3]. MOA-MOH. Zoonotic Disease Prioritization for Inter-sectoral Engagement in Ethiopia, SEPTEMBER 29–30, 2015..

[4]. Romha G, Girmay W. Knowledge, attitude and practice towards anthrax in northern Ethiopia: a mixed approach study. BMC Infect Dis 2020;20:814. 10.1186/s12879-020-05544-z. [PubMed: 33167885]

[5]. Bahiru G, Bekele A, Seraw B, Boulanger L, Ali A. Human and animal anthrax in Ethiopi: A retrospective record review 2009–2013. Ethiopian Veterinary J 2016;20(2):75–85.

[6]. Sterne M, Regnsen EM. Onderspoort J Veterinary Sci 1939;12:9–17.

[7]. Sterne M. The use of anthrax vaccines prepared from avirulent (uncapsulated) variant of Bacillus anthracis. Onderstepoort J Veterinary Sci Animal Industry 1939;13:307–12.

[8]. OIE Terrestrial Manual 2018. Anthrax..

[9]. OIE Manual of standards for diagnostic tests and vaccines. Anthrax. 4th edn., 2004; Pp: 283–294..

[10]. Habing WH. Potency testing of bacterial vaccines for human use. Veterinary..

[11]. Misra RP. Manual for the production of Anthrax and Blackleg vaccines, Food and Agriculture organization of the United Nations (FAO) Animals Production and Health, 1991; Paper 87, Pp: 3–40. FAO, Rome, Italy..

[12]. Miller JH. Determination of viable cell counts: bacterial growth curves. Experiments in Molecular Genetics. In Miller JH.eds 1972, New York: Cold Spring Harbor, 31–36..

[13]. Jula M, Jabbari A. Stability and potency studies of anthrax vaccine (Bacillus anthracis 34F2 Sterne strain) in Iran. Archives of Razi Institute, 2007;62:3, 145–149..

[14]. USDA. Standard Operating Procedure for Bacterial Plate Count. USDA, 2015; Center for Veterinary Biologics, P. O. Box 844 Ames, IA 50010..

[15]. WHO, 2006. Guidelines on stability evaluation of vaccines..

[16]. World Weather Online: https://www.worldweatheronline.com/dodota-weather-averages/et.aspx, Accessed April 2021..

[17]. World Weather Online: (https://www.worldweatheronline.com/dugda-weather-averages/et.aspx), Accessed April 2021..

[18]. Sutton S. The Limitations of CFU: Compliance to CGMP Requires Good Science. J GXP Compliance 2012;16:1.

