

An Old Bug With A New Trick? You Cannot Be Serious!

**Philip A. Lee, MSc FIBMS
Lead Biological Defense Coordinator
Bureau of Public Health Laboratories
Jacksonville, Florida**



Patient Presents: March 1st, 2013

70 y.o. Male patient RG

- Large non-painful swelling on face
 - Developed over past 7-9 days
 - Black eschar
- Resident of Marion County
 - Near Lake County boundary
- No travel history
- Reported touching spot on face when moving a horse
 - Contaminated wound with soil?
- Admitted to Hospital A



Differential Diagnosis



- Brown recluse spider bite
- Cutaneous anthrax
- Ulceroglandular tularemia
- Accidental vaccinia virus
- Necrotic herpes simplex virus
- Glanders (*Burkholderia mallei*)
- Cutaneous skin infection
 - e.g. Orf (parapox virus)
 - e.g. Ecthyma (bacterial)

Antimicrobial Therapy

March 1st

- Vancomycin serum trough levels 10 mg/dL
- Ciprofloxacin 400 mg IV BID
- Doxycycline 100 mg PO BID

March 3rd

- Lesion progression continued
- Added clindamycin 600 mg IV q 8 hrs

• • • • • • • •

March 5th

- No change in medications

• • • • • • • • eschar area

Microbiology – Sentinel Lab

March 4th

Culture isolation

- Gram-positive, broad rod, catalase-positive, spore-forming, aerobe

Genus = *Bacillus*

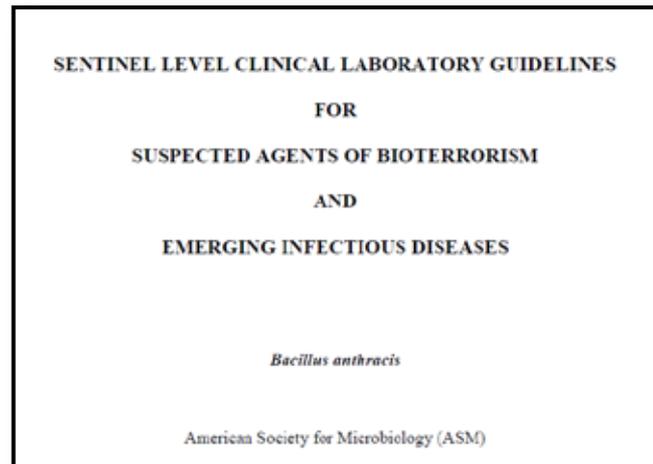
- Ground glass colony morphology

Sentinel laboratory ruled out *Bacillus anthracis*

- Beta hemolytic
- Motile



AMERICAN
SOCIETY FOR
MICROBIOLOGY



Clinical Diagnosis

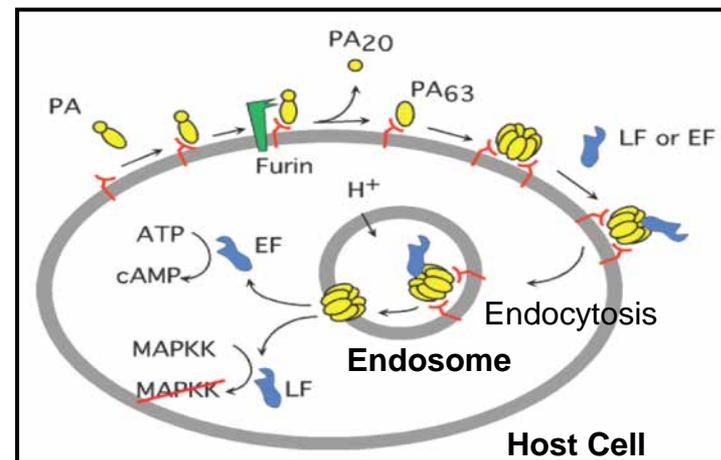
March 4th

- BPHL notified by Medical Epidemiologist at the FL DOH Bureau of Epidemiology
- Physician determined diagnosis

