Advances in Microbiology, **2012, 2, 382-387**
http://dx.doi.org/10.4236/aim.2012.23048 Published Online September 2012 (http://www.SciRP.org/journal/aim)

Bacillus pumilus: Possible Model for the Bioweapon *Bacillus anthracis*

Shannon B. Murphy[1], Merranda D. Holmes[1], Stephen M. Wright[2*]
[1]East Tennessee State University, James H. Quillin College of Medicine, Johnson City, USA
[2]Department of Biology, Middle Tennessee State University, Murfreesboro, USA
Email: *Stephen.Wright@mtsu.edu

Received July 5, 2012; revised August 13, 2012; accepted August 20, 2012

ABSTRACT

The misuse of *Bacillus anthracis* as a bioweapon continues to be a serious concern. Medical personnel and researchers are served well if appropriate non-pathogenic anthrax simulants can be used as countermeasures in preparative planning. While there are several accepted simulants of *B. anthracis*, the addition of another model organism would be beneficial. This investigation was undertaken to evaluate the suitability of *B. pumilus* as a simulant for *B. anthracis*. All organisms were grown on AK Agar #2 to foster sporulation. Optimum conditions for spore formation were determined for *B. pumilus* as well as for currently used anthrax surrogates *B. atrophaeus* and *B. thuringiensis*. Spore dimensions were determined by scanning electron microscopy. Comparative antibody binding studies using commercially available anti-*Bacillus* antisera were completed with the simulants as well as with a negative control organism, *Clostridium sporogenes*. We report that *B. pumilus* sporulated readily (2.9×10^{10} viable spores per plate), had appropriate spore size (1.24 μm × 0.59 μm) and reactivity with anti-*Bacillus* antibodies. The characteristics of *B. pumilus* determined in this study suggest this organism represents a novel, suitable model for *B. anthracis*.

Keywords: *Bacillus pumilus*; *Bacillus anthracis*; Anthrax Simulant; Bioweapon

1. Introduction

The intentional use of a biological agent resulting in morbidity or mortality represents a real and on-going threat [1]. *Bacillus anthracis* is considered a leading candidate as a biological weapon [2]. One of the characteristics of *B. anthracis* that makes it an attractive bioweapon is its ability to form spores in harsh environments and subsequently germinate when conditions become favorable. Survivability of dormant spores is remarkable. Spores of *B. anthracis* recovered from dirt retained lethality in guinea pigs after 60 years [3].

Exposure to aerosolized *B. anthracis* spores could result in inhalational anthrax, a disease that approaches nearly universal mortality if left untreated [4]. The far-reaching implication of bioterrorism involving anthrax in the US was demonstrated by delivery of *B. anthracis* spores through the mail, resulting in over 1000 individuals being considered at risk of exposure [5].

The seriousness of anthrax dictates that investigations into new technologies for detection of anthrax spores continue. Due to the virulent nature of *B. anthracis*, strict adherence to safety protocols must be followed. Inactivation of spores from *B. anthracis* may result in alterations in antigenic structures or changes in targeted nucleic acid sequences that may affect detection [6]. To permit more thorough investigation of possible detection methodologies, it is often desirable to use spores from other *Bacillus* spp. that can serve as models for anthrax spores. The *Bacillus cereus* group, a genetically closely related group of *Bacilli*, is comprised of *B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis* and *B. weihenstephanesis* [7]. From this group, *B. cereus* and *B. thuringiensis* have been used as surrogates for *B. anthracis* [8,9]. Other *Bacilli* have also been used as simulants including *B. atrophaeus* and *B. subtilis* [10-12]. *Bacillus pumilus* has been phylogenetically placed within the branch for *B. subtilis*, an accepted simulant, based on the sequencing of 16S rDNA [13]. Several criteria have been suggested when considering an appropriate organism to model *B. anthracis* including virulence, genetic and morphologic similarity to *B. anthracis*, and how the simulant responds to challenges from chemicals or the environment [14]. *Bacillus anthracis* has been well-characterized and spore dimensions have been reported [11].