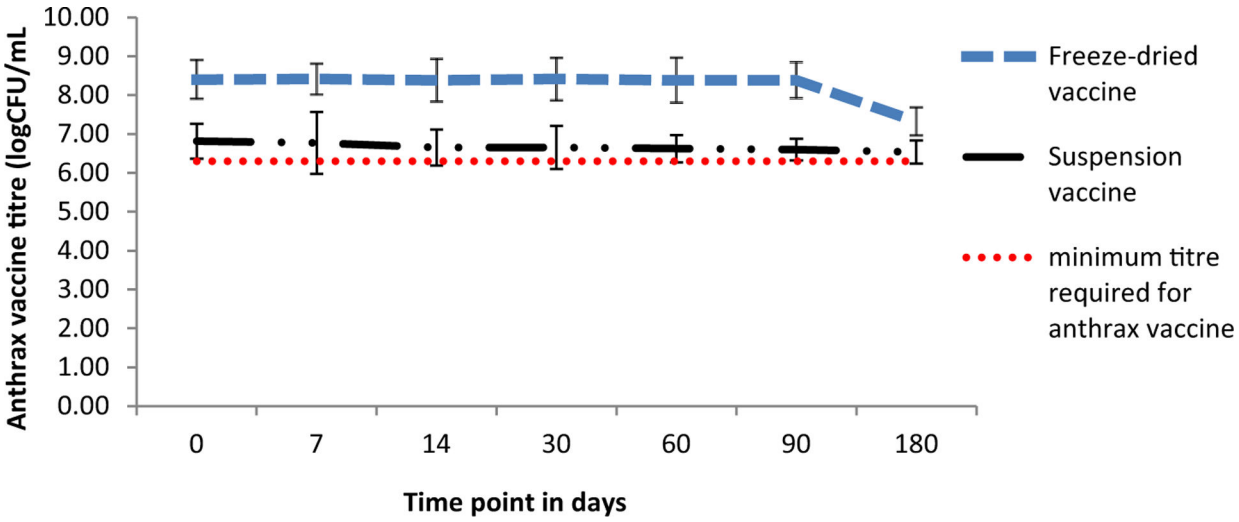

Fig. 1.

Trends in viability of freeze dried and suspension anthrax vaccine formulations stored at 5 °C for a period of 6 months. . Note: The error bars show the 95% CI of each average log CFU/mL. Almost linearly overlapping 95% CI show the lack of significant difference ($P > 0.05$) between the average values of consecutive time points while non-overlapping 95% CI show significant differences ($P < 0.05$).

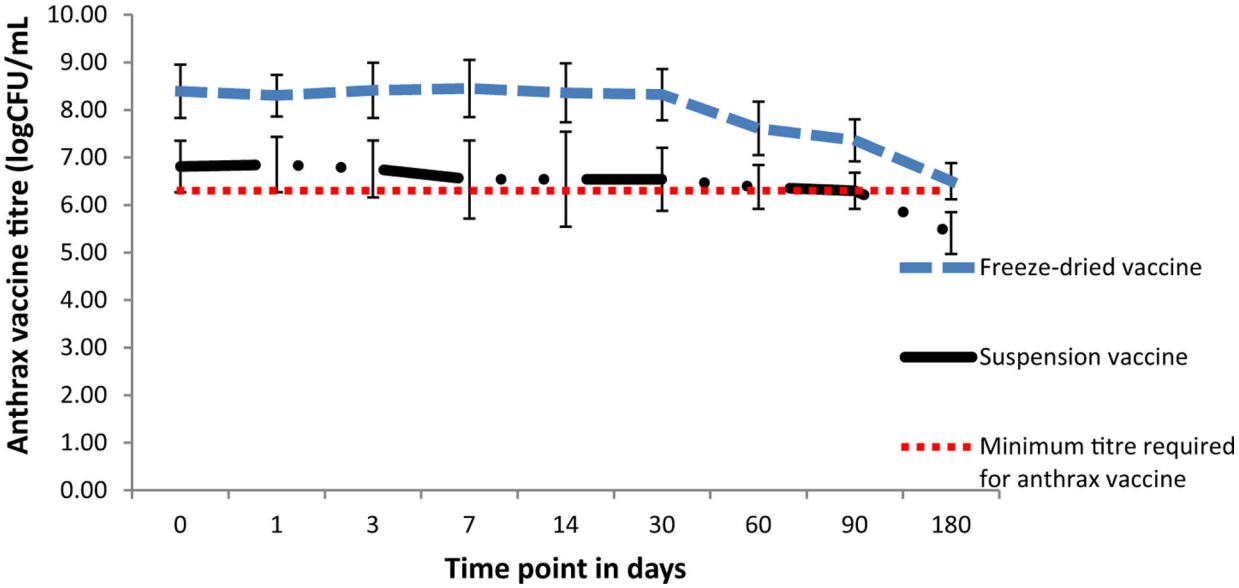

Fig. 2.

Viability of Freeze-dried and suspension anthrax vaccine formulations determined at different time points that were stored at 20 °C for 6 months. . Note: The error bars show the 95% CI of each average log CFU/mL. Almost linearly overlapping 95% CI show the lack of significant difference ($P > 0.05$) between the average values of consecutive time points while non-overlapping 95% CI show significant differences ($P < 0.05$).

