Extraction of PCR-amplifiable genomic DNA from *Bacillus anthracis* spores

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Abstract

Bacterial endospore disruption and nucleic acid extraction resulting in DNA of PCR-amplifiable quality and quantity are not trivial. Responding to the needs of the Hazardous Materials Response Unit (HMRU), Laboratory Division, Federal Bureau of Investigation, protocols were developed to close these gaps. Effectiveness and reproducibility of the techniques were validated with laboratory grown pure spores of *Bacillus anthracis* and its close phylogenetic neighbors, and with spiked soils and damaged samples.

Keywords

B. anthracis, endospore disruption, DNA extraction

Introduction

Molecular biology techniques have been applied to microbial community structure and activity analysis to overcome existing limitations of culture-based classical microbiological methods. Hence, nucleic acids in sufficient quality and quantity need be recovered from the samples of interest. There is no shortage of published nucleic acid isolation and purification protocols (Boom et al., 1990; 1991; Carter and Milton 1993; Clegg et al., 1997; DeLong, 1992; Dojka et al., 1998; Duarte et al., 1998; Faegri et al., 1977; Fuhrman et al., 1993; Goodwin and Lee, 1993; Herrick et al., 1993; Holben, 1994; Holben et al., 1988; Holoman et al., 1998; Jackson et al., 1997; Jacobsen and Rasmussen, 1992; Leff et al. 1995; Lorente et al., 1998; Ogram et al., 1987; 1988; 1994; Olson, 1991; Picard et al.,

1 1992; Porteous et al., 1994; Steffan et al., 1988; Torsvik, 1980; 1995; Torsvik et
2 al., 1990; Trevors and Leung, 1998; Walsh et al., 1991; Zhou et al., 1996), but
3 only a few reports mention bacterial endospores (Kuske et al., 1997; 1998; More
4 et al., 1994; Nicholson and Setlow, 1990). Spore disruption has not been a
5 concern whereas alternative assays and spore detection approaches usually
6 failed due to environmental matrix effects, lack of specificity, or difficulties in
7 achieving spore permeabilization (Bidenko et al., 1998; Blake and Weimer, 1997;
8 Bruno and Yu, 1996; Deng et al., 1997; Fischer et al., 1995; Lebaron et al., 1997;
9 Quinlan and Foegeding, 1997).

10 In the new realm of homeland security and biodefense, validation of the
11 presence of biological warfare agents is crucial in determining the response to
12 the threat. As would have been during the recent willful dissemination of *Bacillus*
13 *anthracis* spores. Here we report on our efforts in designing, adapting, and
14 validating protocols for *B. anthracis* spore disruption, DNA isolation, purification,
15 and quantification for the Hazardous Materials Response Unit (HMRU),
16 Laboratory Division, Federal Bureau of Investigation (FBI).

17

18 Material and Methods

19 Reference to any vendor or manufacturer of equipment, laboratory supplies,
20 chemical or molecular biology kit used in the following methods does not
21 constitute to an endorsement neither should it be considered as a statement of
22 quality or judgment about one product over another. All chemicals and media
23 ingredients were of high purity and quality grad.

Microorganisms and culture media

Table 1 summarizes the microbial cultures that were included in these experiments. *Bacillus cereus* and *B. thuringiensis* were used as the closest phylogenetic neighbors of *B. anthracis*, while *B. globigii* serves often in biodefense experiments as a surrogate for *B. anthracis*.

Cultures obtained were immediately checked for purity and virulence and expanded for storage. Fresh, eighteen-hour cultures were preserved in half-strength nutrient broth (Difco, USA) with glycerol (15% final concentration) and maintained for long-term storage at -86ºC in an ultralow temperature freezer and in the vapor phase of liquid nitrogen. Working stocks of the strains were kept on tryptic soy agar (TBA; BBL, USA) at refrigeration temperature. Stored strains were revived first on TBA medium, checked for virulence by multiplex PCR, and sub-cultured on double-strength modified Schaeffer sporulation medium (2 x SG) (Leighton and Doi, 1971).

