Rapidly Progressive, Fatal, Inhalation Anthrax-like Infection in a Human

Case Report, Pathogen Genome Sequencing, Pathology, and Coordinated Response

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• ***Context.*—Ten years ago a bioterrorism event involving *Bacillus anthracis* spores captured the nation's interest, stimulated extensive new research on this pathogen, and heightened concern about illegitimate release of infectious agents. Sporadic reports have described rare, fulminant, and sometimes fatal cases of pneumonia in humans and nonhuman primates caused by strains of *Bacillus cereus*, a species closely related to *Bacillus anthracis*.**

***Objectives.*—To describe and investigate a case of rapidly progressive, fatal, anthrax-like pneumonia and the overwhelming infection caused by a *Bacillus* species of uncertain provenance in a patient residing in rural Texas.**

***Design.*—We characterized the genome of the causative strain within days of its recovery from antemortem cultures using next-generation sequencing and performed immunohistochemistry on tissues obtained at autopsy with antibodies directed against virulence proteins of *B anthracis* and *B cereus*.**

***Results.*—We discovered that the infection was caused by a previously unknown strain of *B cereus* that was closely related to, but genetically distinct from, *B anthracis*. The strain contains a plasmid similar to pXO1, a genetic element encoding anthrax toxin and other known virulence factors. Immunohistochemistry demonstrated that several homologs of *B anthracis* virulence proteins were made in infected tissues, likely contributing to the patient's death.**

***Conclusions.*—Rapid genome sequence analysis permitted us to genetically define this strain, rule out the likelihood of bioterrorism, and contribute effectively to the institutional response to this event. Our experience strongly reinforced the critical value of deploying a well-integrated, anatomic, clinical, and genomic strategy to respond rapidly to a potential emerging, infectious threat to public health.**

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On September 18, 2001, letters containing spores of the Ames strain of *Bacillus anthracis* were mailed to the offices of news media. Additional letters containing these spores, postmarked October 9, 2001, were sent to 2 US Senators. Five people died, and 17 others were infected but survived.[1–3] This bioterrorism attack resulted in significant new federal support and controls for research on so-called select-agent infectious pathogens, such as *B anthracis*. Importantly, many new specialized laboratories were built to facilitate basic and translational research on select agents. These new facilities, accompanied by significant support from the National Institutes of Health, were also designed to improve training and to assist rapid response to putative or bona fide bioterrorism events or unexpected emergence of new human pathogens.

A decade of accelerated research on *B anthracis* and the very closely related organisms *Bacillus cereus* and *Bacillus thuringiensis* have yielded extensive new information about disease pathogenesis and interspecies and intraspecies genetic variation.[4–21] We now know that, compared with many other bacterial pathogens, relatively little genetic variation exists among strains of *B anthracis* recovered from global sources.[6,8] Similarly, many strains of *B cereus* and *B thuringiensis* are also closely related to one another.[7,12–14,17–19] Although most strains of *B anthracis* and *B cereus* are genetically distinct from one another, genome sequencing and other less-sophisticated genetic analyses have discovered that certain strains of *B cereus* and *B thuringiensis* are phylogenetically more closely related to *B anthracis* than they are to other strains of their same species designation.[12–14] These strains have been reported to contain genes encoding extracellular molecules

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Primers Used to Amplify Genes of Interest			
Gene	**Primer**	**Sequence**	**Use**
plcR	Forward	ATTTATCCATATATTATGCAA	*plcR* gene sequencing for SNP analysis
	Reverse	TTCACATTATTGTAGTGGTAT	
cyt	Forward	AATACATTTCAAGGAGCTA	Identify the presence or absence of the gene encoding the *Bacillus thuringiensis* Cyt toxin
	Reverse	TTTCATTTTAACTTCATATC	
cry	Block 1–5 forward	TATGCWCAAGCWGCCAATYTWCATYT	Identify the presence or absence of the gene encoding the *B thuringiensis* Cry toxin
	Block 1–5 reverse	GGRATAAATTCAATTYKRTCWA	
	Block 2–5 forward	TTTAGATATTGTTGCAWTATKKY	
	Block 2–5 reverse	GGRATAAATTCAATTYKRTCWA	
	Block 1–4 forward	TATGCWCAAGCWGCCAATYTWCATYT	
	Block 1–4 reverse	CATAACGTAGWYTTAYCTKAWT	
lef	Forward	TGCCTTTAATTTATGAGGAAATAAGTAA	Identify the presence or absence of the gene encoding the *Bacillus anthracis* lethal factor
	Reverse	TTTCATATCTTGCCAGCATCCG	
pagA	Forward	GTTATATATTTATAAAAGTTCTGTTTAAAAAGCC	Identify the presence or absence of the gene encoding the *B anthracis* protective antigen
	Reverse	GGTGTCTTGCCTCTGGTG	
cya	Forward	CCATAAAACCGTAAATGTGATTTC	Identify the presence or absence of the gene encoding the *B anthracis* edema factor
	Reverse	TCAAACATGTCGGGGGCATATAA	
glpF	Forward	GCGTTTGTGCTGGTGTAAGT	MLST
	Reverse	CTGCAATCGGAAGGAAGAAG	
gmk	Forward	ATTTAAGTGAGGAAGGGTAGG	MLST
	Forward 3	GAGAAGTAGAAGAGGATTGCTCATC	
	Reverse	GCAATGTTCACCAACCACAA	
ilvD	Forward	CGGGGCAAACATTAAGAGAA	MLST
	Reverse	GGTTCTGGTCGTTTCCATTC	
ilvD_2	Forward	AGATCGTATTACTGCTACGG	MLST
	Forward 4	GCAGAGATTAAAGATAAGGA	
	Reverse	GTTACCATTTGTGCATAACGC	
pta	Forward	GCAGAGCGTTTAGCAAAAGAA	MLST
	Reverse	TGCAATGCGAGTTGCTTCTA	
pur	Forward	CTGCTGCGAAAAATCACAAA	MLST
	Reverse	CTCACGATTCGCTGCAATAA	
pycA	Forward	GCGTTAGGTGGAAACGAAAG	MLST
	Reverse	CGCGTCCAAGTTTATGGAAT	
tpi	Forward	GCCCAGTAGCACTTAGCGAC	MLST
	Reverse	CCGAAACCGTCAAGAATGAT	

Abbreviations: cry, *Bacillus thuringiensis* cytolytic protein; cya, edema factor; cyt, *Bacillus thuringiensis* crystal protein; *glpF*, glycerol uptake facilitator protein; *gmk*, guanylate kinase; *ilvD*, dihydroxy-acid dehydratase; *lef*, lethal factor; MLST, multilocus sequence typing; *pagA*, protective antigen; *plcR*, phospholipase C regulator; *pta*, phosphate acetyltransferase; *pur*, phosphoribosylaminoimidazolecarboxamide; *pycA*, pyruvate carboxylase; SNP, single nucleotide polymorphism; *tpi*, triosephosphate isomerase.

(eg, edema factor, lethal factor, protective antigen [PA], and capsule) and other virulence factors implicated in a clinical phenotype with many features of inhalation anthrax.[9,17–22] (In this manuscript, we will refer to this type of infection as an anthrax-like disease.)

