



Review Committee Report: Inadvertent Shipment of Live *Bacillus anthracis* Spores by DoD

*Committee for Comprehensive Review of DoD Laboratory Procedures, Processes, and
Protocols Associated with Inactivating Bacillus anthracis Spores*

July 13, 2015

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Executive Summary

On May 29, 2015, due to the inadvertent shipment of live anthrax from a Department of Defense (DoD) laboratory, the Deputy Secretary of Defense (DSD) directed the Under Secretary of Defense (USD) for Acquisition, Technology & Logistics (AT&L) to conduct a 30-day review of the Department's safety practices for generating and handling inactivated *Bacillus anthracis* (BA). This independent review was in addition to the concurrent investigation initiated by the Centers for Disease Control and Prevention (CDC). For more than a decade, DoD and CDC have been working closely on optimal oversight of and compliance with the select agent regulations. These regulations impact all U.S. laboratories that work with biological select agents including those supported by the DoD's Critical Reagents Program (CRP), which primarily serves the interagency and the biodefense research community.

USD (AT&L) established a review committee to conduct the comprehensive review mandated by DSD. This document is the Review Committee's report; membership of the committee is provided in Appendix C. The committee was asked to address the following critical areas:

- The root cause for the incomplete inactivation of BA samples at DoD laboratories;
- Why post inactivation viability testing did not detect the presence of live BA;
- Existing DoD laboratory biohazard safety protocols and procedures;
- DoD laboratory adherence to established procedures and protocols; and
- Identification of systemic problems and what steps should be taken to fix those problems.

The United States Department of Defense Chemical and Biological Defense Program (CBDP) develops medical and physical countermeasures to protect the warfighter from chemical and biological threats. BA is an exceptionally resilient organism. This resilience combined with its infectious nature makes BA ideally suited for potential adversaries' biological weapons programs. Therefore it is critical for the Department to have a strong countermeasures program to protect our warfighters against this dangerous organism.

As part of the above process, the DoD routinely inactivates many select agents such as *Bacillus anthracis* (the causative agent of anthrax), *Francisella tularensis* (the causative agent of tularemia), and *Burkholderia mallei* (the causative agent of Glanders disease in horses) through a number of chemical, thermal, or radiation procedures. There are many federal partners and private sector laboratories outside the DoD that depend on DoD's ability to provide them with inactivated pathogens for national security and public safety missions. The four DoD laboratories whose mission involves inactivation of BA are: Edgewood Chemical Biological Center (ECBC) in Maryland, the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) in Maryland, the Naval Medical Research Center (NMRC) in Maryland, and Dugway Proving Ground (DPG) in Utah.

What Happened?

The DoD regularly ships inactivated biological materials for research, development, testing, and evaluation to industry, academia, and other Federal laboratories. On May 22, 2015, a private company notified the CDC that inactivated BA spores in its possession were live. Live BA has the potential to pose a threat to human health and it is only distributed to facilities regulated by the Federal Select Agent Program.

The CDC began an investigation and determined that a BA sample originating from DPG on April 20, 2015, was not fully inactivated and contained viable BA spores. We now know that, over the course of the last decade, 86 facilities in the United States and seven other countries have received low concentrations of live BA spore samples from DPG thought to be completely inactivated. The low numbers of live spores found in inactivated DoD samples did not pose a risk to the general public. Nonetheless, the shipment of live BA samples outside of the select agent program restrictions (at any concentration) is a serious breach of regulations.

What Has DoD Done Since The Incident?

The DoD issued a moratorium on the shipping of inactivated BA from DPG, ECBC, and USAMRIID. NMRC does not currently inactivate or ship BA.

DSD advised any laboratories that may have received inactivated BA from the DoD that they should stop working with it until further instructions are received from DoD and CDC. The CDC promulgated disposition instructions for laboratories who received samples of inactivated BA spore preparations from DPG. The office of the Assistant Secretary of Defense for Nuclear, Chemical, and Biological programs subsequently issued a directive to all DoD laboratories with inactivated BA spores to immediately determine if any inactivated material at their laboratory contained live BA.

The DoD created a website that summarizes current information pertaining to the BA situation at http://www.defense.gov/home/features/2015/0615_lab-stats/.

The DoD opened a hotline that is available for telephone calls 24 hours a day, 7 days a week, and posted the hotline number to the website as a resource for any questions that laboratories or the public may have regarding this incident.

On June 1, the Office of the Assistant Secretary of Defense for Nuclear, Chemical, and Biological Defense Programs established a Task Force to coordinate the DoD response.

On June 5, 2015, the CDC issued updated guidance for testing inactivated BA spores for growth. All DoD laboratories that began testing using in-house protocols restarted testing in conformance with the updated CDC protocol to verify that inactivated samples contain no live BA spores.

The DoD has validated that there are no additional irradiated BA samples of concern that are demonstrating growth using currently available DoD and CDC viability testing protocols.

Root Cause

To perform root cause analysis, the committee visited all four DoD laboratories with capabilities for BA irradiation to interview members of the scientific staff and management. The observations and recommendations in this report apply to all four DoD laboratories conducting this work. The committee also focused on the question of why materials originating from the Dugway Proving Ground (DPG) exclusively exhibited live BA spores in irradiated samples.

For this report, the committee reviewed existing procedures and protocols from all four DoD laboratories that produce and work with gamma irradiated BA spores and the scientific literature pertaining to bacterial spore inactivation.

A key finding by the committee is that there is insufficient technical information in the broader scientific community to guide the development of thoroughly effective protocols for inactivation of spores and viability testing of BA. This has contributed to the creation of protocols that do not completely or permanently sterilize BA with gamma irradiation. The absence of this critical information is a scientific community-wide problem and needs to be addressed with irradiation standards and viability testing procedures. Pending the publication of standards, the Centers for Disease Control and Prevention (CDC) has agreed to initiate a review of the Federal Select Agent Program Authorities to oversee inactivation procedures and consider rule making or other actions to expand the requirements for assurance of adequate inactivation.¹

The DPG is a production facility and its output of inactivated BA materials far exceeds other DoD laboratories that inactivate BA. Several potential causes requiring additional scientific research are suggested. Two additional factors specific to DPG, which may have contributed to the presence of live BA in inactivated samples, include low sampling volume for viability testing and a very short time period between the completed irradiation cycle and start of the viability testing. It is clear that BA spores are particularly difficult to kill² and live spores injured by irradiation may be able to repair their injuries over time.^{3,4}

¹ As described in the Department of Defense and Health and Human Services Memorandum (subject: briefing on DoD Anthrax Sample Investigation) for the President, June 2, 2015.

² Spotts Whitney, Ellen A., *et. al.* "Inactivation of *Bacillus anthracis* Spores" *Emerging Infectious Diseases* 9 (2003): 623-627.

³ Chowdhury, M. S. U., *et. al.* "Influence of Postirradiation Incubation Temperatures on Recovery of Radiation-Injured *Clostridium botulinum* 62A Spores." *Applied and Environmental Microbiology* 32 (1976): 172-178.

⁴ Abshire, Robert L., *et. al.* "Resistance and Recovery Studies on Ultraviolet-Irradiated Spores of *Bacillus pumilus*" *Applied and Environmental Microbiology* 39 (1980): 695-701

Findings

The root cause for the incomplete inactivation of BA samples at DoD laboratories:

A single root cause for shipping viable BA samples could not be identified. DoD personnel appear to have followed their own protocols correctly. However, the committee found inherent deficiencies in protocols for three phases in the production of inactive spores that could lead to non-sterile products: 1) radiation dosing, 2) viability testing, and 3) aseptic operations (contamination prevention). These deficiencies and other factors⁵ contributed to the establishment of protocols that do not completely or permanently sterilize these samples.

Why post inactivation viability testing did not detect the presence of live BA:

There is no single root cause to explain why the BA samples were incompletely inactivated, or why viability testing did not detect live BA spores. Contributing factors that may have resulted in undetected live BA spores during viability testing in DPG's samples include deficiencies in sample sizes and inadequate incubation periods after irradiation.

Existing DoD laboratory biological safety protocols and procedures:

The committee identified existing DoD laboratory safety protocols and procedures in each location. However, these procedures are not standardized amongst the laboratories.

DoD laboratory adherence to established procedures and protocols:

In most cases, the committee observed that DoD laboratories followed their own established procedures and protocols.

Identification of systemic problems and what steps should be taken to fix those problems:

The primary systemic issue responsible for failures in the preparation of inactivated BA spores is the lack of specific validated standards to guide the development of protocols, processes, and quality assurance measures.

Abridged Recommendations

Quality Assurance: Enhance quality control programs at DoD laboratories working with hazardous select agents and other pathogens.

Standardize BA Inactivation Protocols Across Laboratories: All DoD laboratories should follow a common Standard Operating Procedure (SOP) for such practices as irradiation and viability testing.

Institute More Rigorous Quality Procedures: Establish quality assurance (QA) and quality control (QC) procedures for inactivation and viability testing of BA spores.

⁵ The absence of specific scientific community standards for inactivated BA, and continuing scientific uncertainty regarding the survival, injury, and repair of spores exposed to gamma radiation.

Clarify The Conditions Of The Material Transfer Agreement (MTA): Material transfer agreements enable DoD laboratories to communicate potential hazards to the customers and maintain a positive inventory tracking for potential recalls on all select agent inactivated materials.

Perform Preventive Maintenance: All reusable mechanical equipment employed throughout the process should be routinely maintained and calibrated, from mechanical pipettes to the irradiator.

Establish And Manage An Environmental Surface Sampling Program: Some laboratories lack written procedures to document, investigate, and report contamination found outside primary containment areas during environmental persistent agent sampling.