Preparation of spores

In short, cultures were grown on 2 x SG for 48 – 72 h at 37ºC. After reaching high sporulation efficiency (**Figure 1**), the biomass was collected, washed several times with ice-cold sterile water, and harvested by centrifugation. Spores from the resulting pellet were separated by step-density gradient centrifugation in RenoCal-76 (Bracco Diagnostics, USA). Purified spores (**Figure 2**) were freeze-

1 dried and stored at -20ºC. Reproducibly, a batch of twelve 2 x SG plates yielded
2 ~200 mg of pure spores with a spore density of ~10^{12} spores/g.
3
4 *Preparation of spiked soil*
5 Some experiments required the spiking of commercial potting soil with spores
6 of attenuated *B. anthracis* and other bacilli to simulate real-world environmental
7 samples. The potting soil was first sieved using a U.S.A. Standard Testing Sieve
8 set (VWR, USA). The fraction passing through the No.16 sieve (mesh size
9 1.18 mm) was collected, autoclaved at 121ºC and 15 psi for 1 h, and spiked with
10 known amounts of laboratory grown pure bacterial spores. The inoculated soil
11 samples were gently mixed for 15 min at room temperature in a Stomacher,
12 Model 400 Circulator (Seward, USA). The spiked samples were equilibrated at
13 room temperature in the dark for 21 days to one month.
14
15 *Spore damaging treatments*
16 Laboratory grown pure spores and spiked soil samples were subjected to
17 potentially spore damaging treatments to validate the protocols for the extraction
18 of PCR-amplifiable DNA from bacterial endospores. Spores and spiked soil
19 samples were autoclaved at 121ºC and 15 psi for 15 min, boiled at 100ºC for
20 30 min, bleached with 10% household bleach at room temperature for 1 h,
21 treated with Wescodyne (AMSCO, USA) working solution for 10 min at room
22 temperature, and were exposed to sun UV for 2 weeks. Untreated and

uninoculated samples were kept under the same conditions and used as controls in the experiments.

Spore disruption, DNA extraction, purification, and quantitation

Following DNase treatment of the spores with DNA-*free*™ (DNase Treatment and Removal Reagents; Ambion, USA) to assure the removal of contaminating nucleic acids from the spore surfaces, spores were treated by a number of published methods and their combinations as summarized in **Table 2**. Spore disruption was validated by phase contrast microscopy and quantitated by plate counts on tryptic soy agar.

Ultimately, a modified protocol was routinely used for spore disruption of laboratory grown pure spores and of potentially damaged spore samples. In short, 10 mg of pure, DNase-treated spores in a 1.5-ml microcentrifuge tube were washed twice with 500 µl of ice-cold 0.1% sodium pyrophosphate. Spores were collected by centrifugation at 6,000 *g* for 1 min after each washing step. A 5% suspension of Chelex 100 resin (Bio-Rad, USA) was freshly prepared in sterile deionized water (Milli Q; Millipore, USA). The resin was gently mixed in the water by inverting the tube a few times. The spores were resuspended in 600 µl of the 5% Chelex 100 resin suspension. The mixture was incubated at 56°C for 1 h and then kept at room temperature for an additional 1 h. Finally, the mixture was boiled for 8 min at 100°C. Spore disruption was validated by determining the number of colony forming units (CFUs) in chloroform-killed (2.5% v/v) samples on TBA medium.

For spore disruption and DNA extraction, spiked soil samples and potentially damaged soil samples were treated with a modified and optimized protocol of the FastDNA® SPIN Kit for Soil (BIO 101, USA). In short, 100 mg of soil was added to a MULTIMIX 2 Tissue Matrix Tube. The sample was suspended in 978 µl of the sodium phosphate buffer and 122 µl of MT buffer, both provided in the kit, and treated in the FastPrep® instrument at a shaking intensity of 5.5 (or 5.0 if the 1/4" zirconium cylinder was added) 10 times for 30 s, each. Following the ballistic spore disruption, the sample was incubated at room temperature for 1 h. The further steps for DNA extraction, capture, and elution followed the manufacturer's recommendation. Since the standard commercial potting soil is rich in organic matter, the SEPHADEX G-200 spin column separation as described earlier (Kuske et al.1997; 1998) was used to remove humic acids.

