Evaluation of Gamma-Radiation Inactivation of a Bioterrorism Agent, *Bacillus anthracis* Spores, on Different Materials

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Abstract Decontamination of suspected packages, such as sealed envelopes, liquids and tools that are likely contaminated with biological agents is of great importance. In this study, we aimed to determine the gamma radiation dose required for the decontamination of paper, fabric and liquid materials without causing any damage to the structure of these materials. Each study group included 11 pieces of paper, fabric and sterile saline contaminated with 0.8×10^5 virulent *Bacillus anthracis* (*B. anthracis*) spores. These specimens were exposed to doses of 5.49, 11.58, 17.21, 21.75, 27 and 33.1 kilogray (kGy) of gamma radiation from a cobalt-60 source. After irradiation of all the samples, a viability assessment of the *B. anthracis* spores was performed. It was found that full decontamination was achieved with 11.58 kGy on the paper samples and 17.21 kGy on the fabric and liquid samples. It was concluded that a dose of 20 kGy of gamma radiation may be recommended for the inactivation of *B. anthracis* for some surfaces when especially sensitive and valuable materials

cannot be wet decontaminated were exposed. In addition, serologic and molecular assays of the suspected packets can be performed for forensic purposes without damaging existing evidence in a bioterror incident.

Keywords *Bacillus anthracis* · Bioterrorism · Decontamination · Gamma radiation

Introduction

Biological weapons have been extensively used since early historical ages. It was noted that Scythian archers contaminated the tips of arrows with decomposed bodies and blood containing gangrene and tetanus bacteria (*Clostridium perfringens* and *Clostridium tetani*) in the 50th century B.C.E. [1].

Due to the increase in the mailing of contaminated anthrax spores to various official organizations and persons after the September 11th attacks on the World Trade Center and Pentagon for terrorism purposes, the detection, analysis and decontamination of bioweapons has gained a great deal of interest. Decontamination of a person or an environment contaminated with bioweapons requires disinfection and sterilization. Some common methods used in sterilization include heating, filtration, gas application, and the use of chemicals and radiation. Both ionizing and non-ionizing radiation, such as UV rays and gamma rays, may be used for sterilization [2].

Sterilization via radiation with gamma rays has been extensively used in industrial fields. Cobalt-60 (Co^{60}) and/or cesium-137 are generally used for the gamma-radiation procedures, and approximately 200 industrial gamma-radiation centers exist in 50 countries worldwide. Gamma

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radiation was reportedly used in the decontamination of goat skin and hairs in the 1960s and 1970s [3].

Several published papers have mentioned the testing of sterilization methods and some chemical products that were manufactured for the decontamination of *Bacillus anthracis* (*B. anthracis*). Almost all the studies were carried out using various *Bacillus* species instead of *B. anthracis* spores due to its possible human health risks. However, the effect of the same sterilization method on different materials may vary [4].

This study aimed to determine the dose of gamma radiation required for thorough decontamination without causing any damage to routinely used materials, such as paper, fabric and aqueous materials, which are likely to be contaminated in a bioterror attack or in anthrax-contaminated mail.

Materials and Methods

The study was approved by the ethical committee of the Gulhane Military Medical Academy on 06 January 2015.

Preparation of Bacteria

Bacillus anthracis was obtained from stock specimens collected for the Ortatagli et al. study [5]. Bacteria were inoculated on sheep blood agar (RDS, Ankara, Turkey) and incubated for 24 h at 37 °C. Then, the bacteria were kept at 4 °C for 7 days following 24 h of storage at room temperature for sporulation. Spore formation was observed daily by spore staining using the Schaeffer–Fulton spore stain kit (Sigma-Aldrich, Buchs, Swiss) until a 95% sporulation rate was achieved. The number of spores in 0.9% saline was adjusted to 1.6×10^5 /ml by serial dilutions, and this number was used for the liquid control group samples.

Preparation of the Materials and Their Contamination

According to the G*Power V3.1 statistical analysis program, a total of 231 samples were accepted, where 11 samples were distributed to each group by comparing the differences in the dependent groups with two-tailed hypothesis tests and with the acceptance of a 95% confidence interval with an 80% power and 1 effect size.

The paper and fabric specimens were prepared by cutting 8 cm diameter circles and sterilizing them with ethylene oxide. The paper and fabric samples were placed in sterile petri dishes and contaminated by spraying them with 0.5 ml of the spore suspension. For the liquid samples, 1.5 ml of sterile saline in the cryotubes was also

contaminated by adding 0.5 ml of the spore suspension. All the specimens were contaminated with 0.8×10^5 spores. All specimens were separated into groups depending on the applied gamma radiation dose and placed into transparent plastic vessels.

Cobalt-60 Irradiation Procedure

The administration of Co⁶⁰ irradiation was performed at Sarayköy Nuclear Research and Training Center, which was formally under the Turkish Atomic Energy Authority.