Establish Validated Dose Curves: Radiation dose curves should be generated for each BA strain used for production, at the same concentrations used in production and performed on the same irradiator as used for inactivation of spore preparations.

Understand The End Users' Needs: The Chief Science Officer of the lab, Army or Command, should work with individual customers to understand sample requirements and determine the appropriate strains and material needed to support the objectives.

Quantitate Spores Before Irradiation: Spore preparations intended for irradiation should be quantified as precisely as possible to maximize the likelihood of achieving the inactivation levels predicted by kill curves produced with the same strain in the same irradiator.

Peer Review: Establish BA spore inactivation and viability testing protocols that are based on relevant scientific data, standards, and studies conducted to fill knowledge gaps. All protocols and subsequent protocol modifications should be subject to a peer review process, validated and implemented uniformly across similar operations.

Program Management: Program managers should provide adequate laboratory space, equipment and time to conduct relevant safety and surety research for select agents and other pathogens. Program managers should develop a plan to track and document the implementation and long-term sustainability of the recommended corrective actions identified through this review panel as well as all internal and external audits.

Background

On May 22, 2015, the Centers for Disease Control and Prevention (CDC) was contacted by a private company regarding the growth of live *Bacillus anthracis* (BA) from a sample that was purported to be inactivated (*i.e.*, killed bacterial spores). The CDC began an investigation working with the Department of Defense (DoD) laboratories, state officials, and the Federal Bureau of Investigation (FBI). This investigation revealed that on April 20, 2015, inactivated BA samples (Ames strain) originating at the Dugway Proving Ground (DPG) was routed through the Edgewood Chemical Biological Center (ECBC). ECBC sent the inactivated BA material to six different private sector companies on April 29, 2015. This shipment was sent to support a DoD effort to develop a new rapid field-based test to identify biological threats in the environment. One company in Maryland forwarded the BA sample to a subcontractor in Maryland. It was this subcontractor that notified the CDC Select Agent Program on May 22, 2015 of the live BA finding.

The CDC and State health departments recommended post-exposure prophylaxis (PEP) for eight laboratory workers based on aerosol-generating procedures they had performed with the BA sample. Other individuals who were not in the area during the aerosol-generating procedures were deemed not at risk. The CDC also recommended that the three receiving laboratories that worked with the BA sample outside of a biosafety cabinet (BSC) in Biosafety Levels (BSL) -1 and -2 be closed until decontamination procedures were completed.

Over the following days, the DoD identified additional facilities that had received samples containing a small number of viable BA spores from DPG, instead of fully inactivated samples. By May 29, 2015, the extent of this problem, and serious concerns about the DoD laboratory practices performing work with BA, resulted in establishment of a committee for comprehensive review of DoD laboratory procedures, processes, and protocols associated with inactivating spore-forming BA. A more detailed time-line of the events and activities associated with this incident is provided in Figure 1.

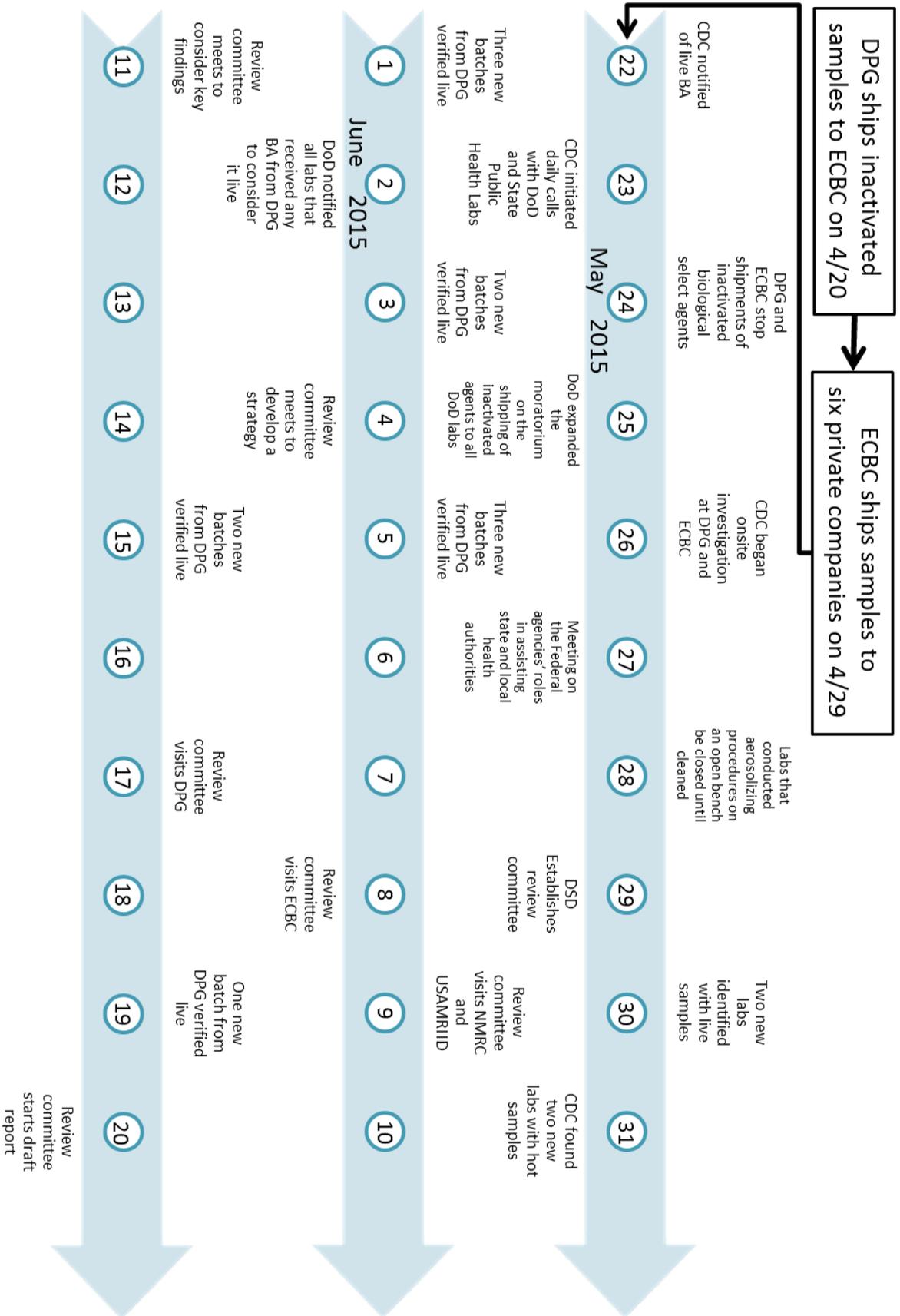


Figure 1: Events and activities time-line for the *Bacillus anthracis* (BA) incident.

Why Is This Work Done At DoD?

The U.S. Department of Defense Chemical and Biological Defense Program (CBDP) develop medical and physical countermeasures to protect the warfighter from chemical and biological threats. To support the development of detection assays/technologies reagents must be tested against the target hazardous agents. These tests are used to evaluate the efficacy of the countermeasures, to verify the accuracy of testing equipment and effectiveness of protective gears. Inactivated agents are used whenever possible in these activities to minimize hazards to the workforce without sacrificing reliability. The DoD routinely inactivates biological agents through a number of chemical, thermal, or radiation procedures. There are many federal partners and private sector laboratories outside the DoD that depend on DoD's ability to provide them with inactivated pathogens for national security and public safety missions. These include the FBI, Department of Homeland Security, the Environmental Protection Agency, and private sector companies, as well as many internal DoD components.

Why Is Irradiation The Method Of Choice?

The use of gamma radiation to sterilize is a common practice for medical devices, parenteral (administration by other than oral route) pharmaceuticals, and raw foods to increase shelf life. This particular form of radiation is high energy, with deep penetration into a wide variety of materials. For biodefense applications, preserving cellular components such as proteins and nucleic acids is necessary for the inactivated material to be used to develop and validate detection assays, and for quality assurance activities such as proficiency testing. One of the challenges faced by the biodefense community is that BA detection methods must accurately and sensitively detect active endospores, yet the detection methods must have safe, non-infectious positive controls for training and test validation. Although the most effective spore decontamination/disinfection methods (chemicals and high heat) do render them inactive, they also degrade/denature the surface antigens which are critical for antibody generation as well as for binding-sites for antibody-based assays.

During empirical research on the effect of inactivation methods on detection, it was found that all methods can affect detection assays⁶, but that gamma irradiation consistently allowed antibody-based tests to remain effective. This was further reinforced in studies by the CDC⁷ and Defense Research and Development Canada Suffield.⁸ Gamma irradiation continues to be the preferred method to inactivate BA endospores in order to retain immunoassay functionality. However, excess radiation does results in dose-dependent and irreversible damage to proteins and nucleic acids. Therefore, the target radiation dose for inactivating spores for research is high enough to

⁶ Dang, Jessica L., *et al.* "Bacillus Spore Inactivation Methods Affect Detection Assays." *Applied and Environmental Microbiology* 67(2001): 3665–3670.

⁷ Dauphin, Leslie A., *et al.* "Gamma Irradiation Can Be Used To Inactivate *Bacillus anthracis* Spores without Compromising the Sensitivity of Diagnostic Assays." *Applied and Environmental Microbiology* 74(2008): 4427–4433.

⁸ Technical Memorandum DRDC, Suffield TM 2005-236, December 2005.

make the product safe to handle, but low enough to preserve essential antigenic properties and nucleic acid integrity.

Which DoD Laboratories Do This Work?

