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## Current status and trends

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# Inactivation and identification of *Bacillus anthracis* spores

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## Abstract

*Bacillus anthracis* endospores must be handled at a biosafety level-3 (BSL-3) containment facility, therefore an appropriate inactivation procedure is essential in order to both detect them in a reliable and rapid way and to avoid human infections from antrax-contamined samples if a biosafety level-3 laboratory is not available. Unfortunately, dormant spores exhibit incredible hardiness against germicidal agents. The most effective methods for inactivating spores are usually the chemical treatment, heat inactivation (boiling, moist heat, dry heat) and irradiation (microwave, UV, gamma, electron beam). The inactivation method used must alter the cell structure as little as possible in order to ensure a correct diagnosis by different techniques. Several kinds of physical and chemical inactivation methods were assessed in order to define the conditions that ensure inactivation of endospores of *B. anthracis* 34F2 Sterne and permit, at the same time, its identification by PCR and immunoassays techniques.

**Keywords:** *Bacillus anthracis*; biowarfare; spores; inactivation; identification

## 1. Introduction

*Bacillus anthracis*, a Gram positive, non-motile, facultative anaerobic and endospore-forming rod-shaped bacterium, is an etiologic agent of anthrax. Animals are infected by contact with soil-borne spores. Humans become infected only accidentally when brought into contact with diseased animals or their waste products. Because of their resistant and high virulence an appropriate inactivation procedure is essential in order to both detect them in a reliable and rapid way and to avoid human infections from antrax-contamined samples.

## 2. Subject

The main challenges of this work were:
1. Viability study of *B. anthracis* spores after chemical and physical inactivation.
2. Effects of different inactivation processes in diagnostic techniques: molecular biological and immunological techniques.

## 3. Material and methods

### 3.1 Spores

*B. anthracis* used in this work was originated from the National Collection of Type Cultures (NCTC) of Public Health England (*B. anthracis* strain 34F2 Sterne NCTC 8234).

Bacterial culture was prepared by growing on tryptic soy broth (TSB) medium for 24 h at 37 °C. Bacilli spores were prepared as previously described by Sancho and Baldrís [1]. Spore counting was carried out as described in the European norm EN 14347.

### 3.2 Inactivation

#### 3.2.1 Chemical inactivation [2]

Formaldehyde (PFA) solutions (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) at final concentrations of 10% and 20% (vol/vol), and trifluoroacetic acid (TFA) solutions [3] (Sigma-Aldrich

Chemie GmbH, Taufkirchen, Germany) at concentrations of 25%, 50% and 80% (vol/vol), were tested. The TFA inactivation was stopped after 5, 15 or 30 minutes by mixing TFA/spore suspension with the neutralization medium (9% Tween 80, 0.9% lecithin, 3% histidine and TSB). PFA-treatment was stopped at 1 h, 2 h, and 3 h by centrifugation at 2,500×*g* for 10 minutes.

#### 3.2.2 Physical inactivation [4]

Gamma irradiation using a Cobalt-60 source at 10-15 KGy-absorbed doses was tested [5].

### 3.3 Identification

#### 3.3.1 Molecular biological techniques [6]

DNA was extracted using the QIAmp DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's protocols. Purified DNA was quantified using the NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., USA). Chromosomal markers *Ba813* have been used to characterize *B. anthracis*. The PCR products were visualized by electrophoresis on a 1% (wt/vol) agarose gel. The gels were stained in ethidium bromide solution 1 mg/ml and documented with the GeneGenius (Syngene). Molecular marker Smartladder (Eurogentec) was included on the gel.

#### 3.3.2 Immunological techniques [7]

Treated and non-treated spores were firstly washed (12,000×*g* 10 min centrifugation and the pellet resuspended in PBS1x, 3 times) and then counted by flow cytometry using BDTruCount™ tubes. An indirect ELISA assay was carried out using an antibody raised against EA1 S-layer protein (SA27) as the primary antibody, and a goat anti-mouse IgG HRP-conjugated secondary antibody. Absorbance was measured at 490 nm (A490 nm). A490 nm data obtained from both paraformaldehyde-treated and trifloroacetic acid-treated spores were analyzed by one-way ANOVA followed by Newman-Keuls test, and those data coming from spores treated with γ-irradiation were analyzed using a Student's t-test.