This investigation was undertaken to determine suitability of *B. pumilus* as a simulant for *B. anthracis*. *Bacillus pumilus* was chosen for study for several reasons, including lack of virulence, ease of growth and sporula-

*Corresponding author.

tion as well as its genetic relatedness to other model organisms. Since spore sizes for *B. pumilus* have not been reported, this study is the first to provide spore dimensions. *Bacillus pumilus* is an environmental organism and has been investigated primarily for commercial application of various enzymes it produces [15,16]. Comparisons were made with other currently used simulants, *B. atrophaeus* and *B. thuringiensis*. *Bacillus pumilus* sporulates readily, is non-pathogenic, has comparable spore size with *B. anthracis* and reacts serologically with other simulants suggesting it merits inclusion as another model organism for *B. anthracis.*

2. Materials and Methods

2.1. Organisms and Spore Preparation

Organisms used in these studies included *Bacillus atrophaeus* (ATCC #9372), *B. thuringiensis* (ATCC #10792) and *B. pumilus* (ATCC #700814). *Clostridium sporogenes* (ATCC #3584) was used as a negative control for antibody binding studies. All organisms were inoculated into 5 ml T-soy broth and incubated overnight at 35˚C. *Clostridium sporogenes* was kept under anaerobic conditions for all manipulations. One hundred µl of each broth was spread onto nutrient-deficient AK Agar #2 plates (Becton, Dickinson and Company, Sparks, MD), sealed with parafilm and incubated at 30˚C. For *B. atrophaeus*, *B. pumilus* and *Cl. sporogenes*, plates were evaluated daily for sporulation through 10 d post-inoculation. Samples from *B. thuringiensis* were evaluated daily through 20 d post-inoculation. Optimum sporulation was considered to have occurred when 90% - 95% of organisms had formed spores. Determination of sporulation was made by Schaeffer-Fulton spore stain and light microscopy.

After appropriate incubation, 10 ml of cold phosphate buffered saline was added to each plate for 5 min to loosen spores. Spores were scraped off plates using a sterile loop. The spore suspension was pelleted by centrifugation in a 15 ml centrifuge tube. The pellet was resuspended in 1 ml of sterile deionized water (dH_2O) and transferred to a 1.5 ml microfuge tube. Spores were washed at least five times with dH_2O. The washed spores were resuspended in 1 ml of dH_2O and heated in a heat block to 65˚C for 30 min to destroy any vegetative cells. After a final wash, spores were resuspended in 1 ml of dH_2O and stored at 4˚C.

Spore counts were determined in two ways. Viable counts were determined by spreading dilutions on T-soy agar plates and counting colonies after overnight incubation. Total direct counts were done in a hemocytometer using a phase-contrast microscope. All counts were done at least three times and averages ± SE were determined.

2.2. Electron Microscopy

In preparation for electron microscopy, an aliquot of each spore stock was washed three additional times in dH_2O and diluted in dH_2O resulting in 10, 100 or 500 spores μl^{-1}. One µl of each dilution was placed on an aluminum specimen stage and allowed to dry completely under a laminar flow hood. The spores were coated with an 8 nm layer of gold (Hummer 6.2 Sputter Coater, Ladd Research, Williston, VT) prior to scanning in a Hitachi SEM 3400 (Hitachi High-Technologies, Tokyo). An accelerated voltage between 15 and 20 kV was applied. A minimum of 100 spores from each organism were evaluated. Image J software was used to measure spores [17].

2.3. Microarray Preparation and Detection

In order to evaluate multiple antibodies binding with spores from different organisms, spore microarrays were developed. In this process, spores from each *Bacillus* spp. and *Cl. sporogenes* were applied to slides as an array. Appropriate spore dilutions of each organism were prepared in spotting solution (ArrayIt, Sunnyvale, CA) such that 100 spores were applied in triplicate as a microarray to an epoxy-coated slide. Microarrays were prepared with a SMP8 pin guided by a SpotBot microarray robot (ArrayIt). The microarray was allowed to air dry at room temperature for at least two h. The slide was heated at 95˚C for 25 min to ensure fixation of the spore samples to the slide. The slide was placed in a UV crosslinker (Spectronics Corporation, Westbury, NY) for two cycles (6500 $\mu J \cdot cm^{2-1}$) to deactivate any remaining epoxy functional groups. The slide was washed with 0.1% sodium dodecyl sulfate (SDS) for 5 min followed by washing with dH_2O for 3 min. ArrayIt blocker was applied to the edge of the slide and a coverslip was placed over it yielding 1 µl blocker mm^{2-1} coverslip. The slide was incubated at room temperature for one h, after which the coverslip was removed for additional SDS and dH_2O washes and drying cycles performed in the same manner as described above.