Work with live and inactivated BA material is focused in four DoD laboratories: The Army Dugway Proving Ground (DPG) in Utah, the Army Edgewood Chemical Biological Center (ECBC) in Maryland, the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) in Maryland, and the Naval Medical Research Center (NMRC) in Maryland. Over the last few years, the funding for BA research has declined and the volume of BA work in many of these laboratories has dropped precipitously. Today, pathogen activities at NMRC are co-located with USAMRIID within the National Interagency Biodefense Campus (NIBC), at Fort Detrick Maryland, to cross leverage capabilities. NMRC has no current BA activities but USAMRIID remains active in BA research. ECBC has a low volume of activities associated with BA, while DPG continues to be the largest producer of BA and other pathogens, largely to support the Critical Reagents Program (CRP) and its customers.

Methods Used In Reviewing This Incident.

The committee for comprehensive review of DoD laboratory procedures, processes, and protocols associated with inactivating spore-forming BA was created by order of the Deputy Secretary of Defense on May 29, 2015 by the Under Secretary of Defense for Acquisition, Technology and Logistics (AT&L). Within AT&L, the Deputy Assistant Secretary of Defense for Nuclear Matters was appointed as the committee chair, to provide an impartial review of the processes used within DoD laboratories.

The committee chair engaged with subject matter experts from interagency partners, academia, and industry. Based on the nature of this incident and relevant experiential or academic credentials needed for this review, a team of experts with specialties in gamma irradiation, select agents, BA, microbiology, biosafety, biosecurity, quality assurance and good laboratory practices was assembled. The committee initially met on June 4, 2015, and was briefed on the inadvertent shipment of live BA samples and the committee's charter. During the initial meeting, the committee was tasked with developing a series of detailed probing questions based on the available facts to identify potential gaps and the likely root cause(s) of the incident. Furthermore, the committee members were asked to implement the charter directed by DSD when constructing their questions.

The committee chair reviewed the questions proposed by individual team members and selected a set of standard questions to be used at every site visit. The committee traveled to and met with the scientific staff and management for ECBC, NMRC & USAMRIID, and DPG on June 8, 9, and 17, 2015, respectively. After site visits, additional questions were sent to each laboratory for further clarification, if needed.

The results presented in this report consolidates the information gathered during the site visits, and presents a review of each lab's procedures, the national/international standards documents, and a review of relevant scientific literature.^{2-4, 6-18} Detailed discussions of site visits are outlined in Appendix A.

⁹ Cook, A. M., *et. al.* "Gamma Irradiation of *Bacillus subtilis* Spores in the Presence of Sugars." *Journal gen. Microbiology* 34 (1964): 185-198.

¹⁰ Hoile, Rebecca, *et. al.* "Gamma Irradiation as a Biological Decontaminant and its Effect on Common Fingerprint Detection Technique and DNA profiling" *Journal of Forensic Sciences* 66 (2010): 171-177.

¹¹ Heninger, Sara J., *et. al.* "Decontamination of *Bacillus anthracis* spores: Evaluation of Various Disinfectants" *Applied Biosafety* 14 (2009): 7-10.

¹² Setlow, P. "Spores of *Bacillus subtilis*: Their Resistance to and Killing by Radiation, Heat and Chemicals" *Journal of Applied Microbiology* 101(2006): 514-525.

¹³ Ghosh, S., *et. al.* "Superdormant Spores of *Bacillus* Species Have Elevated Wet-Heat Resistance and Temperature Requirements for Heat Activation." *Journal of Bacteriology* 191(2009): 5584-5591.

¹⁴ Nelson, F. E. "Factors Which Influence the Growth of Heat-Treated Bacteria" *Journal of Bacteriology* 45(1942): 395-403.

¹⁵ Roberts, Richard E. and Aldous, Elaine "Recovery from Ultraviolet Irradiation in *Escherichia coli*" *Journal of Bacteriology* 57(1949): 363-375.

¹⁶ Stringer, Sandra C., *et. al.* "Contrasting Effects of Heat Treatment and Incubation Temperature on Germination and Outgrowth of Individual Spores of Nonproteolytic *Clostridium botulinum* Bacteria" *Applied and Environmental Microbiology* 75(2009): 2712-2719.

Findings

The Deputy Secretary of Defense tasked the committee to conduct a comprehensive review of DoD laboratory procedures, processes, and protocols associated with inactivating BA spores and to address the following critical areas:

1. The root cause for the incomplete inactivation of BA samples at DoD laboratories;
2. Why post inactivation viability testing did not detect the presence of live BA;
3. Existing DoD laboratory biohazard safety protocols and procedures;
4. DoD laboratory adherence to established procedures and protocols; and
5. Identification of systemic problems and what steps should be taken to fix those problems.

Why Only Samples At DPG Contained Live BA?

As of this writing, only materials originating from the DPG have contained live BA spores in irradiated samples. Other DoD laboratories that inactivate BA have been able to routinely identify live BA in their inactivated samples; subsequently mitigating issues arising from incomplete inactivation. Based on statistics alone, we can predict that sterilization by gamma irradiation will occasionally fail to completely inactivate BA samples. Therefore, it is critical for the post irradiation viability tests to accurately determine if live spores exist in inactivated samples. The key issue with the DPG is not that the irradiation procedures failed; it is that the viability testing did not detect live BA spores in inactivated samples containing live spores.

It is important to emphasize DPG's distinctive production role in the defense biosecurity arena. DPG's primary mission is to provide developmental, operational, and production testing to support the Nation's chemical and biological defense programs. DPG's Chamber and Field Test capabilities provide DoD with the ability to conduct testing of individual protective equipment, contamination avoidance detection systems, and decontamination programs. In order to conduct this mission, DPG produces and uses live and inactivated biological agents. Moreover, DPG is the Nation's primary producer of inactivated select agents and toxins for use in other Chemical and Biological Defense Program research and development efforts. DPG is the largest producer of inactivated BA which is used in numerous research, development, testing and evaluation programs within the U.S. interagency, industry, and academic communities.

The committee studied the irradiation and viability testing procedures used at DPG and noted that the amount of solution extracted from the inactivated sample, to be tested for viability, was the lowest amongst DoD laboratories (5% of the sample is used for viability testing). Additionally, within this production facility, DPG samples experienced a very short time period between the completed irradiation cycle and start of the viability testing. The confluence of large production quantities associated with the DPG, low sampling volume of the inactivated material for viability testing, and a very short time period between the completed irradiation cycle and start of the viability testing may have exacerbated the likelihood of not properly identifying live BA spores in inactivated samples. The development and implementation of ineffective

irradiation and viability testing procedures took place over the last decade; this represents an institutional problem at DPG and does not necessarily reflect on any one individual. Additional details for potential root causes are provided in the following sections.

1. The root cause for the incomplete inactivation of BA samples at DoD laboratories:

A single root cause for shipping viable BA samples could not be identified. DoD personnel appear to have followed their own protocols correctly. However, the committee found inherent deficiencies in protocols for three phases in the production of inactive spores that could lead to non-sterile products: 1) radiation dosing, 2) viability testing, and 3) aseptic operations (contamination prevention). These deficiencies and other factors⁵ contributed to the establishment of protocols that do not completely or permanently sterilize these samples.

Why spores may have survived the irradiation process:

Laboratory workers rely on BA kill curves that are internally generated or published in the scientific literature. However, the conditions used to inactivate the BA samples in DoD laboratories are often not validated by these kill curves. Furthermore, the kill curve studies performed by various laboratories^{7,17,18,19,20} are limited in nature due to inconsistent experimental parameters. There is evidence in the literature that different strains of BA show varying degrees of radiation resistance. This problem is further complicated as various studies are done using different spore concentrations, different sample volumes, different radiation arrangements (*e.g.*, cooled/uncooled samples, single dose/multiple dose treatment), and different measurement units (*e.g.*, MRad/kGy, %survived/cfu).²¹ To illustrate the variability of this data on a single strain of BA, the committee has converted several DoD kill curves and open literature information to a unified set of variables as depicted in Figure 2.

The figure specifically plots the total number of remaining viable spores (to remove variations in volumes and concentrations within irradiation chamber) as a function of gamma radiation dose. To illustrate more clearly some of the landmarks in this figure they have been color coded. The red box denotes the radiation dose region often used by DoD laboratories. The yellow box denotes the limit of detection of the sampling procedures. The red-dot at the top of the ordinate (y-axis) denotes the number of spores

¹⁷ Bowen, Jane E., *et al.* "Inactivation of *Bacillus anthracis* Using a Cobalt Source." *Salisbury Medical Bulletin* (Special Supplement), NO. 87 (1989), 70-72.

¹⁸ Watson, S. and Patience, B. "Irradiation of *Bacillus* Spore Inactivation Methods Affect Detection Assays." *Salisbury Medical Bulletin* (Special Supplement), NO. 87 (1989), 103.

¹⁹ RIID-1994, *Bacillus anthracis* Kill-Curves Established at U.S. Army Medical Research Institute of Infectious Diseases, 1994.

²⁰ RIID-2003, *Bacillus anthracis* Kill-Curves Established at U.S. Army Medical Research Institute of Infectious Diseases, 2003.

²¹ Mega Rad (MRad) and kilo Grays (kGy) are units of absorbed radiation. Colony forming unit (cfu) is used to estimate the number of viable microorganism in a given sample volume.

present in the 1667 lot irradiated at DPG and that was found to contain live spores, after shipping (this is the inactivated BA lot that started the CDC investigation). The blue-hashed circle surrounding the red dot outlines the spore count range irradiated in all DoD laboratories. All other depicted data points, in the gray box, are actual experimental data collected to establish kill-curves. As evident from the figure, it can be seen that: 1) the DoD routinely operates outside validated experimental data for kill curves 2) starting with larger number of spores requires a significantly higher total radiation dose for complete inactivation (*i.e.*, the majority of spores are killed by a small radiation dose but remaining survivors require very high doses for inactivation), 3) the signal-to-noise ratio for low spore counts (for up to 10% sampling) cannot guarantee verification for complete inactivation of the spores, and 4) the results are non-linear.