Here, we report the case of fatal, inhalation anthrax-like disease caused by *B cereus* in a Hispanic man residing in a relatively rural area of Texas. Like several other patients with this type of infection, this patient was a welder.[15–17] The genome of this strain was rapidly characterized by second-generation DNA sequencing within days of its recovery from antemortem cultures. The chromosome of the strain is closely related to a *B cereus* strain that caused an inhalation anthrax-like disease in San Antonio, Texas, and a *B thuringiensis* strain recovered during inspection of a suspected bioweapons facility in Iraq.[6,9,17] The strain contains a plasmid with features similar to virulence plasmids pXO1 and pBCXO1 found in *B anthracis* and some *B cereus* strains, respectively.[18] The organism lacks apparent foreign (non-*Bacillus*) DNA. The genome data permitted us to effectively rule out the likelihood of bioterrorism.

MATERIALS AND METHODS

Polymerase Chain Reaction Analysis for *B anthracis* and *B thuringiensis* Genes

Purified genomic DNA from strain *B cereus* Elc2 was analyzed for genes encoding protective antigen (*pagA*), edema factor (*cya*), and lethal factor (*lef*) encoded by plasmid pXO1 in *B anthracis* and pBCXO1 in *B cereus* strain G9241. Genomic DNA purified from the Ames strain of *B anthracis* was used as a positive control. We also analyzed the strains for the single nucleotide polymorphism in the *plcR* gene encoding a pleiotropic regulator of virulence gene expression.[23–25] The relevant region of the *plcR* gene was amplified with polymerase chain reaction (PCR) primers custom-designed for this strain based on the short-read sequencing data and was characterized by conventional Sanger sequencing using an ABI 3730 instrument (Applied Biosystems [now Life Technologies], Foster City, California). The *cry* and *cyt* genes were analyzed as previously described.[26] The sequences of all PCR primers used in this study are presented in the Table.

Immunohistochemistry Studies

Histologic sections prepared from tissue obtained at autopsy were analyzed using rabbit polyclonal antibodies raised against purified recombinant PA, BslA,[27] BcpA1 (BCE_G9241_4758), and BcpA2 (BCE_G9241_1728).[28] Preimmune serum was used as a negative control. All primary sera were diluted 1:1000, and horseradish peroxidase–labeled horse anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody (Vector Laboratories, Burlingame, California).

Cytokine Analysis

Quantitative multianalyte analysis of 89 cytokines, chemokines, and other bioactive molecules was performed on 4 samples of heparinized plasma collected after the patient was admitted to the referral institution using the human Multi-Analyte Profiles immunoassay (MAP v1.6, Rules Based Medicine, Austin, Texas).

Motility Testing

Motility was assessed by monitoring colony spread after spotting on a semisolid modified Luria-Bertani agar plate (1% bactotryptone, 0.3% yeast extract, 0.5% sodium chloride, 0.3% agar). Motility was scored after incubation overnight at 37°C.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was done by sequence analysis of internal fragments of 7 ''housekeeping'' genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*)[12] (http://pubmlst.org/bcereus, accessed June 23, 2011). The PCR primers used to amplify the target gene segments have been described[12] and are shown in the Table.

Genome Sequencing and Bioinformatic Analysis of the Genomes

The genomes of 3 strains derived from antemortem cultures were sequenced using an Illumina GXII instrument (Illumina, Inc, San Diego, California), and conditions described previously for genome studies conducted with group A *Streptococcus*.[29,30] Genome libraries prepared from each strain were uniquely barcoded to permit subsequent in silico deconvolution.

Bioinformatic analyses of the genome data were conducted by methods described previously for group A *Streptococcus*.[29,30] Genetic content was assessed by analysis of the short-read sequence data using MOSAIK and EDENA assemblers and Tablet viewer[31] (http://bioinformatics.bc.edu/marthlab/Mosaik, accessed June 23, 2011). Other specialized genetic analysis methods are described below.

RESULTS

Case Synopsis

The patient, a 39 year-old, previously healthy, Hispanic man from a rural area of Texas, approximately 75 miles southwest of Houston, presented to the emergency department of a community hospital with a 2-hour history of shortness of breath, massive hemoptysis, and vomiting. He was welding at home and had sudden onset of shortness of breath and cough. After resting briefly, he began experiencing hemoptysis and hematemesis with bright red blood. Review of systems was significant for headache, light-headedness, substernal chest pain, painful breathing, and upper abdominal pain. He denied nausea, diarrhea, sore throat, fever, or chills. He had no previous similar symptoms or significant medical history and was not taking prescription medications. He had never smoked and did not have known exposure to *Mycobacterium tuberculosis*.

On arrival at the emergency department of the outlying hospital, his blood pressure was 93/51 mm Hg, pulse was 115 beats/min, respiratory rate was 20 breaths/min, tympanic temperature was 98.0°F (36.7°C), and oxygen saturation was 95% on room air. The patient was alert and oriented but was anxious and in moderate distress with copious hemoptysis. Bilateral rales and rhonchi were noted throughout the lungs, with decreased air movement, more prominent in the right lung than left lung. There was pleuritic pain on deep inspiration and upper abdominal tenderness to palpation. No skin lesions were noted. Laboratory data were noteworthy for the following values: white blood cell count of 21 000 cells/μL; a hemoglobin level of 18.3 g/dL; a hematocrit of 54.5%; D-dimer and alkaline phosphatase levels of 1574 ng/mL and 161 μg/mL, respectively; a blood glucose concentration of 174 mg/dL (to convert to millimoles per liter, multiply by 0.0555), an aspartate aminotransferase rate of 52 U/L; a total bilirubin level of 1.1 mg/dL (to convert to micromoles per liter, multiply by 17.104); a creatinine level of 1.4 mg/dL (to convert to micromoles per liter, multiply by 88.4); and an abnormally low glomerular filtration rate of 56.42 mL/min. Values for amylase, lipase, creatine kinase, alanine aminotransferase, and troponin levels and the prothrombin time/partial thromboplastin time were within reference range. Analysis of the arterial blood gas showed a pH of 7.42 and values of P_{CO_2}, 34.9 mm Hg; P_{O_2}, 53.4 mm Hg; bicarbonate, 22.90 mEq/L, and Sa_{O_2}, 87.8%. Blood was drawn for cultures.

Chest x-ray and computed tomography (CT) scan showed multicentric pneumonia with a dense consolidation of the right lung; a small, right pleural effusion; and perihilar, hazy, nodular infiltrate of the left lung. The pulmonary vasculature appeared normal. No enlarged mediastinal lymph nodes were noted. Ceftriaxone, 2 g, was administered intravenously. Within 3 hours, the patient's condition deteriorated with oxygen saturation in the low 80s% with respiratory rates around 40 breaths/min. The patient was intubated emergently and transferred to a tertiary care hospital.