The primary monoclonal anti-*B. anthracis* was obtained from Virostat (Portland, ME). All other primary anti-*Bacillus* antibodies were purchased from Tetracore (Rockville, MD). Secondary fluorescently labeled antibodies were acquired from Jackson ImmunoResearch Laboratories (West Grove, PA). Primary antibody was diluted 1:50 in a 1:1 mixture of dH_2O and blocker and added to the array and covered with a coverslip as 1 µl antibody dilution mm^{2-1} coverslip. The slide was incubated at 35˚C for 30 min. Following incubation, the slide was washed with 0.1% SDS and dH_2O as before. The secondary fluorescently labeled antibody was diluted 1:100 and applied, incubated and washed in identical fashion as the primary antibody. The dry slide was analyzed for fluorescence using a confocal laser scanner (Genetix Ltd., Queensway, Hampshire, UK).

3. Results

3.1. Sporulation

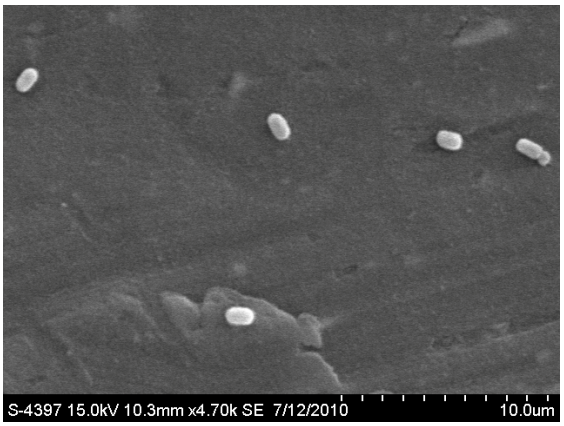
AK Agar #2 proved to be an ideal medium to promote sporulation for most organisms used in these studies. *Bacillus pumilus* yielded the highest number of spores, for both viable plate count ($2.92 \pm 0.19 \times 10^{10}$) and total direct count ($4.58 \pm 0.42 \times 10^{10}$) (**Table 1**). Optimum sporulation occurred on d 6 or 8 post-inoculation for *B. atrophaeus*, *B. pumilus* and *Cl. sporogenes*. *Bacillus thuringiensis* required 16 d post-inoculation to demonstrate 90% sporulation. The number of spores produced by *B. thuringiensis* was the lowest for all organisms (viable: $6.98 \pm 0.13 \times 10^{8}$, direct: $1.19 \pm 0.11 \times 10^{9}$). The percent viability among the *Bacilli* was similar, ranging from 59 - 64. *Clostridium sporogenes* spores exhibited a lower viability at 36%.

3.2. SEM Spore Comparisons

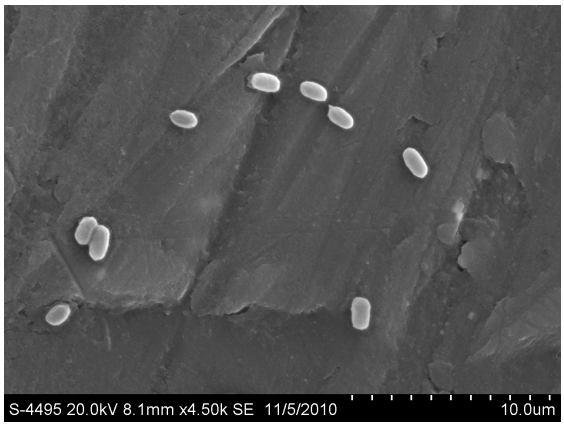
Electron micrographs of each of the *Bacilli* are seen in **Figure 1**. Spores of each *Bacillus* were measured using Image J. Length and width dimensions are summarized in **Table 2**. **Table 2** also lists spore dimensions reported by others. The values for each of the *Bacilli* investigated in this study represent averages ± SE of a minimum of 100 spore measurements. *Bacillus atrophaeus* spores were 1.314 ± 0.018 μm in length and 0.752 ± 0.013μm in width. *Bacillus pumilus* spores were 1.242 ± 0.022 μm

Table 1. Time for optimum sporulation and enumeration of spores.