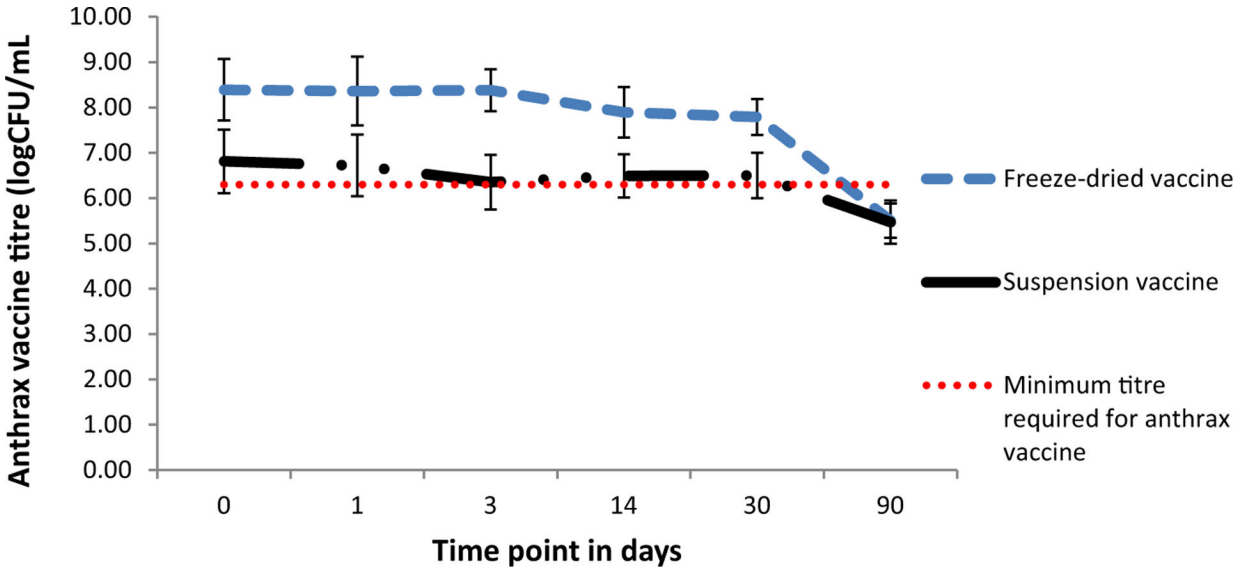

Fig. 3.

Viability of Freeze-dried and suspension anthrax vaccine formulations determined at different time points that were stored at 37 °C for 3 months. . Note: The error bars show the 95% CI of each average log CFU/mL. Almost linearly overlapping 95% CI show the lack of significant difference ($P > 0.05$) between the average values of consecutive time points while non-overlapping 95% CI show significant differences ($P < 0.05$).

Table 1

Temperature and time points used for accelerated potency evaluation of anthrax spore vaccines.

Storage Temperature	Time points at which titre was determined (days)*							
5 °C			7	14	30	60	90	180
20 °C	1	3	7	14	30	60	90	180
37 °C	1	3		14	30		90	

* The time points at the selected temperatures were based on the recommendations of previous studies [13,15].

Table 2

Average titre of freeze dried (three successive batches) and suspension (single batch) anthrax spore vaccine formulations evaluated at different storage temperature and time points.

Vaccine types	Temperature point at which vaccines were stored	Time points at which titre was determined (days)								
		Av. Initial titre	1	3	7	14	30	60	90	180
Freeze-dried	5 °C	2.5×10^8			2.6×10^8	2.4×10^8	2.6×10^8	2.4×10^8	2.4×10^8	2.1×10^7
	20 °C	2.5×10^8	2.4×10^8	2.6×10^8	2.8×10^8	2.3×10^8	2.1×10^8	4.1×10^7	2.3×10^7	3.8×10^6
	37 °C	2.5×10^8	2.3×10^8	2.4×10^8		7.8×10^7	6.2×10^7		3.2×10^5	
Suspension	5 °C	6.5×10^6			6.0×10^6	4.5×10^6	4.5×10^6	4.2×10^6	4×10^6	3.4×10^6
	20 °C	6.5×10^6	7.0×10^6	5.8×10^6	3.5×10^6	3.5×10^6	3.5×10^6	2.4×10^6	2×10^6	2.6×10^5
	37 °C	6.5×10^6	5.2×10^6	4.5×10^6		3.1×10^6	3.2×10^6		3×10^5	