The samples that were divided into groups based on the applied dose were placed in radiating boxes, and the Perspex dosimeters (Harwell Dosimeters Ltd, Oxfordshire, UK) were adjusted for each group separately. Approximately 150 min of irradiation was required for a 5 kGy dose. To achieve the doses of 5, 10, 15, 20, 25 and 30 kGy that were planned for the study, the samples were exposed to gamma irradiation for 150, 300, 450, 600, 750 and 900 min, respectively. Dosimeter measurements were also performed after irradiation.

Assessment of the Gamma Irradiation Effectiveness

To evaluate the spore viability in the control group and in the samples exposed to 6 different gamma radiation doses, sheep blood agar was used for inoculation. The fabric and paper samples were positioned so that their contaminated sides faced the agar surface, and the bacteria were transferred to the sheep blood agar following hatching with a sterile loop. Twenty µl of the liquid samples was dispensed using an automatic pipette and cultured on sheep blood agar. All cultures were incubated at 36 °C, and the petri dishes were examined after 24 h. Then, the bacterial colony counts were standardized as colony-forming units per 1 ml (CFU/ml).

Results

The measurement values obtained by the dosimeters determined the doses received by the samples irradiated by gamma radiation, and the doses were 5.49, 11.58, 17.21, 21.25, and 33.1 kGy instead of the expected values of 5, 10, 15, 20, 25, and 30, respectively.

Sheep blood agar inoculations and formed colony counts were also performed for the viability check of the spores in all the samples of the study group and non-radiated control group (Fig. 1).

In the counts of the control group, the mean colony amounts and standard deviations were 5040 ± 1058 CFUs in the paper samples, 3666 ± 545 CFUs in the fabric samples and 781 ± 212 CFUs in the liquid samples. The

Fig. 1 Colony counts in the viability controls of the spores in the samples

mentioned values were adjusted to standardize the volume per 1 ml, and the spore counts were 1×10^4 CFU/ml, 0.7×10^4 CFU/ml and 1.6×10^5 CFU/ml in the paper, fabric and liquid samples, respectively.

In media irradiated with 5.49 kGy gamma radiation for 150 min, the mean colony values and standard deviations were 287 ± 85 CFU in the paper samples, 30 ± 5 CFU in the fabric samples and 35 ± 16 CFU in the liquid samples.

No growth was obtained on the paper samples when 11.58 kGy radiation was administered within an irradiation period of 300 min. However, 2 of the fabric samples and 5 of the liquid samples showed colony formation. Mean colony counts were standardized to 1 ml, and the standard deviations of all the samples are summarized in Table 1.

No samples in the media irradiated with doses of 17.21, 21.25, 27 and 33.1 kGy of gamma rays had any *B. anthracis* bacterial growth.

Discussion

In recent years, terrorist attacks have targeted masses of innocent civilian people instead of individual assaults against state governors, military staff or top-level

Table 1 Radiation doses during the study and the survival of the *B. anthracis* spores (Colony counts standardized as of 1 ml and standard deviations)

Materials	Radiation doses (kGy)		
	0	5.49	11.58
Paper	$10,080 \pm 2116$	574 ± 170	0
Fabric	7332 ± 1090	60 ± 10	0.36 ± 0.8
Liquid	$156,164 \pm 42,495$	7109 ± 3282	145 ± 201

bureaucrats and led to public panic and chaos. Biological weapons are the mostly likely methods for such a purpose because of their ability to cause massive destruction to humans and their availability and ease of production in sufficient quantities. *B. anthracis* is considered to have the most potential as a biological weapon and is the most likely to be used in terms of its lethal or incapacitating effect because of its appropriate particle size for aerosol forms. Once an attack with *B. anthracis* spores, which may survive in the environment for decades, has occurred, the materials, such as closed envelopes and suspected packages, require decontamination/disinfection as a preventive measure.

Bioterror attacks with *B. anthracis* spores after the September 11th, 2001 events in the USA have demonstrated the real and horrible face of bioterrorism, which is a crime and a real disaster. When compared with chemical weapons, bioweapons are still threatening the world because of the difficulty in detection, poor early-diagnosis, insufficient quarantine measures and severe impact on the public [6, 7].

Living organisms, various surfaces, ambient air, buildings, food sources, coatings, goods, mailed packages and other environmental materials may be considered as contaminated following a biological attack.

In our study, some environmental materials, including paper, fabric and liquid saline solution, which are likely to be infected in a biological attack, were contaminated with *B. anthracis* spores, and the results were evaluated following the disinfection of the media via gamma radiation exposure at six various doses. In a recovery study of the control group, a 1 log loss was observed in the paper and fabric samples. It may be that the porous and rough surface of the paper samples led to an increase in the recovery rate compared with that of the fabric samples.