## 4. Results

### 4.1 Viability study of inactivated *B. anthracis* spores

The chemical inactivation with PFA and TFA solutions at all concentrations and times tested yielded complete absence of bacterial growth of *B. anthracis*.

With regards to gamma irradiation using a cobalt-60 source at 10-15 KGy-absorbed doses, the viability of the spores decreased, but the absence of bacterial growth was not complete.

### 4.2 Effect of different inactivation processes in diagnostic techniques

#### 4.2.1 Molecular biology techniques

*Ba813* gen was detected in the following samples:

- Inactivated samples by gamma irradiation using a cobalt-60 source at 10-15 KGy- absorbed doses (Fig. 1).
- Inactivated samples by PFA solutions at two concentrations assayed (10 and 20%) and at different treatment times (1 h, 2 h and 3 h) (Fig. 2).
- Inactivated samples by TFA solutions at 25 and 50% (not at 80%), when TFA-exposure lasted 5 minutes. No detection was found after either 15 or 30 minutes of TFA-exposure (Fig. 3).

#### 4.2.2 Immunological techniques

A decrease in the immunological-recognition was observed after all treatments (gamma irradiation using a cobalt-60 source at 10-15 KGy-absorbed doses, formaldehyde and trifluoroacetic acid solutions). The results are shown in the Fig. 4, 5 and 6. This immunological-recognition decrease after both formaldehyde and trifluoroacetic acid treatment was not statistically dependent on toxic concentration.

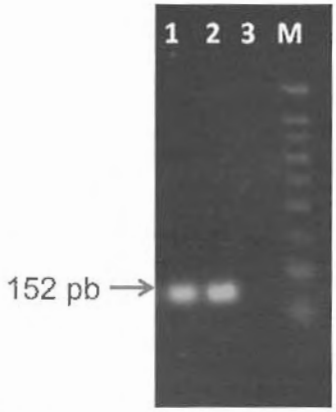



**Fig. 1.** *Ba813* gen amplification in inactivated samples by gamma irradiation.
(M) Molecular weight marker; (1) *B. cereus* spores with *Ba813* gene; (2) *B. anthracis* spores cobalto 60-irradiated; (3) PCR negative control.

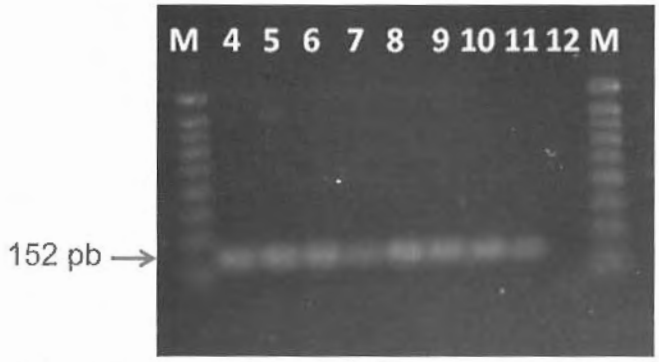



**Fig. 2.** *Ba813* gen amplification in inactivated samples by PFA.
(M) Molecular weight marker; (4) *B. cereus* with *Ba813* gene; (5) *B. anthracis* spores (109 cfu/ml); (6)-(11) inactivated *B. anthracis* spores using PFA solutions at different concentrations and at different times of treatment: (6) 10%, 1 h; (7) 20%, 1 h; (8) 10%, 2 h; (9) 20%, 2 h (10) 10%, 3 h; (11) 20%, 3 h (105 cfu/ml); (12) PCR negative control.




**Fig. 3.** *Ba813* gen amplification in inactivated samples by TFA.
(M) Molecular weight marker. (13)-(21) inactivated *B. anthracis* spores using TFA solutions at different concentrations and at different times of treatment: (13) 25%, 5 min; (14) 50%, 5 min; (15) 80%, 5 min; (16) 25%, 15 min; (17) 50%, 15 min; (18) 80%, 15 min; (19) 25%, 30 min; (20) 50%, 30 min; (21) 80%, 30 min; (22) *B. anthracis* spores treated with neutralization medium without TFA; (23) B. anthracis spores (109 cfu/ml); (24) Bacillus cereus spores with Ba813 gene; (25) PCR negative control.