Microbiology – Reference Lab

March 5th

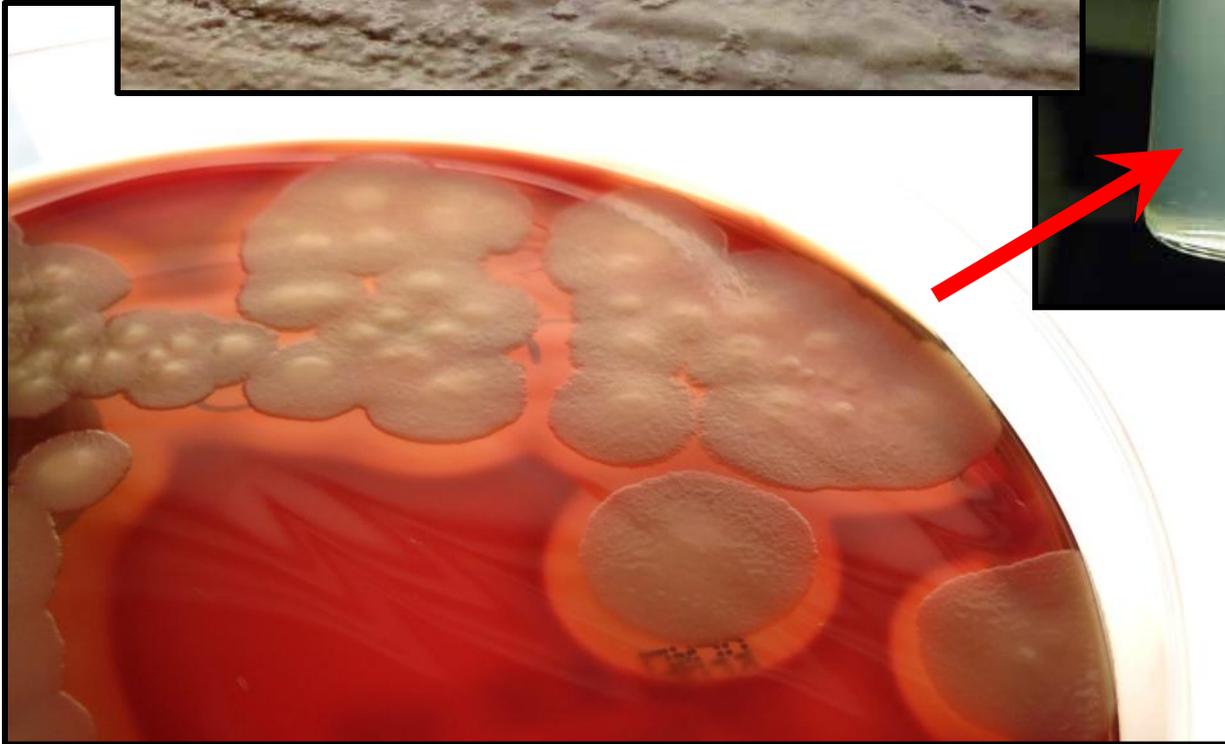
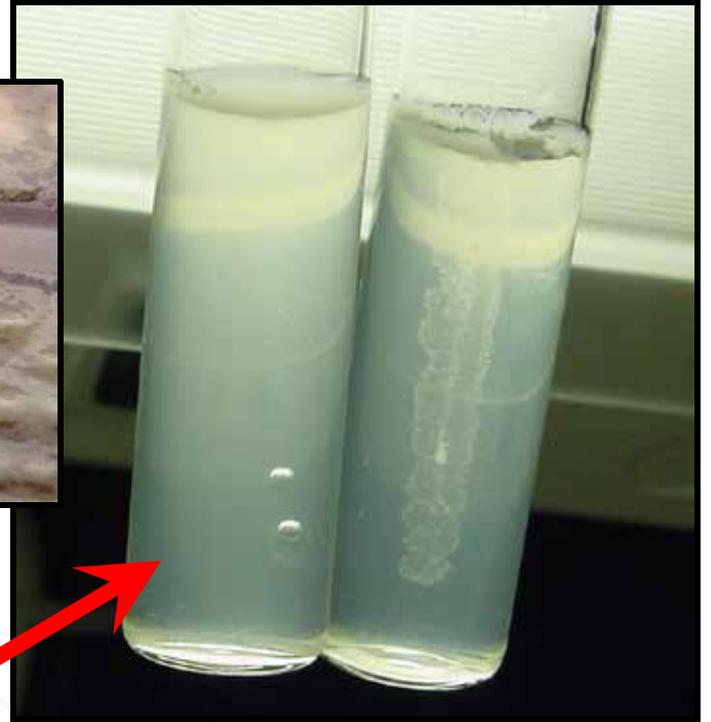
- Specimens received at BPHL-Jacksonville
- Performed LRN *Bacillus anthracis* real-time PCR
 - *B. anthracis* DNA not detected per LRN algorithm
 - Requires detection of 3 *B. anthracis*-specific targets
 - Detected target for *B. anthracis* 182 kb plasmid pXO1
 - Encodes *B. anthracis* toxin genes
 - » Lethal Factor
 - » Edema Factor
 - » Protective Antigen
 - Consulted CDC



Microbiology – Reference Lab

March 6th- 8th

- Conventional microbiology
 - LRN reference level assays confirmed sentinel laboratory results
 - Ruled out *Bacillus anthracis*
 - Isolate identified as *Bacillus cereus*
- Isolate shipped to CDC