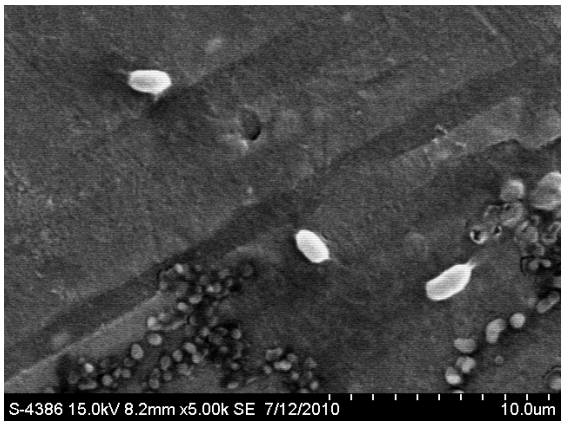
Organism	Optimum Day	Plate Count	Total Count	% Viability
B. atrophaeus	8	$8.66 \pm 0.29 \times 10^{9}$	$1.37 \pm 0.14 \times 10^{10}$	63
B. pumilus	8	$2.92 \pm 0.19 \times 10^{10}$	$4.58 \pm 0.42 \times 10^{10}$	64
B. thuringiensis	16	$6.98 \pm 0.13 \times 10^{8}$	$1.19 \pm 0.11 \times 10^{9}$	59
Cl. sporogenes	6	$1.02 \pm 0.14 \times 10^{9}$	$2.81 \pm 0.14 \times 10^{9}$	36



Bacillus atrophaeus



Bacillus pumilus



Bacillus thuringiensis

Figure 1. Scanning electron micrograph of *Bacillus* spp. spores. The image at the top left is *B. atrophaeus*. Spores from *B. pumilus* are at the top right and *B. thuringiensis* is at the bottom.

Table 2. Comparison of *Bacillus* spore dimensions.

Source	*B. anthracis*	*B. atrophaeus*	*B. pumilus*	*B. thuringiensis*
Buhr [22]		1.21 × 0.68[a]		
Carrera [11]	≤1.26 × 0.81 - 0.86 1.49 - 1.67 × 0.81 - 0.86	1.22 × 0.65		1.61 × 0.80
Fazzini [23]	1.63 × 0.97			
Plomp [21]		1.68 × 0.65		2.17 × 0.94
This study		1.31 × 0.75	1.24 × 0.59	1.58 × 0.75

[a]Dimensions are given as length × width, in μm.

in length by 0.594 ± 0.016 μm in width. *Bacillus thuringiensis* spores were 1.580 ± 0.024 μm in length and 0.754 ± 0.022 μm in width.

3.3. Serologic Reactivity

When evaluating a potential anthrax simulant organism, it is important to consider how the organism lends itself to detection. Binding with antibody through immunoassays is a common approach for detection. Both polyclonal and monoclonal commercially available antibodies were used to evaluate binding among the organisms in this study. All polyclonal antisera bound with the *Bacilli* under investigation (**Table 3**). No binding occurred between any anti-*Bacillus* antisera and the negative control *Cl. sporogenes*. The monoclonal anti-*B. anthracis* was highly specific and failed to bind with any *Bacilli* used in this study. Similarly, monoclonal anti-*B. thuringiensis* was specific and bound only with *B. thuringiensis*. *Bacillus pumilus* displayed reactivity patterns in almost identical fashion as *B. atrophaeus* and even bound with monoclonal anti-*B. atrophaeus*.

4. Discussion

It is likely that the potential for misuse of *B. anthracis* as a bioweapon will continue. This threat requires vigilance on the part of medical and research personnel. Due to biosafety and containment issues, it is highly desirable to use organisms that can serve as model simulants for *B. anthracis* as new detection technologies are developed. *Bacillus pumilus* shows promise as a new anthrax surrogate.

AK Agar #2 was an ideal medium to promote sporulation for *B. atrophaeus*, *B. pumilus* and *Cl. sporogenes*. *Bacillus pumilus* produced the highest number of spores of all organisms in this study. However, AK Agar was not as effective at promoting spore formation for *B. thuringiensis*. With this medium, *B. thuringiensis* yielded the lowest number of spores and required 16 days to achieve 90% sporulation. Successful sporulation of *B. thuringiensis* has been reported using nutrient broth yeast extract agar [9], T-soy agar supplemented with 5% sheep blood [6] and sporulation medium S [18].