On arrival at the referral hospital, his vital signs were as follows: blood pressure 102/50 mm Hg, pulse at 144 beats/min, temperature of 96.0°F (35.6°C), and an Sa_{O_2} of 98% (F_{IO_2} fraction, 100%). The patient's condition worsened after transfer, with blood pressure of 76/52 mm Hg, a pulse of 133 beats/min, a respiratory rate of 24 breaths/min, and an Sa_{O_2} concentration of 79%. Bronchoscopy was performed, revealing profuse bilateral alveolar hemorrhage, which was worse in the lower lobes. The corresponding bronchoalveolar lavage (BAL) was bloody and showed very high numbers of large, uniformly sized, gram-positive rods that stained with Grocott methenamine silver stain and contained minimal neutrophilic reaction (Figure 1, A and B). Many of the bacteria had a negatively staining halo, suggestive of a capsule (Figure 1, C). Cultures of the tracheal aspirate, BAL fluid, and 2 different peripheral blood samples (data not shown and Figure 1, D) grew a pure culture of gram-positive rods, identified provisionally as *Bacillus* species. Urinalysis showed high numbers of red blood cells and white blood cells, with a moderate number of bacteria present, but urine cultures were negative for infection. The serologic test result for human immunodeficiency virus was negative. A comprehensive autoimmune panel, including antinuclear antibody, anti–double-stranded DNA, perinuclear antineutrophil cytoplasmic antibodies, cytoplasmic antineutrophil cytoplasmic antibodies, and anti–smooth muscle antibody titers, was also ordered, and the results were later reported to be negative for antibodies.

Approximately 10 hours after initial presentation at the outlying hospital, the patient remained hypoxic and hypotensive with multiorgan system failure. He was placed on venous-venous extracorporeal membrane oxygenation. Methylprednisolone, 1 g intravenously; piperacillin-tazobactam, 3.375 g every 6 hours; and vancomycin, 1 g every 24 hours, were administered. On day 3, clinical deterioration continued with total opacification of lungs bilaterally, progression of disseminated intravascular coagulation, and development of renal failure, rhabdomyolysis, metabolic acidosis, and abdominal compartment syndrome. The clinical microbiology laboratory presumptively identified the organism as *B cereus*, resistant to ampicillin and ceftriaxone, and susceptible to vancomycin and levofloxacin. Ciprofloxacin, 400 mg intravenously every 12 hours, was added to the regimen. Later

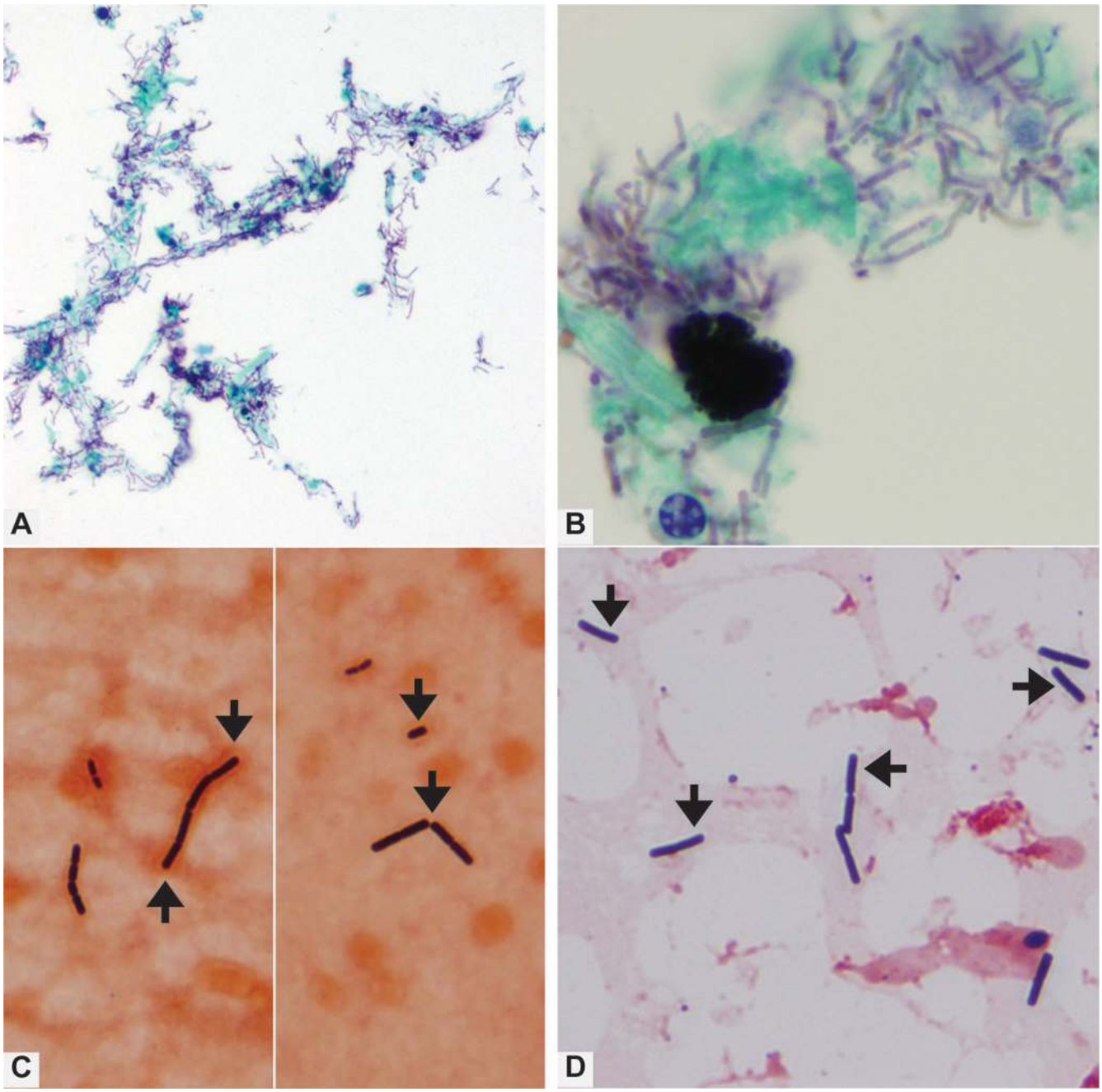

Figure 1. *Bacillus shown in multiple patient specimens. A, The bronchoalveolar lavage (BAL) fluid contained extremely high numbers of rod-shaped bacterial organisms adherent to respiratory epithelial cells. Very few polymorphonuclear leukocytes or lymphocytes were observed (BAL cytocentrifugation preparation). B, Anthracotic pigment is seen alongside the numerous, large, minimally pleomorphic, rod-shaped organisms (BAL cytocentrifugation preparation). C, Many of the gram-positive rods had a negatively staining halo (black arrows, left and right panels) suggestive of a capsule (BAL test). D, Blood collected from multiple anatomic sites grew a pure culture of gram-positive rods with similar morphology, including the presence of a thick capsule (black arrows), to organisms observed in the direct specimens (Papanicolaou stain, original magnifications ×20 [A] and ×100 [B]; Gram stain, original magnifications ×100 [C and D]).*

that evening, the patient had a decompressive laparotomy, and a small portion of ischemic small bowel was removed. The patient expired on hospital day 4, less than 72 hours after initial presentation to the outlying hospital.