DNA concentration was estimated on a 1.2% LE agarose gel (FMC, USA) and quantitatively measured in a model TD-700 fluorometer (Turner Designs, USA) using the Pico-Green® (Molecular Probes, USA) assay as recommended by the manufacturer. The resulting DNA quality was further verified in a routine PCR reaction amplifying the small subunit 16S rRNA-coding sequence with degenerate *E.coli* primers 27f and 1492r (Lane, 1991).

Results and Discussion

Endospores of *Bacillus anthracis* may be attractive to terrorists as weapons of mass destruction because they are easily produced and transported and show resistance to environmental and other forms of degradation.

DNA based assays have proven reliable and reproducible for pure culture isolates or for purified spore preparations. In the field at a suspected crime or terrorist scene, extraction of sufficient DNA and its reliable amplification to detect strain specific sequenced signature regions are still challenging, particularly against an unknown microbial background that also may have been damaged, aged (weathered), or altered.

This project addressed the FBI Laboratory's requirement for the development of improved methods and procedures for processing samples, which are suspected of containing hazardous biological materials, including biological warfare agents. Pure spore preparation protocols and methods for sample preparation, spore disruption, and DNA extraction, recovery and quantitation were developed. The protocols were validated using different strains of *B. anthracis*, its closed phylogenetic neighbors, such as *B. cereus* and *B. thuringiensis*, as well as, bentonite-adsorbed endospores of *B. globigii*, the most widely used surrogate test organism by the intelligence community. Using these techniques PCR-amplifiable DNA was extracted from a variety of bacterial spores. The methods were adapted, improved, or modified for the analysis of spiked soil samples as well as damaged samples.

Pure spore preparation

A protocol for pure spore preparation in the laboratory was adapted. We obtained authentic strains of *B. anthracis* and its close relatives from other investigators in the U.S. and abroad or purchased from reliable culture

collections. Strains were tested for purity and virulence. Attenuated pure cultures were preserved and maintained.

Although the use of the modified Schaeffer's sporulation agar (Leighton and Doi, 1971) made harvesting the biomass more laborious than using a liquid culture, however, much better control over the sporulation event was maintained. Sporulation efficiency was checked by phase contrast microscopy. Spores appeared green and intensively "phase-bright" (**Figure 2**). Harvested spores were purified and stored long-term.

Spore production and purification resulted in a high quality product that was practically free of vegetative cells. The overall mass balance based on both biomass production and viable spore counts showed acceptable losses during the pure spore preparation. It also drew our attention to a strain specific observation: during the washing steps a "lighter" spore fraction was lost time and again. These floating spores had normal microscopic appearance and germinated to regular vegetative cells but could not be harvested by centrifugation.

Sample pretreatment, spore disruption, and DNA extraction

Several sample pretreatment methods were compared to make sure that neither extracellular DNA on the spore surface, nor spore attachment to the soil matrix impair the results. Advantages and disadvantages of both microbial cell fractionation and direct lysis were investigated and ultimately a 0.1% sodium pyrophosphate solution was chosen for the soil samples to enhance the

detachment of spores from the soil matrix. DNA-*free*™ (Ambion, USA) was applied to remove extracellular DNA from the spore surface.

The FBI Hazardous Materials Response Unit's project requirements limited the type of chemicals and laboratory equipment that could be used. Nonetheless, for the purpose of comparison we reproduced and modified a number of published methods for spore disruption and DNA extraction. **Table 2** summarizes these experiments and our findings. Methods and their modification that resulted in efficient spore disruption either yielded badly sheared genomic DNA, used chemicals that could not be considered for this project, or generated hazardous waste, which was not allowed under Sponsor's requirements. Finally, two protocols were modified: one using Chelex 100 resin (Bio-Rad, USA), the other the FastDNA® SPIN Kit for Soil (BIO 101, USA). These protocols were alternatively applied for laboratory grown pure spore cultures and for soil samples.