The percent viability exhibited by the *Bacilli* cultured on T-soy agar following spore formation on AK Agar #2 was similar with an average just over 60%. In our hands, *Cl. sporogenes* was nearly half that at 36%. Yang reported [19] that the viability of *Cl. sporogenes* cultured on T-soy was 30%.

Some variability is evident among reports of spore sizes (**Table 2**). An explanation for this variation may be the amount of water remaining in the spore during preparation [20,21]. Additionally, the method used to determine spore dimensions would impact measurements. Spore dimensions reported by Buhr [22] were based on phase microscopy. Scanning electron microscopy has been used [23, this study] and Carrera [11] used transmission electron microscopy. The measurements made by Plomp [21], resulting in the largest reported spore sizes, were based on atomic force microscopy. Spore dimensions for *B. atrophaeus* in this study (1.31 × 0.75 μm) are in agreement with other reports [11,22] (1.22 × 0.65 μm and 1.21 × 0.68 μm, respectively). Our measurements for *B. thuringiensis* were also similar with dimensions reported by Carrera [11] (1.58 × 0.75 μm vs 1.61 × 0.80 μm). To our knowledge, this is the first report of spore dimensions for *B. pumilus*. One criterion for an appropriate simulant for *B. anthracis* is spore size [14]. Carrera reported [11] that spores of *B. anthracis* fall into two size categories, a larger size of 1.49 - 1.67 μm × 0.81 - 0.86 μm and a smaller spore size of less than 1.26 μm × 0.81 - 0.86 μm. We determined that *B. pumilus* spores represent the smaller sized category with dimensions of 1.24 × 0.59 μm.

There are risks associated with working with *B. anthracis* and inactivation of spores by heat, chemicals or radiation may alter antigenicity [6]. Many detection assays are immunologically centered. An inherent problem with antibody based detection is the high level of cross-reactivity that occurs among *Bacilli*, particularly when using polyclonal antisera [24-26]. Such cross-reactivity was evident in the current study (**Table 3**). Based on our

Table 3. Anti-*Bacillus* antibody reactivity.

Antibody	*B. atrophaeus*	*B. pumilus*	*B. thuringiensis*	*Cl. sporogenes*
Polyclonal anti-*B. atrophaeus*	+++[a]	+++	++	—
Monoclonal anti-*B. atrophaeus*	+++	++	+	—
Polyclonal anti-*B. thuringiensis*	++	++	+++	—
Monoclonal anti-*B. thuringiensis*	—	—	++	—
Polyclonal anti-*B. anthracis*	+	+	++	—
Monoclonal anti-*B. anthracis*	—	—	—	—

[a]+++ = strong binding, ++ = moderate binding, + = weak binding, — = no binding, as determined by fluorescence.

results of antibody binding, there are several noteworthy observations. First, *Cl. sporogenes* appears to be an appropriate negative control spore forming organism, helping avoid false positive results. Secondly, immunoassays used for development of anthrax spore detection must employ specific monoclonal antibodies similar to those used in the current study for *B. thuringiensis* and perhaps *B. anthracis*. Finally, the reactivity patterns shown by *B. pumilus* suggest it could serve as an appropriate model organism, binding with antibody to *B. atrophaeus* in nearly identical manner.

Bacillus pumilus also may represent a useful anthrax simulant since it is rarely implicated as a cause of disease. There is one report of *B. pumilus* being responsible for cutaneous infection in humans [27]. In that report, the authors noted that the lesions were similar to lesions that occur during cutaneous anthrax infection. However, it was also stated that infection of humans by *B. pumilus* was "exceptional". *Bacillus pumilus* seems to represent an appropriate organism to model *B. anthracis*. It sporulates to high titer readily, produces spores of similar size as *B. anthracis* and may be considered a non-pathogenic organism.

5. Acknowledgements

This work was supported, in part, by the Department of Homeland Security through the Southeast Region Research Initiative by contract number 4000086311.

REFERENCES

[1] J. Hang, A. K. Sundaram, P. Zhu, D. R. Shelton, J. S. Karns, P. A. W. Martin, S. Li, P. Amstutz and C.-M. Tang, "Development of a Rapid and Sensitive Immunoassay for Detection and Subsequent Recovery of *Bacillus anthracis* Spores in Environmental Samples," *Journal of Microbiological Methods*, Vol. 73, No. 3, 2008, pp. 242-246. doi:10.1016/j.mimet.2008.02.018

[2] T. V. Inglesby, T. O'Toole and D. A. Henderson, "Preventing the Use of Biological Weapons: Improving Response Should Prevention Fail," *Clinical Infectious Diseases*, Vol. 30, No. 6, 2000, pp. 926-929. doi:10.1086/313794

[3] J. B. Wilson and K. E. Russell, "Isolation of *Bacillus anthracis* from Soil Stored 60 Years," *Journal of Bacteriology*, Vol. 87, 1964, pp. 237-238.