Susceptibility Results

ANTIMICROBIC	MIC (mg/mL)	INTERPRETATION
Amikacin		S
Ampicillin	8	R
Cefazolin	8	S
Cefotaxime	32	I
Ceftazidime	>128	R
Ceftriaxone	16	I
Chloramphenicol	4	S
Ciprofloxacin	• • •	S
Clindamycin	• • •	S
Erythromycin	• • •	S
Gentamicin	0.5	S
Imipenem		

Additional Specimens

Plasma collected March 2nd

– During peak illness

Serum collected March 14th

– Acute

Serum collected April 4th

– Convalescent



CDC Results – National Lab

- Concur *Bacillus cereus*
- Positive for all 3 *Bacillus anthracis* toxin genes
- Negative for pXO2 and pBC218
 - Plasmids encoding capsule genes
- Positive for capsule
 - Mechanism unknown
 - Genome sequencing required
- Multi-locus sequence type ST 78
 - Same as *B. cereus* G9241

CDC Results

Serologic testing

- Anti-Protective Antigen ELISA
 - March 2nd plasma: Not detected
 - March 14th serum: 18.2 µg/ml Anti-PA IgG
 - April 4th serum: 48.0 µg/ml Anti-PA IgG
 - Toxin neutralizing activity

Additional testing

- MALDI-TOF Mass Spectrometry LF assay
 - Sera below LOD (<0.005 ng/ml)
 - March 2nd plasma: 0.819 ng/ml
 - Similar levels to cutaneous anthrax cases

G9241

- Associated with severe pneumonia in a welder from Louisiana in 1994
- Circular plasmid pBCXO1 with 99.6% similarity with the *B. anthracis* toxin-encoding plasmid pXO1
- Capsule encoded by plasmid pBC218
- Similar strains caused two fatal pneumonia cases in Texas metal workers in 2003

Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax

Alax R. Hoffmaster^{1*}, Jacques Ravel^{1†}, David A. Rasko^{1†}, Gail D. Chapman¹, Michael D. Chute¹, Chung K. Marston¹, Barun K. Das¹, Claudio T. Sacchi¹, Collette Fitzgerald¹, Leonard W. Mayar¹, Martin C. J. Maiden¹, Fergus G. Priest¹, Margaret Barker¹, Lingxia Jiang¹, Regina Z. Cer¹, Jennifer Rilstone¹, Scott N. Peterson¹, Robbin S. Woyant¹, Darrall R. Galloway¹, Timothy D. Read^{1§}, Tanja Popovic^{1*}, and Claire M. Fraser^{1**}

¹Epidemiologic Investigations Laboratory, Meningitis and Special Pathogens Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, NE, Atlanta, GA 30333; ²Microbial Genomics and Pathogen Functional Genomics Resource Center, Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850; ³Visa Peter Medawar Building for Pathogen Research and Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom; ⁴School of Life Sciences, Heriot Watt University, Edinburgh EH14 4AS, United Kingdom; and ⁵Biological Defense Research Directorate, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910

Communicated by John B. Robbins, National Institutes of Health, Bethesda, MD, April 5, 2004 (received for review March 24, 2004)

Bacillus anthracis is the etiologic agent of anthrax, an acute fatal disease among mammals. It was thought to differ from *Bacillus cereus*, an opportunistic pathogen and cause of food poisoning, by the presence of plasmids pXO1 and pXO2, which encode the lethal toxin complex and the poly- γ -D-glutamic acid capsule, respectively. This work describes a non-*B. anthracis* isolate that possesses the anthrax toxin genes and is capable of causing a severe inhalation anthrax-like illness. Although initial phenotypic and 16S rRNA analysis identified this isolate as *B. cereus*, the rapid generation and analysis of a high-coverage draft genome sequence revealed the presence of a circular plasmid, named pBCXO1, with 99.6% similarity with the *B. anthracis* toxin-encoding plasmid, pXO1. Although homologues of the pXO2 encoded capsule genes were not found, a polysaccharide capsule cluster is encoded on a second, previously unidentified plasmid, pBC218. A mouse challenged with *B. cereus* G9241 confirmed the virulence of this strain. These findings represent an example of how genomics could rapidly assist public health experts responding not only to clearly identified select agents but also to novel agents with similar pathogenic potential. In this study, we combine a public health approach with genome analysis to provide insight into the correlation of phenotypic characteristics and their genetic basis.