Spore disruption was monitored and validated qualitatively based on the loss of spore microscopic refractility, and quantitated by viable spore counts. Phase contrast microscopy proved to be rapid and convenient: the loss of refractility (**Figures 3-4**) is a very practical means of monitoring spore intactness. Intact bacterial endospores are green and intensively "phase bright". As spore disruption proceeds, the spores turn dark. Eventually, only spore walls, "ghosts", are visible under the phase contrast microscope.

Many forms and ways of DNA capture and purification were tested. The silica binding matrix (BIO 101, USA), the DNA binding columns containing a nylon

membrane and a glass matrix (BIO 101, USA), and the silica-gel membrane in the QIAamp and QIAquick spin columns (QIAGEN, USA) were the most practical and efficient under the conditions tested. The resulting DNA was PCR amplified via a standard protocol routinely used in our laboratory.

DNA concentrations were determined fluorometrically using PicoGreen® (Molecular Probes, USA). The influence of RNA contamination on the DNA quantitation was also investigated. It was found that the amount of interference (up to 10% of the fluorometer reading) was dependent upon the amount of RNA in the sample (tested range spread from 5% – 95% RNA). Due to its fragile nature, it is likely that the majority of the contaminating RNA will degrade during spore DNA extraction. If this is not the case, treatment with DNase-free RNase (Roche Diagnostics, USA) provides an easy solution.

DNA extraction from spiked and damaged samples

To validate that the developed methods were applicable to real world samples, spiked soil samples were prepared. Commercially available potting soil was mixed with spores under controlled laboratory conditions resulting in 10^7-10^8 spores per mg of spiked soil. Potting soil was chosen because it was rich in enzymes-inhibiting organic matter, therefore, the need for organic matter removal and the usefulness and ease of the removal protocol could also be tested.

Following a pretreatment, total genomic DNA was extracted from spiked and control soil samples using the modified FastDNA® SPIN Kit for Soil (BIO 101, USA). In most cases, there was no need for additional organic matter removal

after the procedure. If, however, humic acids needed to be removed from the DNA sample, the SEPHADEX G-200 spin column separation proved to be simple and successful. DNA was quantitated and found to be ~80% lower than computed for the amount of spores present in the samples. Nonetheless, DNA quantity and quality was verified to be sufficient for PCR amplification.

Laboratory grown pure spores, spiked soil samples, and controls were exposed to autoclaving, bleaching, disinfectants, and sunshine UV. Following the treatments, we determined the viable plate counts, and using the already described procedures, extracted PCR amplifiable DNA from the samples. The treatments successfully lowered the viable plate counts, damaged and decreased the concentration of genomic DNA (**Figure 5**). Bleaching had the most destructive effect on spore viability and DNA concentration, while the effect of 2-week exposure to sun UV was not noticeable.

Conclusions

The project provided our laboratory with the opportunity to realize a major gap in molecular biology techniques, *i.e.*, current published protocols do not satisfy the need for reproducible bacterial endospore disruption. Consequently, it must be assumed that genomic DNA extracted from environmental or forensic samples most likely does not contain representative spore DNA.

Here, we developed and validated protocols for laboratory scale pure spore sample preparation, spore disruption, DNA extraction, and purification. Sample pretreatment and DNA quantitation methods were also tested. All methods were

tested with spiked soil samples before and after different types of spore damaging treatments.