Autopsy Findings: Gross Pathology

An autopsy performed shortly after death revealed edema of the head, neck, lips, bilateral eyelids, and upper and lower extremities. There were scattered ecchymoses and multiple papules ranging in diameter from 0.3 to 1.0 cm on the upper and lower extremities. Multiple serous fluid–filled vesicular bullae were present on the right upper extremity, confirmed to be present before death by medical staff. The heart weighed 440 g (reference range, 260–360 g) and had a serosanguinous pericardial effusion (150 mL) and scattered epicardial petechiae. The lungs were markedly edematous and hemorrhagic bilaterally, with a combined fresh weight of 2850 g (reference range, 700–1000 g). Serosanguinous pleural effusions were present bilaterally (right, 500 mL; left, 100 mL), despite

well-positioned and patent bilateral chest tubes, and the right lung had extensive pleural adhesions. No gelatinous edema of the mediastinum was present. The tracheobronchial tree was hyperemic. The liver was diffusely mottled with extensive fatty change, congestion, and necrosis. There was mild splenomegaly with a spleen weight of 220 g (reference range, 125–195 g). The greater curvature of the stomach, small intestine, and large intestine had patchy, dusky ischemic changes with mucosal necrosis. The kidneys had mildly congested medullae. The prostate was diffusely dusky, and there was significant scrotal edema. The brain weighed 1470 g, had subarachnoid hemorrhages, and was edematous. Cultures were obtained from bilateral lung parenchyma, bilateral pleural fluid, blood, kidney, liver, spleen, hilar lymph nodes, and bullous skin lesion fluid. All specimens, except the skin lesion fluid, grew a *B cereus*–like organism. *Cryptococcus neoformans* grew in cultures from the right upper lobe lung parenchyma and in blood drawn from the aorta.

Autopsy Findings: Histopathology

Representative sections from the lung, stained with hematoxylin–eosin, had extensive areas of necrosis, hemorrhage, and intra-alveolar edema (Figure 2, A and B). The alveolar septa showed necrosis, and the alveolar airspaces contained focal alveolar macrophages, including some with anthracotic pigment. The bronchi, bronchioles and membranous bronchioles had denuded epithelium and extensive wall hemorrhage (Figure 2, A and B). Consistent with the imaging studies and gross pathology examination, the right lung was more severely affected than the left lung. Many gram-positive, rod-shaped bacteria were present in the alveolar spaces and the pleura (Figure 2, C and D). These bacteria were seen in the hematoxylin-eosin sections of all lung lobes, most prominently in the right upper lobe. Many of the bacteria had a negatively staining halo, suggestive of a capsule (Figure 2, E). Edema, hemorrhage, and mild, acute and chronic inflammation were also identified in the pleura, extending into the interlobular septa (Figure 2, F). The tracheal mucosa was necrotic with denuded epithelium, and the mucosal surface had diffuse hemorrhage with fibrin deposits and groups of acute inflammatory cells.

The splenic red pulp was expanded and congested, with prominent hemophagocytosis (Figure 3, A and B). There was an increase in immature, hypolobated eosinophils. Lymphoid follicles were sparse and no collapse of germinal centers was noted, which has been reported in humans and nonhuman primates with inhalation anthrax.[3,10] No evidence of hematologic malignancy was observed.

Several organs showed findings potentially related to hemodynamic compromise and lack of perfusion. For example, sections of the gastrointestinal tract, including stomach, small bowel, and large bowel, had changes consistent with ischemia, including patchy areas of mucosal, coagulative necrosis with underlying congestion and edema. Focal areas of organisms morphologically consistent with *Candida* species were seen in the ischemic stomach epithelium. Acute tubular necrosis was present in both kidneys with widespread, coagulative necrosis of the tubules, predominately proximal, whereas other tubules showed prominent vacuolization (Figure 3, C). Some collecting ducts contained blood and inflammatory cells. Focal fibrin thrombi were also seen in some of the glomeruli. The adrenal glands showed diffuse cortical congestion with hemorrhage and necrosis. The liver had sheets of necrosis and diffuse sinusoidal congestion (Figure 3, D). Areas of viable hepatocytes had macrovesicular and microvesicular steatosis. No significant inflammation was present in the portal triads. No increased fibrosis, increased iron stores, or globules positive for periodic acid–Schiff were revealed by special stains. The heart showed focal, recent hemorrhage. The prostate parenchyma had focal glandular necrosis.

Conventional Microbiologic Characterization

All isolates cultured from patient samples were β-hemolytic on blood agar plates, and the colonies had a ground-glass appearance. The organisms also grew on chocolate agar and were positive for catalase and lecithinase. The organism was susceptible to vancomycin and levofloxacin and was resistant to ampicillin and ceftriaxone. All organisms were presumptively identified as *B cereus* based on these criteria. The BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, Maryland) identified the organism as *B cereus*. The organisms were motile, as assessed by growth in semisolid agar, and encapsulated, as shown by India ink staining (Figure 4, A and B).

Cytokine, Chemokine, and Biomarker Analysis

The host response to the severe pneumonia and overwhelming sepsis was assessed by measuring 89 cytokines, chemokines, and biomarkers in 4 plasma samples collected for routine diagnostic procedures at approximately 24-hour intervals after the patient was transferred to the referral institution. Consistent with the clinical impression and autopsy findings, the data were consistent with a vigorous systemic response. Granulocyte colony stimulating factor (approximately 300-fold), interleukin-10 (approximately 40-fold), interleukin-1 receptor antagonist (approximately 35-fold), myeloperoxidase (approximately 18-fold), CD40 antigen (approximately 3-fold), and interleukin-16 (approximately 2-fold) were markedly elevated (relative to the upper limit of their reference range) in each sample tested. Also consistent with previous reports of biomarkers measured in patients with sepsis and in animal models of inhalational anthrax, there were increased plasma levels of endothelin-1 (ET-1), tissue inhibitor of metalloproteinase 1 (TIMP-1), macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β. Possibly explaining the numerous immature, eosinophilic leukocytes observed in the spleen tissue collected at autopsy, eotaxin-1, a chemoattractant of eosinophils, was elevated (approximately 5-fold). Also consistent with the clinical diagnosis of disseminated intravascular coagulation, clotting factor I (fibrinogen) and factor VII (proconvertin) were decreased, and type-1 plasminogen activator inhibitor 1 was markedly elevated.

Genome Sequencing and Bioinformatic Analysis

The investigative group evaluated all available clinical and pathology data the morning after the autopsy was performed, and the literature was reviewed. Initial PCR analysis of the isolate recovered from the antemortem blood culture revealed that the organism had the genes encoding PA and lethal factor. Four main possibilities