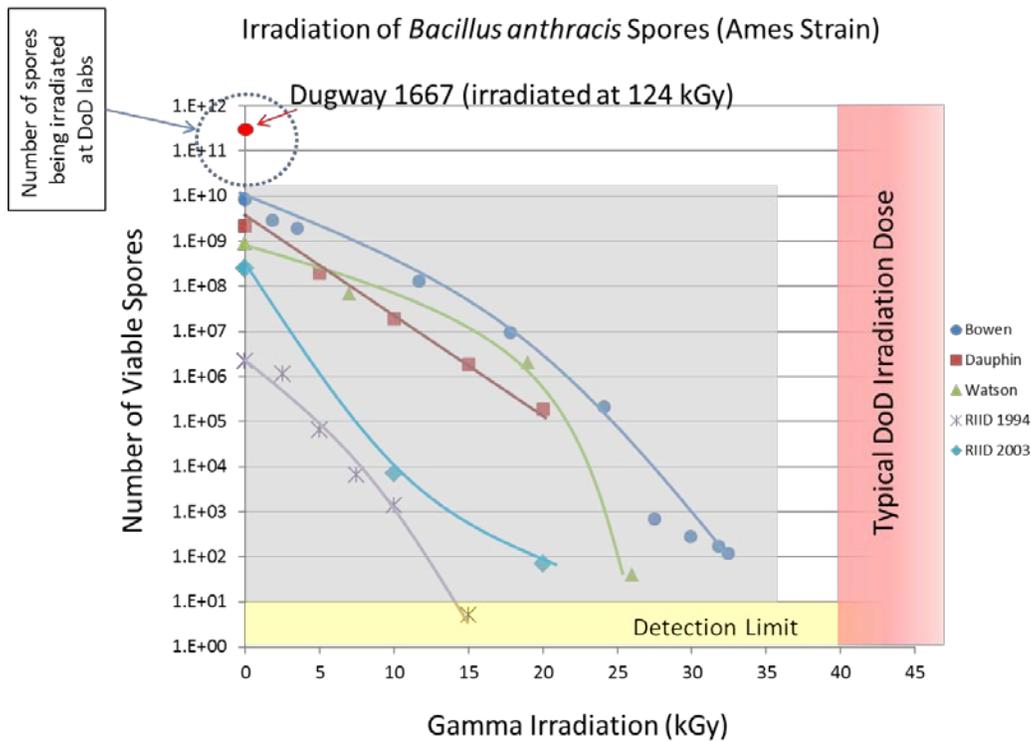


Figure 2: Consolidated kill curve data from multiple sources.

Coupled with published observations that injured spores are capable of germinating and repairing damage, it can be concluded that the end-to-end inactivation process for BA has not been adequately characterized and validated by the scientific community.

2. Why post inactivation viability testing did not detect the presence of live BA:

There are a variety of factors that contribute to the inability of post inactivation viability testing to detect live BA spores. According to the DoD laboratories, gamma irradiation with an absorbed dose of 40 kGy is expected to result in an 8 to 10 log reduction in viable spores in the suspension. If the concentration of spores is 10^9 / ml, an 8 log reduction would result in 10

viable spores/ml. One possibility is that sampling 5% of an irradiated batch may randomly result in no viable spores extracted for viability testing. An alternative explanation is that radiation damaged spores may exhibit a significantly slower growth rate relative to un-injured spores. There is information in the literature indicating that injured, but not inactivated spores of *Bacillus* species as well as other spore forming bacteria have delayed germination times. When these injured bacteria are able to germinate, they will repair the injury. The small sample sizes taken for viability testing and short incubation periods (based on viable spores not injured), may have resulted in a viability testing protocol that is prone to error.

In a preparation of spores that had not been highly purified, cellular debris and inactivated spores could serve as a source of nutrients for damaged, but not inactivated spores in the population. These damaged spores could, given time, begin to germinate, repair damage, and develop into live cells in the preparation. This hypothesis needs to be carefully tested, but results from the CDC on one tube of lot AGD0001667 that was held at ambient temperature over a weekend in a warehouse lends some credence to this hypothesis. This sample, when tested, showed growth that was deemed “too numerous to count”, whereas other tubes from the same lot that were either frozen or refrigerated showed counts of 200-300 cfu/ml (130 cfu/ml, when retested at DPG) , suggesting that allowing the sample to rest at ambient temperature promotes recovery of the injured spores. These types of experiments have not been reported in the literature and therefore represent a significant knowledge gap in DoD laboratories’ understanding of the optimal conditions for viability testing. It is recommended that a series of experiments be performed to ensure that all DoD laboratories working with BA use conditions that allow for the capture and growth of any injured spore.

Knowledge gaps regarding BA spore recovery mechanisms post irradiation along with insufficient sampling are thought to be major factors contributing to the failure of the viability testing.

Why cross-contamination of sterile inactivated spores cannot be ruled out:

After irradiation (see Appendix A, DPG discussion), the spores were returned to the same room in which they were produced and in which positive control samples were handled. Common equipment was used for handling both viable and nonviable materials. These practices create openings for contaminating sterile irradiated spores with viable spores. In addition, because all select agents are handled in the same room, any sterile product is susceptible to contamination with any other organism in the program. The only significant contamination control practice was the use of separate biological safety cabinets (BSC) for work with viable and nonviable agent. However, BSCs do not offer full containment, if used improperly, and the cross-contamination potential is similar to the open bench when using common pipettes for sterile and non-sterile products. Ideally, viability tests should be conducted in facilities separate from production and positive

control areas. If space is inadequate, engineering controls should be adopted to prevent cross-contamination.

3. Existing DoD laboratory biological safety protocols and procedures:

The committee identified existing DoD laboratory safety protocols and procedures in each location. However, these procedures are not standardized amongst the laboratories. The committee observed some deviations from protocols related to the inactivation process existed between researchers, and these deviations did not appear to undergo a peer review process. These procedures should be standardized within a reasonable timeframe for similar operations.

4. DoD laboratory adherence to established procedures and protocols:

In most cases, the committee observed that DoD laboratories followed their own established procedures and protocols. Some procedures lacked technical rigor and documented pedigree in some laboratories. The consequence of potential procedural deviations is not always apparent to the researcher.

5. Identification of systemic problems and what steps should be taken to fix those problems:

The primary systemic issue responsible for failures in the preparation of inactivated BA spores is the lack of specific validated standards to guide the development of protocols, processes, and quality assurance measures. Standards for irradiation sterilization (not specific to BA) as well as additional relevant standards exist, as delineated below. Ensuring DoD laboratories adopt some of these guidelines will significantly address many of the existing quality assurance (QA) and quality control (QC) deficits:

- ISO Guide 34:2009 (E) General requirements for the competence of reference material producers
- ISO 17025:2005 General requirements for the competence of testing and calibration laboratories
- ANSI/AAMI/ISO 11137-1:2006/(R)2010 Sterilization of health care products— Radiation—Part 1: Requirements for development, validation, and routine control of a sterilization process for medical devices
- ANSI/AAMI/ISO 11137-2:2013 Sterilization of health care products— Radiation—Part 2: Establishing the sterilization dose
- ANSI/AAMI/ISO 11137-3:2006/(R) 2010 Sterilization of health care products— Radiation—Part 3: Guidance on dosimetric aspects
- ANSI/AAMI/ISO 11737-2: 2009/(R)2014 Sterilization of medical devices – Microbiological methods – Part 2: Tests of viability performed in the definition, validation and maintenance of a sterilization process

- United States Pharmacopeia 71 Sterility Tests

All laboratories relied more on historical practices than validating the processes used. This resulted in development of independent protocols with fundamental differences, as well as potential weaknesses. BA is an agent with high radiation resistance requiring rigorous validation through all protocols and processes. Standards cited above, specifically the ANSI/AAMI/ISO 11137 series prescribe how to validate the irradiation process and develop a sterility assurance level for each product type treated. Although these standards were developed for medical devices, many elements of them are applicable and should serve as a roadmap for DoD laboratories.

The committee acknowledges the finite probability of some small number of spores surviving an irradiation treatment. These numbers may be so low that the demonstration of sterility can only be proven by sampling the entire lot (which is clearly not an acceptable solution). However, proper validation of the processes can minimize the inherent risks. Rigorous testing and performance monitoring on a routine basis, including all equipment used, will ensure best practices are consistently used. This includes incorporation of preventive maintenance on all equipment and performing calibrations on a routine basis. The general standard for certification frequency is yearly, with more frequent certifications required if local conditions dictate a greater need (extremely high use, aged equipment, etc.).

Another potential systemic source of error is that the irradiation process has too many unknowns. Issues such as volume, temperature and titer (concentration in term of colony forming unit per milliliter or cfu/ml) of spores subjected to irradiation vary between runs. Titers ranged from 10^6 cfu/ml to 10^{10} cfu/ml, without an understanding of how factors such as concentration will affect the process. Use of a common radiation dose without demonstration of its effectiveness across this 10,000-fold range provides opportunity for error.