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17

Table 1 Summary of microbial strains included in the experiments

Species	Strain
B. anthracis	UM23C1-1
	UM441c9
	4229 (pOX2+)
	7702 (pOX1+)
	9131 (no plasmid)
	delta NH-1
	delta V770-1
	delta Sterne-1
	delta VIB-1
	delta Ames-1
	VNR-1-delta 1
	NNR-1-delta 1
	NNR-1
	ANR-1
	Ames pXO1- pXO2-
	Ames pXO1+ pXO2-
	Ames pXO1- pXO2+
B. cereus	BGSC* 6A1 (type strain)
	NRRL B-3711
	AH512
B. globigii	BGSC 11A1
	JFT
B. thuringiensis	BGSC 4A1
	BGSC 4R1
	BGSC 4Y1

* BGSC – Bacillus Genetic Stock Center (Department of Biochemistry, College of Biological Sciences, Ohio State University)

Table 2 Summary of different spore disruption treatments*

#	Treatment**	Comments
1.	wet grinding w/50 mg 1-µm zirconium beads in 10% SDS, LN cooling, manual grinding	species-dependent changes
2.	dry grinding w/three sizes of glass beads, 50 mg each, 2 models of Wig-L-Bug	difficult transfer, DNA sheared
3.	UltraClean Soil DNA Kit (MoBio, USA)	inefficient spore disruption
4.	10% 2-mercaptoethanol, 45˚C, 120 min	not effective
5.	same as #4, 90 min	not effective
6.	same as #5, 1,000 µg/ml lysozyme in STET buffer***	not effective
7.	same as #6, all three sizes of glass beads, 100 mg, each	debris only
8.	7.2 M urea, 45˚C, 120 min	not effective
9.	same as #8, 90 min	not effective
10.	same as #9, 1,000 µg/ml lysozyme in STET buffer***	few dark spores
11.	same as #10, all three sizes of glass beads, 100 mg, each	debris only
12.	50 mM dithiotreitol pH 8.5, 45˚C, 90 min	few dark spores
13.	same as #12 pH 10.5	few dark spores
14.	same as #13, 500 µg/ml lysozyme in STET buffer***	50% dark spores
15.	7.2 M urea, 10% 2-mercaptoethanol, 45˚C, 90 min	more dark spores
16.	3.6 M guanidine hydrochloride pH 2.8, 45˚C, 60 min	effective
17.	same as #16, 10% of 2-mercaptoethanol, 45˚C, 60 min	effective
18.	same as #17, 1,000 µg/ml lysozyme in STET buffer***	effective
19.	same as #18, all three sizes of glass beads, 100 mg, each	DNA sheared
20.	10% of 2-mercaptoethanol, 8 M urea, 65˚C, 15 min	effective
21.	0.15 M N-ethylmaleimide, 1 h, 65˚C, 2,000 µg/ml lysozyme in 0.15 M NaCl and 0.1 M EDTA	toxic but very effective
22.	same as #21, 2 sizes of glass beads, 4 x 1 min	toxic but very effective
23.	50% mercaptoacetic acid, 8 M urea, 10 mM EDTA,	toxic but effective
24.	500 µg/ml lysozyme in 10 mM Tris, 10 mM EDTA, 1 mg/ml dithiotreitol [alkaline lysis]	toxic but effective

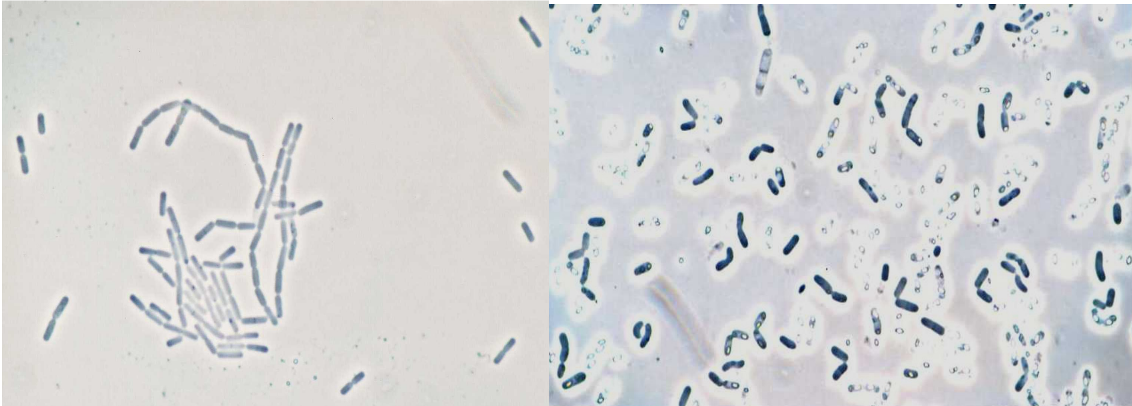
25.	FastDNA kit (BIO 101, USA) w/modifications	very effective
26.	protocol by Ogram (1998) [PEG precipitation]	somewhat effective
27.	protocol by Kuske et al. (1997) [phenol-chloroform]	toxic but effective
28.	QIAamp Tissue Kit (QIAGEN, USA) protocol combined w/bead beating	somewhat effective