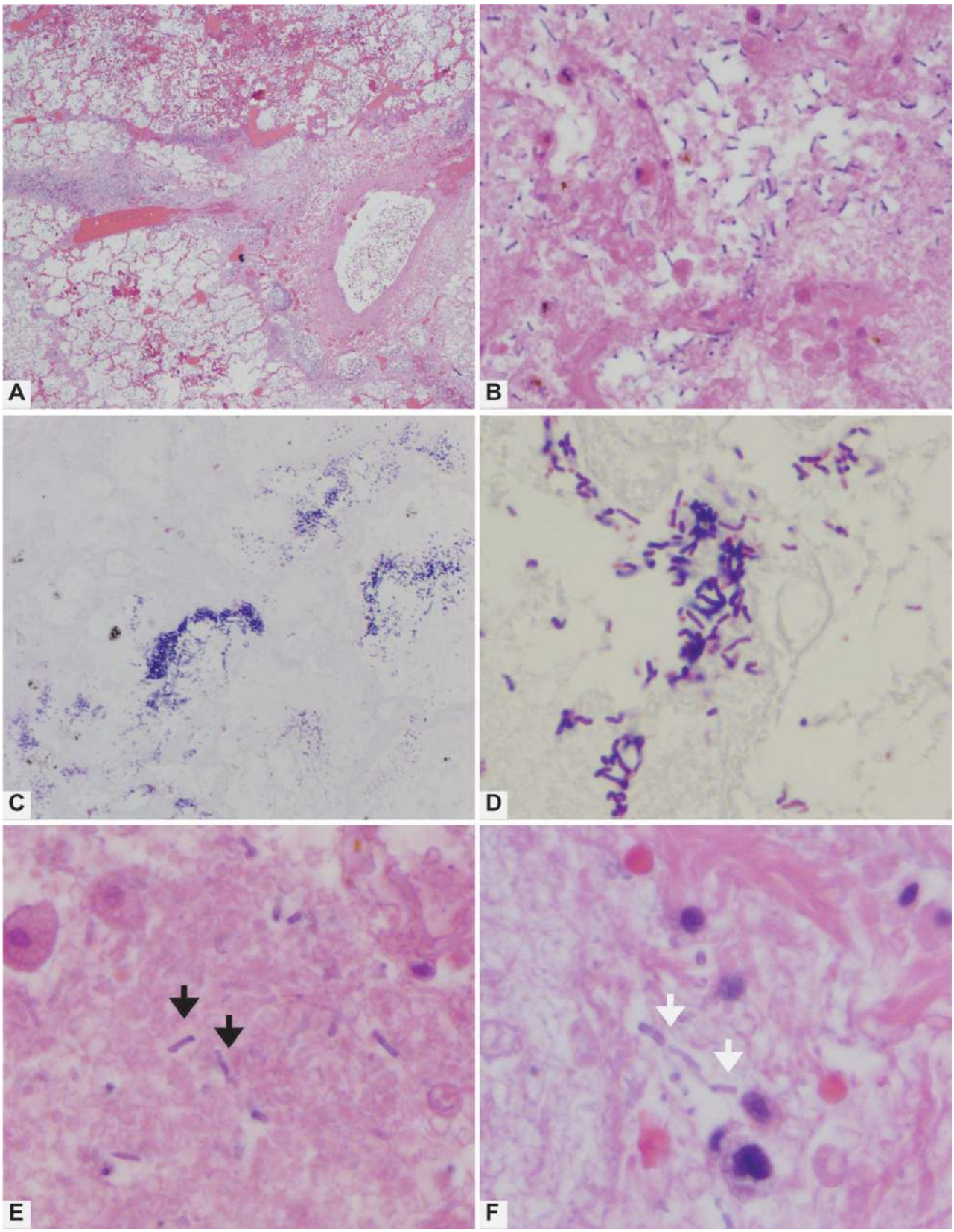

Figure 2. *The autopsy confirmed the diagnosis of severe pneumonia and sepsis. A, The alveolar septa are moderately thickened and congested. Edema, hemorrhage, and mild inflammation were also identified in the pleura, extending into the interlobular septa. B, Microscopic examination of all lung lobes, particularly the right upper lobe, demonstrates extensive edema and hemorrhage within both the alveolar and bronchial air space. Many rod-shaped bacteria and very few acute inflammatory cells were identified at higher magnification. C, Extremely high numbers of gram-positive bacteria in the lung. D, Although most of the organisms were strongly gram-positive, a small subset showed a gram-variable staining pattern. E, Many of the organisms had a negatively staining halo (black arrows) suggestive of a capsule. F, Bacteria (white arrows) were also present in the pleura (hematoxylin-eosin, original magnifications ×2 [A], ×40 [B], and ×100 [E and F]; Gram stain, original magnifications ×10 [C] and ×100 [D]).*

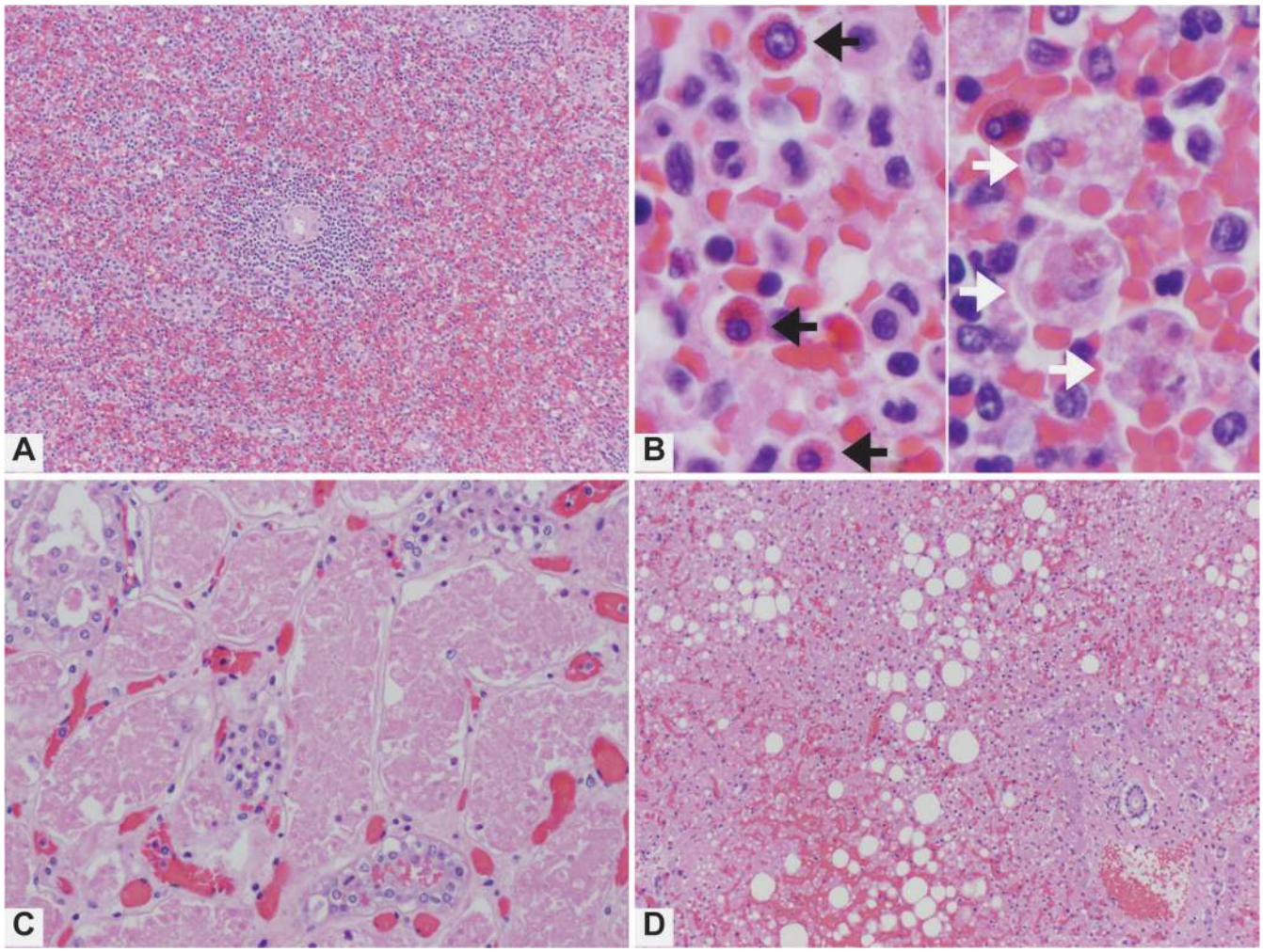

Figure 3. *The autopsy examination identified histologic features of severe, disseminated infection and generalized hypoperfusion. A, The splenic red pulp was markedly expanded, and the white pulp was paucicellular with depletion of lymphocytes and germinal centers. B, Numerous monolobated, immature, eosinophilic leukocytes (black arrows, left panel) and prominent hemophagocytosis (white arrows, right panel) were observed in the spleen. C, Mild congestion and acute tubular necrosis were observed in the kidney. D, Congestion, patchy necrosis, and microvesicular and macrovesicular steatosis were present in the liver (hematoxylin-eosin, original magnifications ×10 [A and D], ×100 [B], and ×40 [C]).*