Our study also showed that the recovery obtained from the inoculation of various media resulted in 80–90% of the existing contamination, while colony counts were found in the liquid samples from the petri dishes. Those findings indicated that the use of liquid samples might allow more

precise results to be obtained during the disinfection and sterilization studies. The use of various media would simulate the environments one can face during routine life.

Studies involving the diagnosis and inactivation research of *Bacillus spp.* have previously been performed using non-pathogenic species, mainly *Bacillus atrophaeus* (*B.atrophaeus*), due to the high pathogenicity and infectivity of *B. anthracis* [4, 8]. In studies of inactivation, some chemicals, heat and UV radiation have been administered, and liquid media have generally been used for this purpose [9, 10].

Virulent *B. anthracis* spores were used instead of a *B. anthracis* simulant in the present study. For the medium of the infecting area, an aqueous medium and fabric, paper and saline, which could possibly be contaminated in a bioterrorism event, were used to reflect a real attack.

One of the pioneering studies on the inactivation of *B. anthracis* spores was carried out by Horne et al. [11]. They used goat hairs contaminated with *B. anthracis* spores and found that all of the spores were inactivated by 15 kGy.

Niebuhr et al. [12] applied 10 megaelectronvolts (MeV) of radiation produced by a linear electron accelerator with doses of 2.5, 5.5, 8.3, 9.8, 15.1, 20.4 and 24.1 kGy on *B. anthracis* Sterne 34F2 (an animal vaccine species) spores that were mixed with milk powder and placed into envelopes to simulate an anthrax bioterror attack, such as suspicious packages. They observed the inactivation of all the spores at a radiation dose of 15.1 kGy, and the radiation dose (D_{10}) required to decrease the amount of spores to 10 logs in their analyses was determined to be 3.35 ± 0.02 kGy, and they determined a dose of 40 kGy was necessary for the inactivation of 10^{12} spores.

In another study, Helfinstine et al. [13] used 10^8 *B.atrophaeus* spores that were absorbed in a polycarbonate membrane filter located in a mail envelope and applied 5 MeV of radiation generated by a Dynamitron electron beam accelerator with doses of 5, 10, 15, 20, 25, 30, 35 and 40 kGy, which resulted in a D_{10} value of 1.53 ± 0.03 kGy. They also reported a 14 kGy dose of radiation was required to inactivate $8 \log_{10}$ of spores.

In a similar study performed by Auslender et al. [14], kaolin contaminated with 10^6 *Bacillus cereus* (*B.cereus*) and *Bacillus thuringiensis* (*B.thuringiensis*) in an envelope was subjected to radiation at doses of 1, 5, 10, 20, 30, 40, 50, 62.5, 87.5, 100, 125, 200, 250, 300 and 400 kGy, which were generated by a 2.4 MeV ILU-6 Impulse linear accelerator, and it was found that a dose of 8.5 and 22 kGy was required for complete inactivation of the *B.thuringiensis* spores and *B.cereus* spores, respectively.

Similar results were found for various concentrations of bacteria spores in inactivation studies that were performed using radiation from different types of sources with different energy levels. However, our study showed a

radiation dose of 11.58 kGy was adequate for complete sterilization in addition to a 94.30% decrease in the spore amounts with a dose of 5.49 kGy. The gamma radiation obtained from a Co^{60} source with 1.33 MeV energy was applied on paper samples contaminated with 1.6×10^5 CFU/ml of *B. anthracis* spores for complete inactivation.

After the preparation of 11.05×10^8 CFU of *B. anthracis* spores following the freeze-dry method in glass ampoules, Natalia et al. [15] found a 95.37, 99.58 and 99.99% decrease in spores when the spores were subjected to Co^{60} source gamma radiation at doses of 10, 20 and 30 respectively.

Dang et al. [9] applied a Co^{60} source for gamma radiation at doses of 10, 15, 20, 25, 30 and 35 kGy on 3 ml of sterile water containing 10^8 CFU/ml *B. anthracis*, and no bacterial growth was noted in the samples dosed with 20 kGy and more.

In our study, the number of *B. anthracis* spores in the liquid samples contaminated with 1.6×10^5 CFU/ml spores decreased by 95.51 and 99.74% with doses of 5.49 and 11.58 kGy, respectively. Complete inactivation was observed in samples irradiated with 17.21 kGy.

In the contaminated fabric samples, complete decontamination was obtained with 17.21 kGy of gamma radiation, and the number of living spores decreased by 99.18 and 99.97% when doses of 5.49 and 11.58 kGy, respectively, were applied.

Conclusion

Although studies have mostly reported that 3 kGy was the lowest and 30 kGy was the highest dose adequate for the inactivation of 10^6 – 10^8 spores, 15 or 20 kGy with an extended security level would be acceptable as optimal doses to inactivate any type of bacterial spores contaminating the media.

Thus, the present study concludes that 15 kGy gamma radiation is sufficient to decontaminate *B. anthracis* spores on all exposed surfaces when sensitive and valuable materials cannot be wet decontaminated.

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