Viability testing also has systemic issues. The sample size (5-10%), taken for testing post irradiation, may be too low to allow for detection of low numbers of viable or injured spores. In addition, the specific culture media used has to enable the growth of stressed spores. Two standards cited above, ANSI/AAMI/ISO 11737 and the United States Pharmacopeia title 71 Sterility Test, provide specific guidance on conducting viability testing on sterilized medical devices, parenteral drugs, and other products and materials. In addition, CFR 21 Food and Drug Administration require the viability test method be validated, and culture media used subjected to growth promotion testing prior to use. Although these documents are not specific to BA, they can serve as a starting point for DoD laboratories and can be tailored to meet the specific requirements.

Recommendations

The body of this report contains detailed recommended corrective actions for implementation to be considered by the Army, in collaboration with the Navy. In broad terms, the committee's recommendations can be distilled into three categories: enhancement of quality assurance, a more extensive scientific peer review process, and improvements in program management for inactivation and viability testing of BA.

Because of significant differences in irradiation procedures and viability testing for inactivated BA samples, it is possible that this level of inconsistency exists in other high hazard operations. The DoD should review all select agent procedures at the DoD laboratories. The committee also found variance in each of the chains of command between the laboratories. This resulted in inconsistent promulgation of safety best practices. It is prudent that the Army, Navy and Office of the Secretary of Defense review the chain of command and pursue consistent safety practices.

The Chemical, Biological Defense Programs (CBDP) should ensure sufficient funding is available for research related to the development of standardized irradiation and viability testing protocols. The Office of Secretary of Defense (OSD) should oversee Army and Navy adherence to the implementation plan and timelines.

Prior to implementing the committee's other recommendations, DoD should continue its moratorium on the production and shipment of inactivated BA, except as required for development of standardized irradiation and viability testing protocols. Production of inactivated BA spores should resume only after implementation of peer reviewed and validated procedures for irradiation and viability testing.

The committee appreciates differences in operations between research and development facilities and production facilities. Although research facilities should be provided flexibility to explore the boundaries of science, the production facilities must operate in a repeatable and reliable fashion to ensure quality products. For some common operations such as inactivation and viability testing of BA, common procedures must be utilized. The committee's findings are divided into three categories and for each category, detailed recommendations are provided.

- A) **Quality Assurance:** Enhance quality control programs at DoD laboratories working with hazardous select agents and other pathogens. Establish consistent expectations regarding equipment maintenance, calibration, record keeping, experimental controls, environmental monitoring, and sample preparation. Conduct root cause analyses for any procedures that result in anomalous outcomes. Procedures must stay within the validated experimental design parameters. Provide accurate descriptions of sample contents and storage and usage instructions for samples shipped to outside institutions.
 - a. **Standardize BA Inactivation Protocols Across Laboratories:** All DoD laboratories should follow a common Standard Operating Procedure (SOP) for such practices as irradiation and viability testing. This should be defined by the

highest standard necessary for any one of the laboratories using the most complex sample.

- i. Institute requirements to destroy any sample that deviates significantly (*e.g.*, 10, 20, 30, 50% etc.) from standard irradiation conditions. If the sample fails the viability test, it should be logged, any anomalies of the sample recorded, and the sample should be destroyed. A root-cause analysis should be initiated to help understand what factors contributed to the failure.
 - ii. Empirically identify optimal media and additives for recovery of injured BA spores. Determine whether spores injured by gamma radiation can recover (*i.e.*, repair damage) in the formulation in which it is shipped as well as the optimal temperature for recovery, and whether or not a shock is required to induce germination (*e.g.*, heat, freeze-thaw cycle). When performing positive growth controls on media, inoculate with low spore counts (10 to 100 cfu) to simulate low level spore survival in irradiated samples.
 - iii. Adopt rigorous procedures to obviate cross-contamination of sterile product with viable agent.
- b. **Institute More Rigorous Quality Procedures:** Quality assurance (QA) and quality control (QC) procedures for inactivation and viability testing of BA spores were lacking across all DoD laboratories. Information on what should be routine, such as noting how often a sample failed viability testing, was often absent within the laboratories. The lack of a mature QA/QC program, and corresponding incomplete documentation, makes determining the root cause of the problem impractical.
- c. **Clarify The Conditions Of The Material Transfer Agreement (MTA):** The MTA for inactivated BA spore preparation should clearly prescribe the risks associated with use of the product, such as indicating that the sample passed viability testing at the limits of detection. Further qualifications within the MTA could include sample storage, number of freeze-thaw cycles recommended and requirements in the event the CRP needs to recall the lot. The samples should have a declared shelf-life or use-by date. At a minimum, the MTA should be signed prior to shipment and be required for each lot.
- d. **Enhance Preventive Maintenance:** Reusable mechanical equipment used throughout the process should be routinely maintained and calibrated, from mechanical pipettes to the irradiator. Given the complete dependence of the processes on key instruments, it was surprising that no systematic preventive maintenance is performed. For example, ensuring the irradiator and dosimeter are performing to specification is vital to operations. Both these instruments are calibrated to National Institute of Standards and Technology (NIST) specifications at installation, indicating the importance of the calibration process. Even minor issues such as changes in humidity can affect these instruments.

Performing yearly certifications by qualified professionals should be mandated for everything from pipettes to the dosimeter.

- e. **Establish And Manage An Environmental Surface Sampling Program:** Some laboratories lack written procedures to document, investigate, and report contamination found outside primary containment areas during environmental persistent agent sampling. The procedures, at a minimum, should include notification protocols, including notification of the Safety Manager of the sample locations, results and follow-up actions taken.
 - f. **Establish Validated Dose Curves:** Radiation dose curves should be generated for each BA strain used for production, at the same concentrations used in production and performed on the same irradiator as used for inactivation of spore preparations. In order to accurately assess the correct amount of radiation to kill BA spores effectively at various concentrations, radiation kill curves should be generated for the specific BA spore strain being produced.
 - g. **Understand The End Users' Needs:** The Chief Science Officer of the lab, Army or Command, should work with individual customers to understand sample requirements and define the appropriate strains and material needed to support the objectives. For example, avirulent or attenuated strains should be used where applicable to meet the intended use and goal. Many of the intended uses of these materials can be easily fulfilled by such a substitution. In some cases there might still be a need to inactivate virulent strains to fulfil a legitimate use.
 - h. **Quantitate Spore Before Irradiation:** Spore preparations intended for irradiation should be quantified as precisely as possible to maximize the likelihood of achieving the inactivation levels predicted by kill curves produced with the same strain in the same irradiator. Spore preparations can be accurately enumerated only if they contain negligible numbers of viable vegetative cells and do not exhibit significant clumping. Therefore, preparations should be examined microscopically prior to spore enumeration to determine if they meet predetermined standards for the content of phase-bright and phase-dark spores, vegetative cells, and debris. If preparations do meet standards, clumping should be reduced by the use of surfactants and disruptive techniques (*e.g.*, sonication) to maximize the accuracy of counts.
- B) **Peer Review:** Establish BA spore inactivation and viability testing protocols that are based on relevant scientific data, standards, and studies conducted to fill knowledge gaps. All protocols and subsequent protocol modifications should be subject to a peer review process, validated and implemented uniformly across similar operations. Ensure internal and external audits have a scientific component to assess the validity of proposed procedures and assure a science based foundation for proposed methodologies, changes and deviations. Improve collaboration between DoD laboratories and with the broader BA research communities to promote information sharing, peer review, and promulgation of best practices and lessons learned.

- a. **Periodic Scientific Reviews Of Protocols And Procedures:** The rate of advancement in science is accelerating and often these advancements will have material impact on existing protocols and procedures. To ensure that the DoD laboratories are taking advantage of the most up to date knowledge, external Subject Matter Experts (SMEs) should be engaged periodically to review current best practices and determine if new information requires updating of protocols and procedures.
- b. **Assemble A Group of (Internal Or External) Irradiation SMEs:** To identify and determine the studies that needs to be done to better understand the effects and limitations of irradiation for inactivation of BA spores. This should include an investigation of the following variables: irradiator model variations; dose of radiation required to inactivate BA spores; inter-strain susceptibility to radiation exposure; concentration of spores and dispersion (evenly dispersed versus clumped etc.) when exposed to radiation; and broth types for spore suspensions.
- c. **Assemble A Group Of (Internal Or External) Viability SMEs:** To determine the best approach for implementation of viability tests for inactivated BA spores. This should include: when to subject the irradiated material for viability testing; what type of broth should be used to support and monitor recovery of damage spores; what temperature should be used for incubation of these samples and for how long; how much of the irradiated material should be subjected to viability tests; what type of agar plates should be used for growth monitoring; what temperature should be used for incubation of plates and for how long; what positive controls and negative controls should be implemented to support viability testing; and remedial action notification processes for viability test failures.
- d. **Audits And Inspections:** DoD laboratories that utilize select agents should be inspected by audit teams comprised of competent microbiologists, scientists, and credentialed subject matter safety experts. Inspections should be meaningful with a focus on technically reviewing the processes and procedures for handling select agents. These audit teams should be led by the major command surety program managers and comprised of subject matter experts with operational experience in select agents and toxins.
- e. **Assure Material Integrity:** The materials subjected to irradiation must maintain their integrity for the specific intended use and application. Overexposure of materials to radiation may compromise the sample integrity and reduce its potency to support the specific intended use.
- f. **Provide Independent Viability Verification:** Employ an independent laboratory to verify a sample is inactivated prior to shipment. Implement a process for performing a secondary viability test prior to shipping or distributing materials from the production laboratories to customers. This independent confirmation will reduce anomalies or limitations associated with the production or viability testing process.