were considered, including that the strain was (1) a conventional (common) *B cereus* organism, (2) a natural variant of *B cereus* that produces one or more anthrax-related toxins, (3) an unusual natural variant of *B anthracis*, or (4) a human-engineered form of one of these two species or another closely related species, such as *B thuringiensis*. The PCR results effectively ruled out possibility (1) above. We concluded that the only definitive way to differentiate among the remaining 3 possibilities was to sequence the genome of the causative organism. Given the substantial public health and biosecurity implications of several of these possibilities and the on-site genomics capacity and expertise of the Houston investigative group,[29,30] we also concluded that the effort and cost required to sequence the genomes were very well justified.

Three isolates were chosen for analysis, including one organism each grown from antemortem blood, tracheal aspirate, and BAL specimens obtained at the referral hospital. We chose to sequence the genome of 3 isolates initially predominantly as a hedge against an isolated technical problem resulting in the lack of genome sequence data if only one isolate were processed for analysis. We also opted to have 2 experienced investigators (S.B.B. and A.R.F.) create the sequencing libraries independently, also as a hedge against an unanticipated technical problem. A second reason for sequencing the genomes of 3 separate isolates was our interest in beginning to assess the degree, if any, of genome sequence divergence in organisms recovered from distinct anatomic sites. Intrahost genetic variation is well known to occur in certain RNA viruses, such as the human immunodeficiency virus,[32] and has also been described in the highly polymorphic gene encoding the streptococcal inhibitor of the complement secreted by group A *Streptococcus*.[33] However, that issue has not been assessed at the full-genome level in bacterial isolates cultured at the same time from different sites of one patient.

Generation of the genome sequence data with an Illumina GXII instrument was completed 8 days after the autopsy and 6 days after the decision was made to perform this analysis. The genome analysis run yielded approximately 4 000 000 to 5 000 000 reads of high-quality sequence data for each isolate (total of 23 964 180 high-quality reads for the 6 strain libraries). We focused our

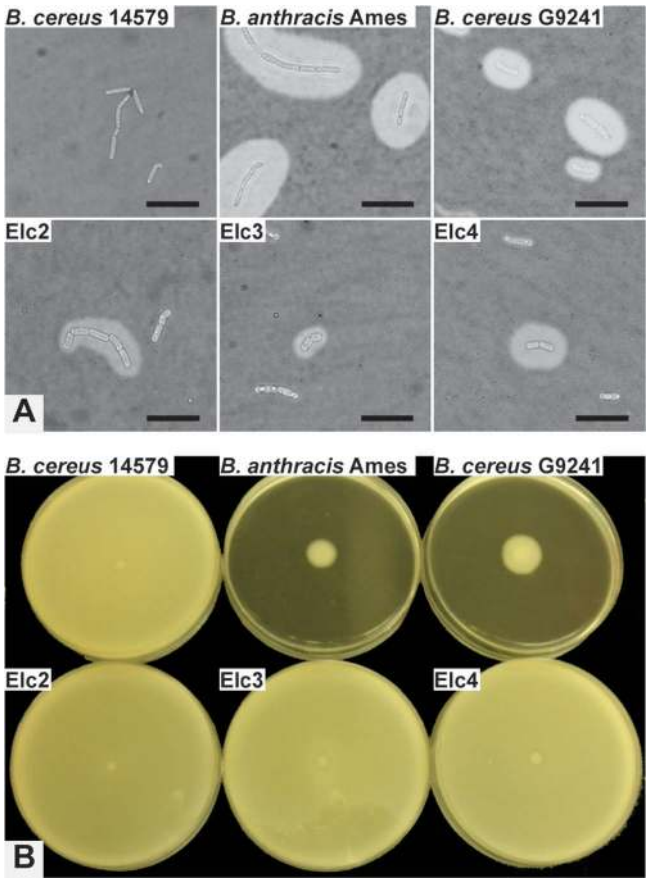

Figure 4. *Phenotypic characterization of* Bacillus cereus *strains recovered from the patient. A, India ink stain showing capsule production by* Bacillus *strains. Images for the Elc isolates suggest a mixed population with encapsulated and unencapsulated forms (original magnification ×100). B, Motility assay from growth on semisolid agar. Abbreviations: 14579,* B cereus *strain American Type Culture Collection (ATCC) 14579; Ames,* Bacillus anthracis *Ames strain; G9241,* B cereus *strain G9241; Elc2, Elc3, Elc4, three* B cereus *isolates cultured from different specimens of the source patient. The strain designations are the same in both A and B.*

initial bioinformatic analyses on the genome data obtained for the isolate recovered from the blood culture performed when the patient arrived at our facility. Of note, the greatest number of high-quality reads (5 159 932) was generated for this isolate. This organism was designated Elc2. Reads were mapped to all *B anthracis* ($n = 5$ strains), *B cereus* ($n = 10$ strains), and *B thuringiensis* ($n = 5$ strains) reference sequences available in the National Center for Biotechnology Information Complete Microbial Genome Database. The Elc2 shared the greatest similarity, in increasing order, to reference genomes of *B anthracis* Ames Ancestor (Amerithrax event),[2] *B cereus* 03BB102 (fatal pneumonia, San Antonio, Texas),[16] and *B thuringiensis* Al Hakam, recovered in a suspected bioweapons facility in Iraq.[9] The data (Figure 5, A through C) showed that approximately 77% of reads mapped to the reference chromosome of each of these *B cereus* and *B thuringiensis* isolates, whereas only about 70% of the reads mapped to the chromosome of the *B anthracis* reference. The pattern of mapped reads (Figure 5, A) was consistent with the idea that the Elc2 isolate lacked 3 of the 4 prophages invariantly reported in the chromosome of the Ames Ancestor and all other *B anthracis* strains.[34,35] The pattern more closely resembled that expected for either of the other 2 reference genomes analyzed (*B cereus* O3BB102 and *B thuringiensis* Al Hakam) (Figure 5, B and C). We next mapped the DNA sequencing reads to plasmids found in each of these 3 reference genomes and the additional one of *B cereus* strain G9241, which was isolated from a patient with a fatal inhalation anthrax-like infection in Louisiana in 1994.[15,16] Consistent with the PCR amplification of *lef*, *cya*, and *pagA* gene segments, 11.3% of the Elc2 sequence library reads mapped to pXO1, the plasmid that encodes these critical *B anthracis* virulence factors (Figure 5, D). Similarly, 11.4% of all reads mapped to pBCXO1, a toxin-encoding plasmid found in *B cereus* G9241 that is 99.6% identical to pXO1. However, exceedingly few of the reads (0.05%) mapped to pXO2 (Figure 5, E), and 0.76% mapped to pBC218, a large plasmid in *B cereus* strain G9241 that is required for full virulence in mouse infection.[20] These results suggest that Elc2 has a pXO1-like plasmid that encodes the tripartite anthrax exotoxin but lacks a pX02-like plasmid, a finding also supported by PCR (Figure 5, F). We note that the higher fold-coverage of the plasmid data relative to the chromosomal data (×50 coverage for the reads mapped to the chromosome of either *B cereus* 03BB102 or *B thuringiensis* Al Hakam, compared with ×200 coverage for the reads mapped to either pXO1 or pBPXO1) (Figure 5) suggests that the Elc2 isolate contained a higher proportion of plasmid copies relative to the chromosome.