*10 mg of spores of different strains of *Bacillus anthracis*, *B. cereus*, *B. globigii*, and *B. thuringiensis* treated

**unless stated otherwise, subsequent DNA extraction followed the QIAamp Tissue Kit (QIAGEN, USA) protocol and bead beating with Mini Bead Beater 8 (BioSpec Products, USA)

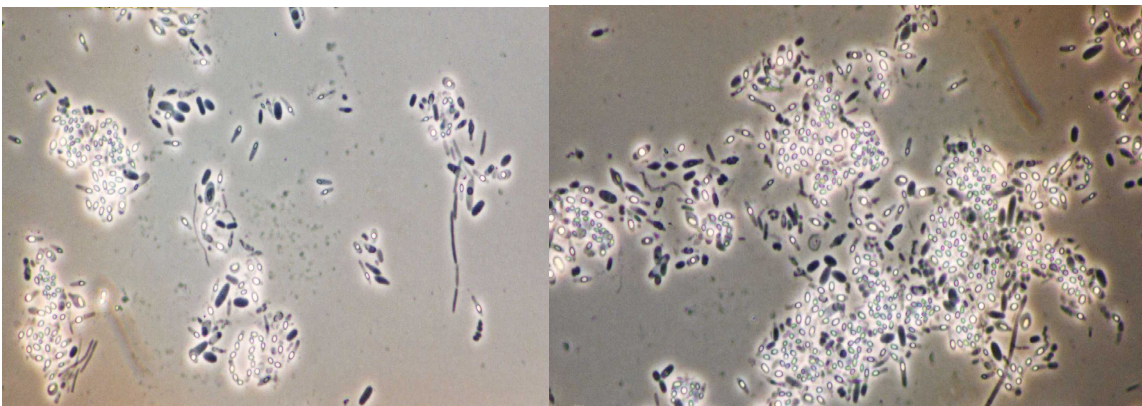
***STET buffer contains 0.1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, and 5% Triton X-100

1 **A** **B**



2

3 **C** **D**



4

5

6 **Figure 1** Pure culture of a differentiating *B. anthracis* strain showing

7 vegetative cells (A) and cells and spores (B-D) (1,000 x

8 magnification)