Bacillus cereus and Bacillus anthracis are members of a closely related phylogenetic cluster referred to as the *B. cereus* group. Although classification of *B. anthracis* and *B. cereus* as separate species based on molecular analysis has been questioned, they differ in phenotype and in the diseases they cause (1–4). *B. cereus* is ubiquitous in nature and is an opportunistic pathogen. In individuals with underlying conditions, immunocompromised individuals, or patients recovering from surgery, *B. cereus* has been known to cause a variety of infections, including endophthalmitis, bacteremia, septicemia, endocarditis, salpingitis, cutaneous infections, pneumonia, and meningitis (5). In contrast, *B. anthracis* is the etiologic agent of anthrax, an acute fatal disease among mammals, which in recent years has become known for its use as a biological weapon (6). The genes encoding the major anthrax toxins and the poly- γ -D-glutamic acid capsule are located on two virulence plasmids, pXO1 (182 kb) and pXO2 (96 kb), respectively, and are required for full virulence (7–9).

Materials and Methods

Genomic Sequencing. Genomic DNA extraction of *B. cereus* G9241 was performed as the Centers for Disease Control and Prevention. Random shotgun sequencing was undertaken by using a strategy similar to previous projects (10). Random insert libraries of 2.5–4.5 kb and 4.5–9.0 kb were constructed, and 93,562

high-quality sequences were obtained (817-bp average read length). A 13.5 \times coverage draft genome sequence was obtained and assembled by using the Celera assembler (11). The assembly consisted of 131 contigs that were ordered and concatenated with our prior knowledge of the *B. anthracis* and *B. cereus* genome sequences (10, 12). The resulting 5,296,464-bp sequence was annotated through the Institute for Genomic Research Bioinformatics pipeline as reported (13). The genomic features of this molecule are described in Table 2, which is published as supporting information on the PNAS web site.

Capsule Visualization. *B. cereus* was streaked onto trypticase soy agar containing 5% sheep blood (Becton Dickinson Microbiology Systems) and incubated in ambient atmosphere overnight at 37°C. *B. anthracis* was streaked onto trypticase soy agar containing 0.8% sodium bicarbonate and incubated in 5% CO₂ at 37°C. Cells from a single colony were dispersed into 5 μ l of water on a microscope slide and covered with a coverslip. A single drop of India ink (Remel, Lenexa, KS) was applied to the edge of the coverslip and allowed to diffuse into the sample. The cells were visualized with a \times 100 oil-immersion objective (14).

Biochemical and Microbiological Phenotypic Characterization. Motility was observed by wet mount of cells grown in heart infusion broth and by using Motility Test medium with triphenylcarazolium chloride (Remel). Susceptibility to γ -phage (15) was performed by adding 5 μ l of γ -phage to the first and second quadrants of a 5% sheep blood plate streaked for isolation and incubated at 37°C overnight. The *B. anthracis* specific two-component direct fluorescent Ab (DFA) assay was performed as described by De et al. (16). Sequencing of the 16S rRNA gene was performed as described by Sacchi et al. (17).

Comparative Genomic Hybridization (CGH). CGH was performed as described for other *B. cereus* isolates by Read et al. (10).

Multiple Locus Sequence Typing. Phylogenetic tree of members of the *B. cereus sensu lato* group was generated based on partial nucleotide sequences of seven housekeeping genes, totaling

Abbreviations: CGH, comparative genomic hybridization; PA, protective antigen; DFA, direct fluorescent Ab.

Data deposition: The Whole Genome Shotgun project reported in this work has been deposited in the GenBank database (accession no. AA030000000). Theoretical derived from this paper is the first version, AA030000000.

*A.R.H., H.D., D.A.R., T.D.R., T.P., and C.M.F. contributed equally to this work.

**To whom correspondence should be addressed. E-mail: on-train@cdc.gov.

© 2004 by The National Academy of Sciences of the USA.

www.pnas.org/cgi/doi/10.1073/pnas.0402414101

PNAS | June 1, 2004 | vol. 101 | no. 22 | 8449–8454

Hoffmaster AR, Ravel J, Rasko DA, et al. 2004. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. PNAS. 101:8449–54.



Conclusion

First report of a *Bacillus cereus* isolate harboring *B. anthracis* toxin genes associated with a naturally occurring cutaneous anthrax-like infection with demonstrated Lethal Factor toxemia and a detectable humoral response to the toxin with *in vitro* toxin neutralizing activity.

Acknowledgements

- Danielle Stanek, DVM
Medical Epidemiologist
Florida Department of Health
Bureau of Epidemiology
- Nancy Pickens, MPH & George Churchwell, MPH
Florida Department of Health
Bureau of Public Health Laboratories
- Alex Hoffmaster, PhD & Chung Marston, PhD
Zoonoses and Select Agent Laboratory
Bacterial Special Pathogens Branch
Division of High-Consequence Pathogens & Pathology
Centers for Disease Control and Prevention