Five different MLST schemes have been developed for the *B cereus* group of related organisms.[5] More than 1100 strains have been analyzed by these methods, resulting in large public databases (http://mlstoslo.uio.no/ and http://pubmlst.org/bcereus/, accessed on June 23, 2011). We performed MLST analysis on Elc2, first by analyzing the genomic data, and subsequently, by Sanger-sequencing internal fragments of 7 housekeeping genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*).[12] We discovered that the MLST of the Elc2 isolate was identical to *B cereus* MLST sequence type ST108 at all 7 of these loci (Figure 6). Importantly, ST108 is closely related to *B anthracis* strains and several *B cereus* and *B thuringiensis* strains that have caused fatal cases of fulminant pneumonia.

A key single nucleotide polymorphism that differentiates all *B anthracis* strains from closely related organisms, such as *B cereus*, is a nonsense mutation in the gene (*plcR*) encoding a pleiotropic virulence regulator that controls expression of a large number of virulence factors.[23,24] This single nucleotide polymorphism creates a premature stop codon in *B anthracis*, whereas the other closely related *Bacillus* strains have a wild-type allele. Thus, we next analyzed the DNA sequencing data using MOSAIK (http://bioinformatics.bc.edu/marthlab/Mosaik, accessed June 23, 2011) to determine the *plcR* allele present in Elc2. The data suggested that Elc2 had the wild-type *plcR* allele, a finding that was confirmed by conventional Sanger sequencing of the relevant PCR-amplified region of the *plcR* gene (data not shown). Subsequent analysis of the genome sequencing data for the isolates cultured from the BAL and tracheal aspirate by these same methods confirmed the above findings and conclusions (data not shown).

A complete description of the genome of this *Bacillus* organism will be presented elsewhere (manuscript in preparation).

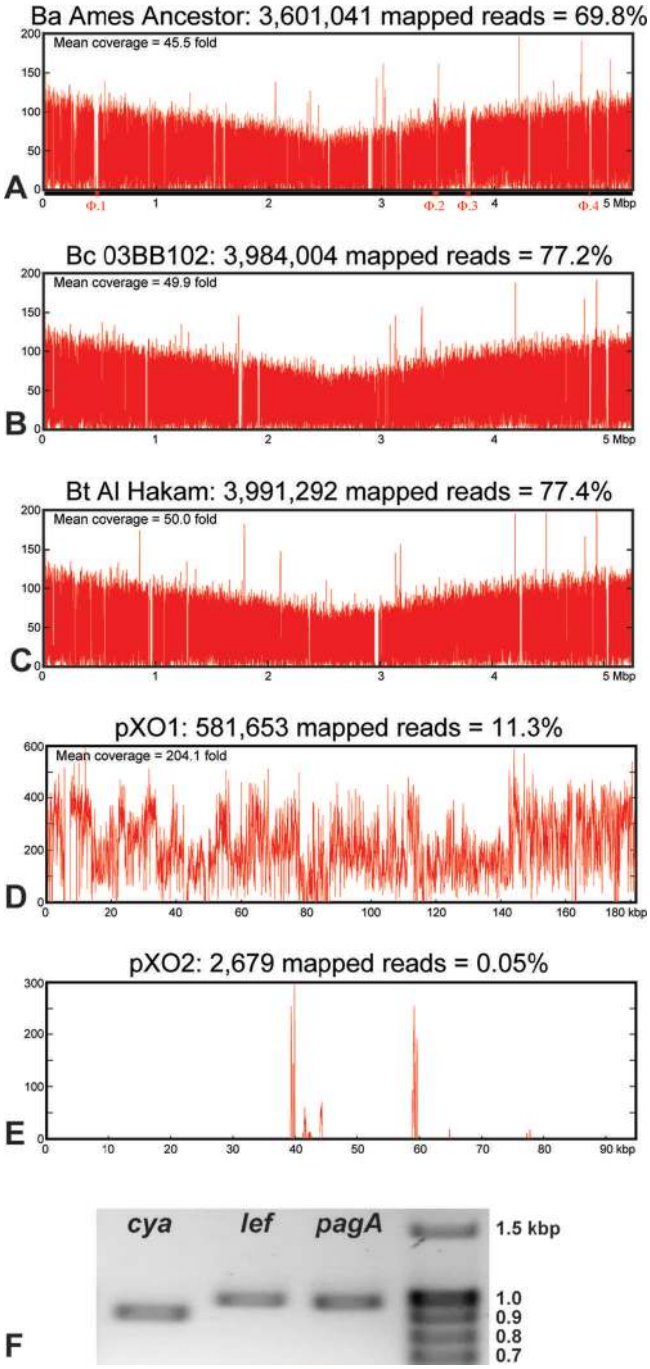

Figure 5. *Genetic analysis of* Bacillus *strain Elc2 cultured from blood. Approximately 5 000 000 high-quality sequencing reads were obtained from* Bacillus *strain Elc2 cultured from the blood of the patient. The DNA sequencing was done with an Illumina GXII instrument (Illumina, Inc, San Diego, California). The reads were electronically mapped to all 20 available* Bacillus *group genomes (*B anthracis, B cereus, B thuringiensis, B mycoides, *and* B weihenstephanensis*) deposited in the National Center for Biotechnology Information Complete Microbial Genome Database (Available at: http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi, accessed June 23, 2011) using Mosaik. Shown are depth-of-coverage plots for the Elc2 short-read sequences mapped to the indicated reference chromosomes for* B anthracis *Ames Ancestor (A),* B cereus *03BB102 (B),* B thuringiensis *Al Hakam (C), the* B anthracis *plasmid pXO1 that encodes the tripartite anthrax toxin (D), and the* B anthracis *plasmid pXO2 that encodes a poly-D-glutamic acid capsule (E). Text in figure shows the percentage of the total approximately 5 million sequencing reads that mapped to each chromosome or plasmid. The x-axis refers to the position along the >5 000 000-bp (5 Mbp) chromosome of each strain or plasmid (kbp, kilobase pair). The panel for the Ames Ancestor strain shows the location of 4 prophages on the x-axis. Also shown on the y-axis are the fold-coverage values for the chromosomes and the plasmids. The Elc2 reads, mapped throughout the pXO1 sequence, demonstrate the presence of a pXO1-family plasmid in the Elc2 genome. Inspection of the Elc2 reads mapped to pXO1 showed deep coverage across the* cya, lef, *and* pagA *genes encoding anthrax toxins edema factor, lethal factor, and protective antigen, respectively. Very few reads mapped to plasmid pXO2 (E). Displayed in (F) is an agarose gel image with polymerase chain reaction–amplified products corresponding to genes (*cya, lef, *and* pagA*) encoding* B anthrax *toxins. There was very high, mean fold-coverage for the Ames Ancestor chromosome (45-fold to 50-fold [A]) and plasmid pXO1 (about 200-fold [D]).*

Immunohistochemical Studies

Immunohistochemical study of tissues obtained from patients infected during the 2001 anthrax attack showed that several well-known extracellular anthrax virulence factors are made in vivo.[3] However, analogous studies of patients with fulminant *B cereus* or *B thuringiensis* infections have not been conducted. Inasmuch as the genome analyses found that the infecting strain had genes encoding several extracellular virulence molecules produced by anthrax, we tested the hypothesis that these factors were made in vivo in this patient. Consistent with the hypothesis, immunohistochemical analysis of specimens prepared from multiple lung lobes revealed material reactive with specific rabbit antibodies raised against purified PA; BslA, an extracellular protein involved in adhesion of *B anthracis* to host cells[27]; and the pilin subunits BcpA1 and BcpA2[28] (Figure 7, A through D). No reactivity was observed in any tissue section treated with preimmune (control) sera (Figure 7, E).