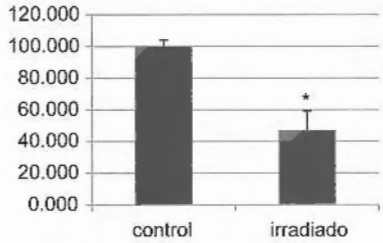



**Fig. 4.** Immunological-recognition alteration in spores treated with cobalt-60 source at 10-15 KGy-absorbed doses *versus* non-treated ones. Results expressed as percentage of control average A490 nm from indirect ELISA. Different from saline: **P*<0.001.

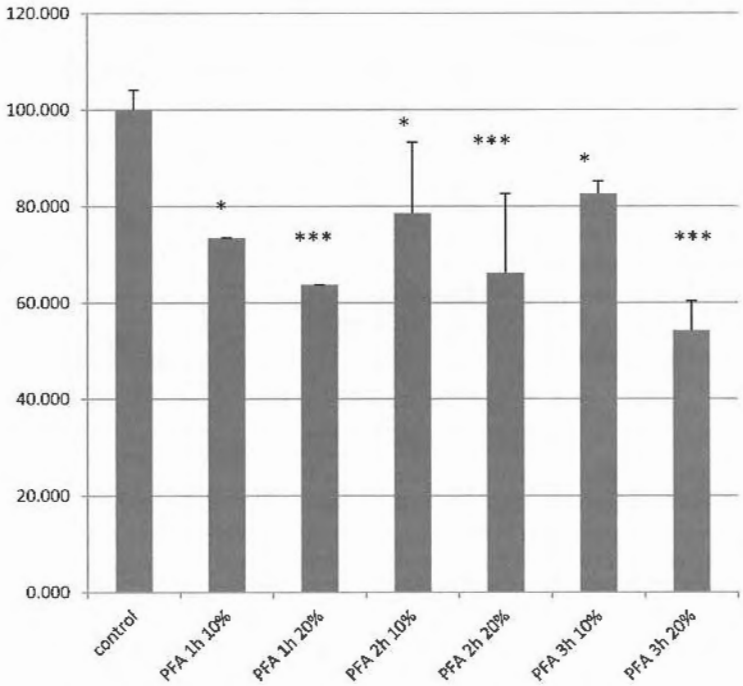



**Fig. 5.** Immunological-recognition alteration in PFA-treated spores versus non-treated ones. Results expressed as percentage of control average A490 nm from indirect ELISA. Different from saline: **P*<0.05, ***P*<0.01, ****P*<0.001.

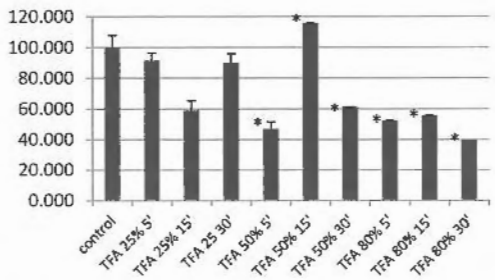



**Fig. 6.** Immunological-recognition alteration in TFA-treated spores *versus* non-treated ones. Results expressed as percentage of control average A490 nm from indirect ELISA. Different from saline: **P*<0.001.

## 5. Conclusions

According with the obtained results, the following conclusions are drawn:

- Absence of bacterial growth of *B. anthracis* spores is complete when they are inactivated using formaldehyde solutions at 10 and 20% (v/v) and trifluoroacetic acid solutions at 25, 50 and 80% (vol/vol) at all tested times.
- Both chemical and physical inactivation methods tested to inactivate *B. anthracis* spores do not interfere with the diagnostic by molecular biology techniques.
- Diagnostic by immunological techniques is altered in every case. However, inactivation using a cobalt-60 source at 10-15 KGy-absorbed doses, as along with some TFA–treatments, seems to alter the most.
- Both treatments with 10% PFA and 25% TFA are proved to be better than the other assessed inactivation methods with regards to both sporicidal and non-altered diagnostic capabilities.

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