- C) **Program Management:** Program managers should provide adequate laboratory space, equipment and time to conduct relevant safety and surety research for select agents and other pathogens. Program managers should develop a plan to track and document the implementation and long-term sustainability of the recommended corrective actions identified by this review committee as well as all internal and external audits.
- a. **Prioritize R&D For Those Pathogens Where Information is Lacking:** A problem that was endemic to all DoD laboratories visited was the failure to recognize the importance of knowledge gaps. Unknown variables throughout the irradiation and viability processes make a root cause analysis problematic. Performing key experiments that can define effects of variables such as; spore concentration, irradiation temperature, type of media, sampling size for validity testing should be researched to define more definitively appropriate parameters for the protocols needed.
 - b. **Minimize Cross-Contamination:** Ensure laboratory space associated with production of inactivated spores is separate from laboratory space that performs viability tests in order to avoid cross-contamination. Live materials and inactivated materials should be handled in separate laboratory space or in space with sufficient engineering controls and practices to obviate cross-contamination.
 - c. **Revise Death Certificates:** Instead of using "Kill or Death Certificate", rename the certification of inactivation to "Viability Test Results" with information about the sampling approach and the length of incubation period. An express statement of the detection limit of the viability testing protocol should be included, as well as a statement that it is the responsibility of the receiving laboratory to ensure that the work conducted using this material is executed in a safe and contained manner minimizing any potential risk to their laboratory scientists. Additionally, if any growth is observed by the receiving laboratory, it is the responsibility of the receiving party to notify the sender and other appropriate parties immediately, and secure the sample.
 - d. **Track Inactivated Select Agents With The Same Procedure Used For Live Select Agents:** This electronic tracking mechanism will enable a better sample custody in future for potential recalls if necessary.
 - e. **Establish An Oversight Group To Insure Implementation Of Corrective Actions:** For the corrective actions to be effective and persistent, an internal group should actively engage in tracking standards, processes and procedures throughout the laboratories.

Appendix A: Laboratory Visits and Discussions

ECBC Visit

The review Committee visited ECBC on June 8, 2015. During this visit, the committee chair provided an overview of the live BA shipment incident and the purpose of the visit and openly discussed aspects of the work done at the laboratory with committee members. Further, the committee received a demonstration on how samples are loaded in the ECBC cobalt-60 irradiator. During the demonstration, dose curves (mapping of the exposure rate within the chamber) were shown with an emphasis placed on the relevance and importance of location of the sample. The following sections provide a synopsis of what was discussed during the tour and visit.

Preparations of Inactivated BA Spores:

ECBC produced three batches of inactivated BA spores. Two preparations of spores, produced in 2006 and 2007, passed viability testing. ECBC indicated that these two batches were created for the National Bioforensic Analysis Center (NBFAC), to support an electron microscopy research study. The remaining spore preparation, which was made in 2014, was destroyed following viability testing that demonstrated viable spores were present. This batch differed from the others in that the spores were centrifuged prior to irradiation with the supernatant (excess liquid on top of sample pellet) removed. The resulting wet pellet was then irradiated (as opposed to spores held in suspension). The assumed inactivated sample was successfully identified as still viable by the viability testing and it was destroyed.

Irradiation Methodology:

ECBC uses a Cobalt-60 gamma irradiator for inactivation. The primary function of the irradiator is to treat unknown samples prior to analysis for chemicals; it is not used for routine production of inactivated spores. The inactivation gamma radiation dose was determined from work published by the United Kingdom Ministry of Defense Chemical Defense Establishment as well as kill curve experiments conducted at ECBC using *Bacillus globigii*, an organism that is claimed to be more difficult to kill with radiation than BA. Both studies showed that a complete kill was achieved with a dose of 40 kilo Grays (kGy), ECBC made it policy to use a dose that is 30% higher, or 54 kGy. This decision was made to increase confidence in the treatment of the unknown samples prior to chemical testing.

To conduct the procedure, the radiation safety officer (RSO) calculates the position of the container of spores to be irradiated in the chamber, as well as the time of exposure to achieve the necessary dose, factoring in the age of the radiation source (Cobalt-60). The particular model of irradiator used contains a turntable to rotate materials undergoing irradiation in front of three gamma radiation sources; however, validation of the dose received is not performed. ECBC scientists observed cellular debris and clumping of the spore suspensions after irradiation was

performed. ECBC does not maintain control charts to identify the frequency of off-normal operations by their irradiation instruments.

Viability Testing:

ECBC takes 10% of the irradiated lot of spore material for viability testing. This sample size was determined by a statistician factoring in infectious doses, including consideration of organisms that have a low infectious dose. According to ECBC, to obtain a range of 6-7 log reduction (*e.g.*, 1 log reduction is 90% killed, 2 log is 99%, 3 log is 99.9%, etc.), this sample size is considered statistically relevant. Viability testing is conducted as soon as possible after irradiation, which is not always within the same workday, but is always within 24 hours. The microbial culture medium used for the test is Trypticase Soy Agar (TSA), a solid growth medium used in petri dishes. The viability tests were incubated for five days at 37°C. The viability test procedure does not utilize methods to recover stressed organisms such as culturing through broth media followed by plating on solid media, or richer media containing higher levels of nutrients.

Additional Comments:

The personnel at ECBC were highly cooperative and willing to answer questions from the committee. The Standard Operating Procedures (SOP) addressed safety, and incorporated thorough risk assessments. The committee noted that ECBC used statistical analysis to determine the size of sample (10%) to be taken for viability testing. However, the assumptions for this analysis focused on infectious dose, and possibly did not take into account stressed spores or cells. Additionally, 10% sampling provides 95% confidence, which may be statistically relevant, but insufficient for verification of complete inactivation. Direct plating was performed for viability testing, whereas enrichment through broth culture followed by plating may have been more optimal. The incubation time of five days also presents an opportunity for improvement. In the pharmaceutical industry, a standard practice is to incubate viability test broths and plates for 14 days. In addition, growth promotion testing of the culture media prior to use in (or in parallel with) viability testing is required to ensure that growth of small numbers of viable organisms are detected. Use of positive and negative controls for the viability test is another opportunity for improvement.

In discussions, the committee pointed out that kill curves, in different volumes/concentrations and various suspension matrices, should be performed with all strains of BA that could be subject to inactivation by irradiation. It has been observed that different strains have varying resistance to radiation, and as part of process validation these experiments need to be performed.⁷ It was also pointed out that germination curves for injured spores show a longer time to germinate compared to non-injured spores. In an irradiated spore preparation, low numbers of surviving spores could use residual cellular debris and broken spore debris as a nutrition source. Continuing discussions on nutrients revealed that Trypticase Soy Agar is not a rich medium as

compared to Sheep Blood Agar or Brain Heart Infusion Agar and it may not provide the optimum growth conditions.

When provided opportunity to comment on improvements, ECBC identified three areas: perform independent verification of radiation dose by using alanine test strips, utilize broth enrichment as a component of viability testing, and lastly, incorporate a dwell (rest) time post irradiation before performing viability testing. Research papers, using different irradiation techniques, have shown that irradiated spores that are injured but not killed may recover and become viable after a period of rest.^{3,4,15}

USAMRIID Visit

The review committee visited USAMRIID and NMRC on June 9, 2015. Due to the co-location of these two facilities, it was more efficient to meet with both teams at the same time. The committee chair provided an overview of the live BA shipment incident and the purpose of the visit. The committee members were allowed to freely discuss various aspects of the work done at the laboratory. The following sections provide a synopsis of what was discussed during the committee's visit with USAMRIID staff.

Preparations of Inactivated BA Spores:

The original number of inactivated batches reported by USAMRIID was overestimated due to a misunderstanding of the request for information. USAMRIID provided a count of any preparation containing BA, including both vegetative cells and spores. As a research institute, USAMRIID does not routinely irradiate BA spores. The irradiated BA spore preparations were used to create antibodies or to support activities in the Diagnostic Systems Division. The spore preparations used to produce antibodies were sent to an outside vendor to inject into rabbits (for antibody production). None of the rabbits injected with these BA spore preparations became ill or died from infection (thus illustrating that if any live spores remained, they were present at a concentration below infectivity to the rabbit). The current estimate of inactivated BA spore preparations produced at USAMRIID is about 26 (<10 samples since 2009). During the discussion with the technical staff, there were inconsistent answers about how often irradiated samples test positive. One researcher used a generic answer of 2-3% (no documentation was provided); others pointed out that at USAMRIID some samples are purposefully subjected to a sub-lethal dose for research purposes and are expected to fail the viability tests. In general, USAMRIID uses exceptionally purified spores with minimal contaminants (typically >99% pure).

Irradiation Methodology:

USAMRIID conducted kill curve studies on Ames and Colorado strains of BA, more than a decade ago. The study subjected samples to gamma radiation doses up to 40 kGy with the results indicating spore inactivation at 20 kGy. For inactivation purposes, the decision was made to use 40 kGy as the dose for spores. Earlier in this report we showed that the current operational procedures used by USAMRIID (and all other DoD laboratories) are outside of the historical experimental kill curves produced at USAMRIID and captured in published research.^{7,17-20}

To conduct the procedure, the radiation safety officer (RSO) calculates the position of the container of spores to be irradiated in the chamber, as well as the time of exposure to achieve the necessary dose, factoring in the age of the radiation source (Cobalt-60). USAMRIID routinely freezes their samples and maintains this state by employing an ice/dry-ice bath in which the samples sit throughout the irradiation. Independent verification of the dose received is not

performed. USAMRIID does not maintain control charts to identify the frequency of off-normal operations by their irradiation instruments.

Viability Testing:

Inactivated spores are prepared in small batches (up to 15 ml) based on experimental needs. Post irradiation, 10% of the batch is plated on Sheep Blood Agar (SBA). A 48-hour incubation time was chosen due to BA being a fast growing organism. Broth cultures are used if greater volumes than the small batch are prepared.