[4] T. J. Cieslak and E. M. Eitzen, Jr., "Clinical and Epidemiologic Principles of Anthrax," *Emerging Infectious Diseases*, Vol. 5, No. 4, 1999, pp. 552-555. doi:10.3201/eid0504.990418

[5] M. S. Traeger, S. T. Wiersma, N. E. Rosenstein, J. M. Malecki, C. W. Shepard, P. L. Raghunathan, S. P. Pillai, T. Popovic, C. P. Quinn, R. F. Meyer, S. R. Zaki, S. Kumar, S. M. Bruce, J. J. Sejvar, P. M. Dull, B. C. Tierney, J. D. Jones, B. A. Perkins and the Florida Investigation Team, "First Case of Bioterrorism-Related Inhalational Anthrax in the United States, Palm Beach County, Florida, 2001," *Emerging Infectious Diseases*, Vol. 8, No. 10, 2002, pp. 1029-1034. doi:10.3201/eid0810.020354

[6] J. L. Dang, K. Heroux, J. Kearney, A. Arasteh, M. Gostomski and P. A. Emanuel, "*Bacillus* Spore Inactivation Methods Affect Detection Assays," *Applied and Environmental Microbiology*, Vol. 67, No. 8, 2001, pp. 3665-3670. doi:10.1128/AEM.67.8.3665-3670.2001

[7] N. J. Tourasse, E. Helgason, O. A. Okstad, I. K. Hegna and A. B. Kolsto, "The *Bacillus cereus* Group: Novel Aspects of Population Structure and Genome Dynamics," *Journal of Applied Microbiology*, Vol. 101, No. 3, 2006, pp. 579-593. doi:10.1111/j.1365-2672.2006.03087.x

[8] E. Helgason, O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna and A.-B. Kolsto, "*Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*—One Species on the Basis of Genetic Evidence," *Applied and Environmental Microbiology*, Vol. 66, No. 6, 2000, pp. 2627-2630. doi:10.1128/AEM.66.6.2627-2630.2000

[9] V. G. DelVecchio, J. P. Connolly, T. G. Alefantis, A. Walz, M. A. Quan, G. Patra, J. M. Ashton, J. T. Whittington, R. D. Chafin, X. Liang, P. Grewal, A. S. Khan and C. V. Mujer, "Proteomic Profiling and Identification of Immunodominant Spore Antigens of *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*," *Applied and Environmental Microbiology*, Vol. 72, No. 9, 2006, pp. 6355-6363. doi:10.1128/AEM.00455-06

[10] E. T. Arakawa, N. V. Lavrik and P. G. Datskos, "Detection of Anthrax Simulants with Microcalorimetric Spec-

troscopy: *Bacillus subtilis* and *Bacillus cereus* Spores," *Applied Optics*, Vol. 42, No. 10, 2003, pp. 1757-1762. doi:10.1364/AO.42.001757

[11] M. Carrera, R. O. Zandomeni, J. Fitzgibbon and J.-L. Sagripanti, "Difference between the Spore Sizes of *Bacillus anthracis* and Other *Bacillus* Species," *Journal of Applied Microbiology*, Vol. 102, No. 2, 2007, pp. 303-312. doi:10.1111/j.1365-2672.2006.03111.x

[12] M. Laue and N. Bannert, "Detection Limit of Negative Staining Electron Microscopy for the Diagnosis of Bioterrorism-Related Micro-Organisms," *Journal of Applied Microbiology*, Vol. 109, No. 4, 2010, pp. 1159-1168. doi:10.1111/j.1365-2672.2010.04737.x

[13] S. Porwal, S. Lai, S. Cheema and V. P. Kalia, "Phylogeny in Aid of the Present and Novel Microbial Lineages: Diversity in *Bacillus*," *PLoS One*, Vol. 4, No. 2, 2009, p. e4438. doi:10.1371/journal.pone.0004438