9

A **B**

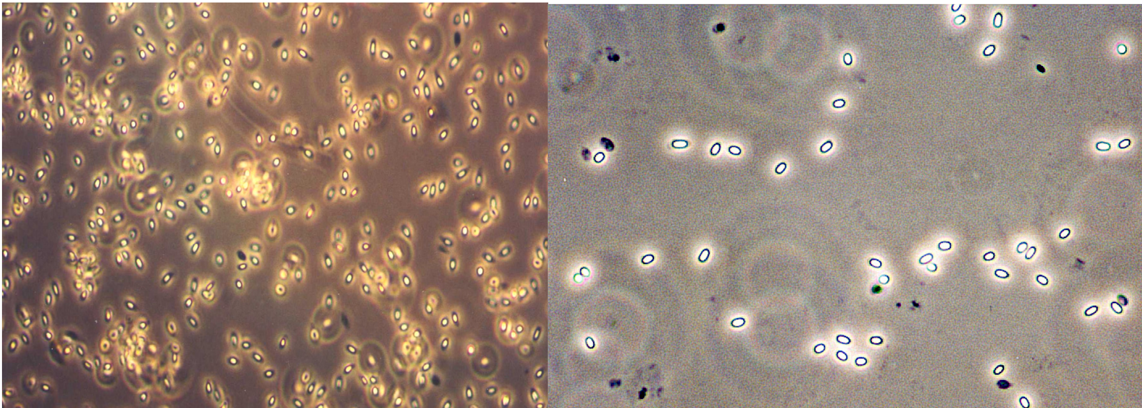
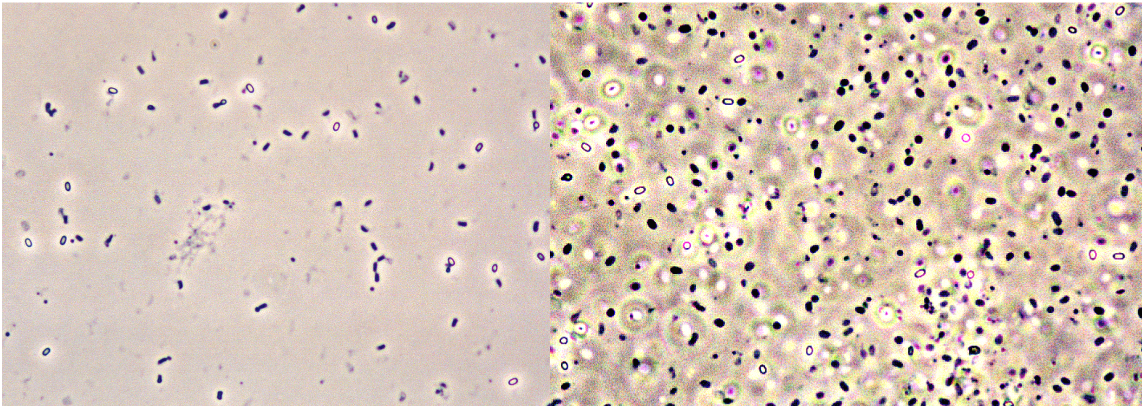
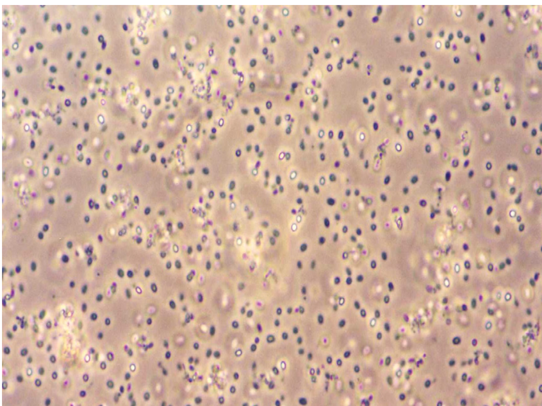


Figure 2 Fully mature bacterial endospores of *B. thuringiensis*, (A) *B. anthracis* (B) imaged by phase contrast microscopy (1,000x magnification)

1 **A** **B**



2
3
4 **Figure 3** Chelex 100 resin treated _B. anthracis_ spores after 1 h of incubation
5 at 56°C. Spores are mostly dark and lost refractility (1,000x
6 magnification)
7
8 **A** **B**



9
10
11 **Figure 4** _B. anthracis_ spores after ballistic disruption (after 3 min [A] and
12 5 min [B]) lost refractility and turned dark (1,000x magnification)
13

1 1 2 3 4 5 6 7

2

3 **Figure 5** Genomic DNA extracted from *B. anthracis* spores before (lanes 2-

4 4) and after autoclaving (lanes 5-7). Lane 1 is the 1-kb size

5 standard (300 ng of DNA).