USAMRIID described a small batch as 3-4 ml of inactivated spores per rabbit for antibody production. Up to 15 ml is produced to support testing on up to 5 rabbits per test. Spore titer (concentration) for these small batches is determined by direct plate count, most of the time on SBA. This is the same lot of SBA used for titer determination during viability testing.

USAMRIID conducts viability testing as soon as possible post irradiation, within 24 hours. It is noteworthy that the researcher doing the spore preparation work for antibody production chose to conduct viability testing by taking 50% of the preparation as the sample.

Thorough contamination mitigation is implemented prior to conducting viability testing. The biological safety cabinet (BSC) is decontaminated with 10% stabilized bleach, or Hype-Wipe® disinfecting towels with bleach, or freshly prepared bleach. As part of this contamination mitigation, positive controls, media checks and other steps where viable BA is grown are conducted in areas that are physically separated from where viability testing is conducted.

Additional Comments:

USAMRIID viability testing on irradiated spore preparation demonstrated an estimated failure rate of <3% (*i.e.*, >97% of samples showed complete gamma inactivation). The preparations showing growth during viability testing were associated with the transition to a new irradiator. When a committee member asked for the result of a root cause analysis, it was stated that no root cause analysis was performed. During subsequent discussions, it appears that a potential source of irradiation failure was identified by USAMRIID personnel. The new irradiator used a turntable to expose the material to the cobalt source. The RSO stated that JL Shepard, the manufacturer of this irradiator (484R), has a problem with the turntable stopping during the irradiation run. There is no real-time mechanism to identify when during the irradiation cycle the turntable stops functioning. In one instance, the RSO advised the researcher of a turntable failure (hence the sample was not uniformly irradiated), and when viability testing was performed, growth was observed. The spore preparation was subjected to repeat irradiation in an Atomic Energy of Canada Ltd Gammacell 220 irradiator that drops the material into a chamber surrounded by radiation sources. Viability testing showed no growth after this treatment. The two other preparations showing growth during viability testing were also associated with the transition; improper placement of sample in the chamber of the model 484R was suggested as the cause.

In an open discussion, the committee discussed various aspects of spores under pH stress, germination time and the influence of heat stressing. The U.S. Department of Agriculture has evaluated this phenomenon and recommends extending incubation times when testing for viability. The CDC DSAT is now requiring all second checks for viability to be conducted in enriched media for 14 days (based on June 5, 2015 updated guidance for testing inactivated BA spores).

USAMRIID staff recommended post irradiation microscopic evaluation of spore preparations, and it was pointed out that spore preparations from Dugway Proving Ground are “rough preps”, with cellular and spore debris present. It was hypothesized (in the absence of experimental data) that this debris can degrade over time to possibly form a nutritional supplement that would support germination and growth. In a follow up note to the committee chair, USAMRIID officials elaborated that:

“Recent interagency discussions has identified that clumping noted in Dugway spore preparations may be a significant contributing factor to both failed inactivation and delayed growth during viability testing. Samples held at room temperature have shown a 3 log increase in colony counts and we know that clumps dissociate over time releasing spores from their center into solution. Micrographs have also shown very large spore clusters mixed with debris in these preparations. This could result in an apparent increase in viable spores when it is actually an increase in availability of previously existing spores that were trapped within a clump.”

Concern was also expressed regarding low titers (concentration) of viable spores in an irradiated preparation, and the probability of their detection through sampling.

In response to a question from a committee member as to the feasibility of using an attenuated BA strain rather than inactivated BA spores, USAMRIID identified a number of possible alternatives. During discussions with USAMRIID’s staff, it became clear that recipients of inactivated spores should be informed that there is no guarantee of zero germinating spores in the preparation.

NMRC Visit

The review committee visited NMRC on June 9, 2015. Due to the co-location of NMRC with USAMRIID, it was more efficient to meet with both teams at the same time. The committee chair provided an overview of the live BA shipment incident and the purpose of the visit. The committee members were allowed to freely discuss various aspects of the work done at the laboratory. It is important to note that NMRC does not have current funding to pursue BA research activities and accordingly, they are responsible for verification of legacy samples prepared as early as 2005. In its current location, NMRC has BSL-3 laboratories at USAMRIID and operate under USAMRIID's Select Agent Registration. NMRC adheres to USAMRIID's BSL-3 standard operating procedures. The following sections provide a synopsis of what was discussed during the committee's visit with NMRC staff.

Preparations of Inactivated BA Spores:

The initial number of inactivated BA spore preparations reported by NMRC represented double counting; the actual number of preparations is less than 40. These preparations were used to develop antibodies at both an outside vendor as well as the USAMRIID Large Animal Research Facility, and to conduct testing of immunological assays under development. As part of Defense Base Realignment and Closure (BRAC), all NMRC BA work was moved to USAMRIID in 2011. There is a memorandum of agreement in place and NMRC works under USAMRIID's operational and safety protocols.

Irradiation Methodology:

Spore preparations under the NMRC protocol used the JL Shepherd & Associates Co-60 Model 109 Irradiator, which surrounds the material being treated with source rods. The dosage used by NMRC was a minimum of 32.5 kGy. The NMRC protocol used multiple runs at lower doses but achieved a final absorbed dose of 40-50 kGy. The spore suspensions were frozen solid for each irradiation run. Up to 400 ml spore suspensions were irradiated. NMRC currently plans to use the facility at USAMRIID for their sample irradiation. In their current location, USAMRIID does not maintain instrument quality control and performance charts. In their previous location, NMRC did not maintain control charts to identify the frequency of off-normal operations by their instruments. At the time, the irradiator used by NMRC was under the control of Walter Reed Army Medical Center (WRAMC). Any records relating to this irradiator would have been held by WRAMC rather than NMRC.

Viability Testing:

The NMRC protocol for viability testing had two different test routines detailed, one for gross contamination and another for low levels of contamination, with sample sizes defined based on the amount of material to be tested. In tests for gross contamination, the maximum sample to be taken was defined as 1-5%. Inoculation was on plate media, chosen from a list of media in the

protocol (the protocol states not to use Tryptic Soy Agar-TSA). A noteworthy element of the protocol is that a media growth promotion test is conducted with the viability test. In tests for low levels of contamination, a 10% sample of the material is inoculated into broth and incubated followed by plating. The brain heart infusion broth used for BA is supplemented with 10% serum to create a rich medium.

Additional Comments:

When NMRC produced inactivated BA spores, the documentation of viability testing provided to the recipient did not state that the preparation was sterile, but that viable spores were below the limit of detection. Viability tests had to be read by two people who would sign off on the documentation of the procedure.

NMRC routinely used multiple radiation doses to preserve antigen structure. Up to a week between runs could occur, in part due to scheduling time in the irradiator. Similar to USAMRIID's observation, when inactivated BA spores were sent for antibody production there was no report of inoculated animals becoming ill or dying.

DPG Visit

The review committee visited DPG on June 17, 2015. The committee chair provided an overview of the live BA shipment incident and the purpose of the visit. During the visit, the committee noted that the staff was very concerned about the recent events. The committee members were allowed to freely discuss various aspects of the work done at the laboratory. The Biological Safety Officer for the Life Sciences Division (LSD) at DPG opened the meeting with a presentation on Post safety and the agenda of the laboratory visit.

The committee visited Building 2032 to observe the two gamma cell irradiators utilized by LSD. Irradiator 484R has been in operation since 2012. This irradiator has 3 cobalt source rods in the rear of chamber and a rotating turntable. Samples are irradiated at chamber temperature without ice (DPG's in-house research has shown no significant changes in sample temperature during irradiation process). Alanine stripes are placed on opposite sides of the vial holding the sample (primary container). Alanine forms a very stable free radical when subjected to gamma radiation. These free radicals yield electron paramagnetic resonance signals that are dose dependent, thus allowing an accurate measurement of the radiation dose. Irradiation failures are communicated electronically to laboratory personnel (usually the principal investigator). However, control charting or monitoring of these failures is not performed.

Next, the committee visited Baker Laboratory which now houses a state of the art biological aerosol test chamber. Lastly, the committee visited the Life Sciences Test Facility (LSTF) in Building 2029. LSTF houses the Critical Reagents Program (CRP) Antigen Repository. As of this reporting, all positive BA spore lots were produced on behalf of the CRP. The CRP Antigen Repository consists of a sample packaging and shipping lab, -70°C sample storage freezers, two BSL-2 laboratories for production and characterization of non-Select Agent material, and a BSL-3 sample preparation and viability testing lab. The following sections provide a synopsis of what was discussed during the committee's tour and visit.

Preparations of Inactivated BA Spores:

DPG produces inactivated biological agents in support of the DoD Critical Reagents Program. These materials are provided to customers to support research and development, quality assurance, and test and evaluation activities. When the committee asked "how often do irradiated samples fail the viability test?" the answer was about 2-3%. DPG communicated that no root cause analysis was performed on these anomalies. At the end of our discussion, the committee members asked for documentation on failed viability tests of irradiated samples. The documentation provided shows that since 2012, there have been a total of 19 BA inactivations. From these, four samples failed the viability test (21% failure rate). About 10% were attributed to a failing turntable in the irradiator. Lack of supporting documents for many of the answers to critical questions during the committee's visit pointed to poor record keeping in a critical production laboratory.

Irradiation Methodology:

Two different irradiators have been used at DPG. The first, a Gammacell 220 was taken out of service in 2012, and a JL Shepherd & Associates Co-60 Model 484 with a two inch turntable was placed in service in the same year.