[14] D. L. Greenberg, J. D. Busch, P. Keim and D. M. Wagner, "Identifying Experimental Surrogates for *Bacillus anthracis* Spores: A Review," *Investigtive Genetics*, Vol. 1, No. 1, 2010, pp. 4-16. doi:10.1186/2041-2223-1-4

[15] K. S. Ibrahim, J. Muniyandi and A. Karutha-Pandian, "Purification and Characterization of Manganese-Dependent Alkaline Serine Protease from *Bacillus pumilus* TMS55," *Journal of Microbiology and Biotechnology*, Vol. 21, No. 1, 2011, pp. 20-27. doi:10.4014/jmb.1009.09001

[16] R. Reiss, J. Ihssen and L. Thony-Meyer, "*Bacillus pumilus* Laccase: A Heat Stable Enzyme with a Wide Substrate Spectrum," *BMC Biotechnology*, Vol. 11, No. 9, 2011, pp. 9-20. doi:10.1186/1472-6750-11-9

[17] National Institutes of Health, "Image J: Image Processing and Analysis in Java". http://rsb.info.nih.gov/ij/

[18] M. Carrera, R. O. Zandomeni and J.-L. Sagripanti, "Wet and Dry Density of *Bacillus anthracis* and Other *Bacillus* Species," *Journal of Applied Microbiology*, Vol. 105, No. 1, 2008, pp. 68-77. doi:10.1111/j.1365-2672.2008.03758.x

[19] W.-W. Yang, E. N. Crow-Willard and A. Ponce, "Production and Characterization of Pure *Clostridium* Spore Suspensions," *Journal of Applied Microbiology*, Vol. 106, No. 1, 2009, pp. 27-33. doi:10.1111/j.1365-2672.2008.03931.x

[20] A. J. Westphal, P. B. Price, T. J. Leighton and K. E. Wheeler, "Kinetics of Size Changes of Individual *Bacillus thuringiensis* Spores in Response to Changes in Relative Humidity," *Proceedings of the National Academy of Sciences*, Vol. 100, No. 6, 2003, pp. 3461-3466. doi:10.1073/pnas.232710999

[21] M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin, "The High-Resolution Architecture and Structural Dynamics of *Bacillus* Spores," *Biophysical Journal*, Vol. 88, No. 1, 2005, pp. 603-608. doi:10.1529/biophysj.104.049312

[22] T. L. Buhr, D. C. McPherson and B. W. Gutting, "Analysis of Broth-Cultured *Bacillus atrophaeus* and *Bacillus cereus* Spores," *Journal of Applied Microbiology*, Vol. 105, No. 5, 2008, pp. 1604-1613. doi:10.1111/j.1365-2672.2008.03899.x

[23] M. M. Fazzini, R. Schuch and V. A. Fischetti, "A Novel Spore Protein, ExsM, Regulates Formation of the Exosporium in *Bacillus cereus* and *Bacillus anthracis* and Affects Spore Size and Shape," *Journal of Bacteriology*, Vol. 192, No. 15, 2010, pp. 4012-4021. doi:10.1128/JB.00197-10

[24] P. Longchamp and T. Leighton, "Molecular Recognition Specificity of *Bacillus globigii* Spore Antibodies," *Letters in Applied Microbiology*, Vol. 31, No. 3, 2000, pp. 242-246. doi:10.1046/j.1365-2672.2000.00808.x

[25] A. P. Phillips and K. L. Martin, "Investigation of Spore Surface Antigens in the Genus *Bacillus* by the Use of Polyclonal Antibodies in Immunofluorescence Tests," *The Journal of Applied Bacteriology*, Vol. 64, No. 1, 1988, pp. 47-55. doi:10.1111/j.1365-2672.1988.tb02428.x

[26] J. J. Quinlan and P. M. Foegeding, "Monoclonal Antibodies for Use in Detection of *Bacillus* and *Clostridium* Spores," *Applied and Environmental Microbiology*, Vol. 63, No. 2, 1997, pp. 482-487.

[27] D. Tena, J. A. Martinez-Torres, M. T. Perez-Pomata, J. A. Saez-Nieto, V. Rubio and J. Bisquert, "Cutaneous Infection Due to *Bacillus pumilus*: Report of 3 Cases," *Clinical and Infectious Diseases*, Vol. 44, No. 4, 2007, pp. 40-42. doi:10.1086/511077