Irradiation dosage was based on literature and historical information. The dosage received during runs was determined by taping two alanine dosimetry strips, on opposite sides, to the outside of the tube containing the spore suspension. The tube(s) is then placed inside a polypropylene secondary container with an O-ring sealed lid. Upon loading, the turntable is turned on via a switch on the control panel, the door is closed and the irradiation process is initiated. The run is performed at ambient temperature, and logged in a notebook. When the run is over, the door is opened, and turntable operation is confirmed visually; however, this is not often documented. If any anomalies such as a turntable failure are observed, then the anomaly is communicated to the researcher. The alanine strips are subsequently read in a Bruker e-scan alanine dosimeter reader and any anomalies are recorded in the instrument log and reported to the principal investigator. The DPG technician observed about 10% of the irradiated samples had some form of anomaly.

The time of exposure is derived annually by a technician taping five test strip inside a glass beaker and running it through hourly intervals in the irradiator. At the end of each time point, a single test strip is removed and analyzed on the dosimeter. When all time points are completed, the doses are plotted against time. This plot is used to determine time of exposure to achieve dose. This plot is never compared to the expected dose from the Co-60 source(s) based on the known decay kinetics. This is a built-in control that should be used to verify the dosimeter calculations. The dosimeter is periodically checked, once in 2012, and again recently. When asked if the e-scan dosimeter requires routine calibration, the DPG technician responded that calibration was not required to his knowledge. It was not determined if the Bruker e-scan was calibrated and if it met the National Institute of Standards and Technology (NIST) traceability criteria upon initial installation.

Empirically, DPG observed that the difference in radiation exposure of the outside of the secondary container and the inside was 10-20 kGy which was consistent with the irradiator iso-dose curves. Consequently, DPG discontinued taping alanine strips to the secondary container and began using strips taped to opposite sides of the primary container.

Approximately 50 irradiation runs are performed a year including virus preparations for internal research use and certain culture media; it was estimated that approximately 500 irradiation procedures conducted to inactivate bacterial agents have been performed in the history of the Critical Reagents Program.

Viability Testing:

Irradiated samples are often tested immediately after irradiation, typically within 30 minutes. The protocol initially provided to the committee, WDL-BIO-147 was not the protocol used to prepare CRP inactivated antigens. A hard copy of the protocol CRPAR-WI-007 was provided to the committee during the meeting. This protocol calls for a 5% sample of the irradiated material to be inoculated into 2X nutrient broth (the concentration of nutrients are twice the normal), incubated at 34⁰ C for 48 hours. Two hundred microliters of inoculated broth is then plated across 10 TSA plates and the plates are incubated for a minimum of 48 hours. It was noted that for the pre-2012 irradiation samples, an average incubation time for the samples was 12.9 days and for the new instrument, the average was 13.3 days. Positive (undiluted) and negative controls are performed at the same time in different biological safety cabinets in the same room. DPG procures the culture media from an ISO accredited source, and does not perform growth promotion testing on new lots. Based on what the committee learned at other DoD laboratories, this procedure should be revisited to include best-in-class practices.

Additional Comments:

At the request of the committee, DPG reviewed past records and found that lots of inactivated BA spores that contained viable material were produced using both the decommissioned and currently active irradiator.

From the laboratory walk through the committee learned that with the exception of the irradiator facility, all other elements of the select agent production process occurs in the same BSL-3 laboratory. Agent propagation, sporulation, and viability testing procedures are performed in the same laboratory space that is shared with other inactivated biological agent work, without dedicated equipment (aside from two biological safety cabinets). The committee noted that this approach could introduce cross-contamination. Furthermore, key reagent transfer equipment such as mechanical pipets is also shared. Although it is unknown at this time if cross-contamination occurred with DPG samples, it cannot be ruled out. DPG acknowledges this issue and plans to have dedicated laboratory space for viability testing when a new laboratory building is commissioned.

A committee member noted the discrepancy on the death certificates for two lots of inactivated spores (AGD0001151 and 1667) where protocol WDL-BIO-147 Rev1 Ch1 is cited, when the actual protocol in use was CRPAR-WI-007 Ver 5. DPG acknowledged that they recently had this error pointed out and that it would be corrected.

DPG personnel stated that the typical irradiation time to achieve 40 kGy exposures is 240 minutes at the current decay of the Co-60 source in the model 484 irradiator. It was pointed out that lot AGD0001667 had a radiation dose of 124.02 kGy noted on the death certificate. Review of the records showed that the lot had been irradiated twice after growth occurred on the viability test after the first run. The time for the first run was noted as 220 minutes, and the second run

was 240 minutes. The alanine test strips showed a cumulative dose of 124.02 kGy which is what was reported. Upon showing no growth on the second viability test, the lot was approved for release March 18, 2014. No effort was made to investigate the anomaly of the dosimeter showing 124.02 kGy under exposure conditions that are expected to produce a total exposure around 80 kGy.

DPG personnel noted in the retesting experiments currently underway, multiple *Bacillus* species including *B. anthracis* strains that were inactivated and archived have come up positive (*i.e.*, viable spores are present in inactivated samples).

The review committee was unable to identify if there had been a similar event with non-CRP program samples. Typically, 100% of non-CRP batches are sent to customers, or used for internal R&D purposes, with nothing archived, thus no samples remain at DPG to retest. Archived samples (specifically data tied to variations in production methods) could help gain insight in achieving best laboratory practices when striving for complete inactivation results.

After the visit, the committee received some additional information from DPG regarding their spore preparation, reiterating that all laboratory equipment is surface decontaminated before and after each use to minimize cross-contamination.

Appendix B: Commonalities and Differences in Procedures

The following table outlines the variability in irradiation procedure used at DoD laboratories.

Comparison of gamma irradiation procedures for inactivation of *B. anthracis* spores

Laboratory	Irradiation						
	Dose (kGy)	Dose Determination	Starting Titer (cfu/ml)	Expected log Kill	Irradiator	Starting Volume (ml)	Temp Control
ECBC	54 ^(a)	Calculated using time/decay rate of isotope and calibration data from instrument install	Max: 10 ⁸ cfu/ml	>8	JL Shepard and Assoc. 484R-2 (Turntable)	Not defined	Controlled by irradiator
USAMRIID	40 ^(b)	Not performed ^(b)	10 ⁸ - 10 ¹⁰ ^(b)	10 ^(d)	JL Shepard and Assoc. 484R (Turntable) Gammacell 220 (Surround)	8-15	Cold ^(d) or frozen prior to irradiation ^(b)
NMRC	Min: 32.5 Typical: 40-50 ^(b)	Not stated in protocol	5.9x10 ² - 6.9x10 ⁹ with majority 5x10 ⁸ -5x10 ⁹ ^(b)	6-8	JL Shepard & Assoc. Model 109 (Surround) ^(b)	<= 400 ^(b)	Frozen prior to irradiation
Dugway	38-42	Alanine dosimeter test strips	10 ⁸ - 10 ¹¹	10	JL Shepard and Assoc. 484R-2 ^(c) (Turntable)	20-30 ^(c)	Not stated in protocol

- (a) Specified in 6/8/15 meeting at ECBC
- (b) Specified in 6/9/15 meeting at USAMRIID
- (c) Specified in 6/17/15 meeting at DPG
- (d) Information provided after site visit

The following table outlines the variability in viability testing used at DoD laboratories.

Comparison of viability testing procedures for gamma irradiated *B. anthracis* spores

Laboratory	Sterility Testing									
	Sampling	Culture Media		Incubation						
	Sterility Sampling %	Broth	Solid Media	Broth to Plate?	Direct Plating	Incubation Temp (°C)	Incubation Time (hrs)	Positive control?	Negative control?	
ECBC	10%	None ^(a)	Tryptic Soy Agar ^(a)	No	100 ul/plate	25-37 "appropriate growth temp"	72 ^(a)	No	No	
USAMRIID	10-50% ^(b)	None identified	Blood Agar ^(b)	None identified	None identified	37	48	No	Yes ^(b)	
NMRC	Small vol: 1-10 µL Large vol: 1-5%	Brain Heart Infusion Broth +10% serum	Blood Agar, Brain Heart Infusion Agar, Nutrient Agar, Mueller-Hinton agar	1 µl and 200µl are plated	Broth: >=72 Plates: 72	35-37	72	Yes	Yes	
Dugway	5%	25 ml 2X nutrient broth	Tryptic Soy Agar	200 µl/10 plates	No	34	96 – 336 ^(c)	Yes	Yes	

- (a) Specified in 6/8/15 meeting at ECBC
- (b) Specified in 6/9/15 meeting at USAMRIID
- (c) Specified in 6/17/15 meeting at DPG

Appendix C: Primary and Support Member of the Review Committee

*Committee Members Visited DoD Laboratories

<u>NAME</u>	<u>ORGANIZATION</u>
Dr. Vahid Majidi*, Committee Chair	OASD(NCB)/NM
Dr. Douglas Beecher*	FBI
Dr. Gigi Gronvall*	UPMC
Ms. Donna Hudson*	Consultant/SAIC
Dr. Alan Jacobson*	DTRA
CAPT Malcolm Johns USPHS*	DHS
Dr. Richard Meyer*	Consultant/Tauri Group
Dr. William Palmisano*	ATEC
Mr. Geoffrey Phillips*	USAMRMC
Mr. Scott Winter*	DTRA
SSA John Woodill*	FBI
Dr. Malin Young*	SNL
Ms. Lisa Foddrill	DHS
Dr. John Jensen	USDA
Mr. Joseph Kozlovak	USDA
Dr. James Lindsay	USDA
Dr. Brendan Niemira	USDA
Dr. Segaran Pillai	DHS
Dr. James Rogers	USDA
Ms. Susan Weekly	Consultant
Dr. David White	USDA