COMMENT

Strains of *B cereus* and close genetic relatives that cause fulminant pneumonia and other severe invasive infections in humans and wild chimpanzees have been described.[17–19,36,37] Although exceedingly rare, infections in humans attract significant interest because they can mimic some clinical features of anthrax, thereby raising substantial public health and bioterrorism concerns. This patient, like several others described in the literature,[16,17] was a welder living in a relatively rural area. The explanation for the strong association between a history of welding and other forms of metalwork, rural environments, and Texas and Louisiana[16,17,36] is not known, but welding can predispose a person to severe lung infections.[38] The fulminant course of this patient's infection, together with the growth of *C neoformans* from the lung parenchyma and his human immunodeficiency virus–negative status, suggest that an underlying pulmonary defense dysfunction contributed to disease pathogenesis.

Our study adds to the concept that rapid, full-genome analysis of microbial pathogens is an important component of contemporary infectious diseases response and investigation.[39,40] Full-genome analysis is an especially important issue for pathogens with significantly restricted levels of naturally occurring genetic variation, such as *B anthracis* and related organisms. The ability to sequence bacterial genomes economically and accurately, and to define isolates based on all gene content and genetic

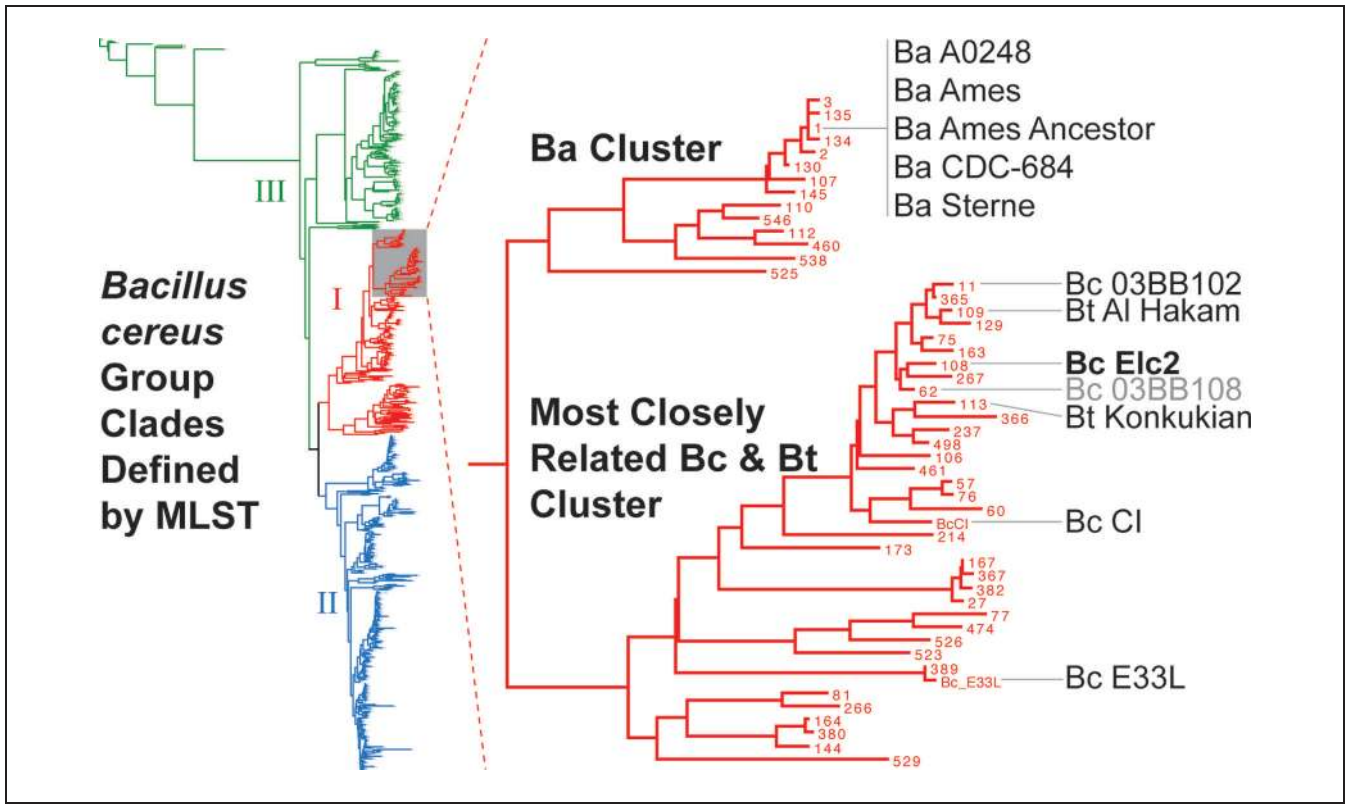

Figure 6. *Estimates of genetic relationships among* Bacillus *group strains. The estimates are based on multilocus sequence type data obtained for 7 loci, analyzed by the neighbor-joining method. The left side shows a dendrogram with 3 main genetic clades that are color-coded in green (top), red (middle), and blue (bottom). The right side shows an expanded view of the portion of the dendrogram containing the sequence types corresponding to* Bacillus anthracis *strains (top) and to organisms most closely related to the* B anthracis *strains (bottom). The fuchsia-colored numbers at the end of the branches of the dendrogram refer to sequence types using the nomenclature found at the multilocus sequence typing (MLST) Web site (http://pubmlst.org/bcereus, accessed June 23, 2011), based on the 551 sequence types present in this database. Strains for which full-genome sequence data are available in public databases are shown in the dendrogram. Strain* Bacillus cereus *Elc2 is sequence type 108, as shown, and is part of a genetic cluster that is most closely related to* Bacillus thuringiensis *strain Al Hakam, recovered in Iraq from a suspected bioweapons facility, and* B cereus *strain 03BB102 isolated from a fatal human inhalation anthrax-like case that occurred in San Antonio, Texas, in 2004. The Elc2 is very closely related to sequence type 62 strain 03BB108 shown in grey. The 03BB108 strain was isolated from the environment in San Antonio, Texas, in association with the inhalation anthrax-like case (its genome sequence has not been determined). Abbreviations: Ba,* B anthracis*; Bc,* B cereus*; Bt,* B thuringiensis.

variation, permits rapid elucidation of close genetic relatives. In the context of this event, the full-genome data permitted us to effectively rule out the likelihood of bioterrorism. That is, the genome data revealed no evidence that this organism had been genetically altered in the laboratory for malicious purposes. We will report elsewhere, in more detail, findings related to the genomic analysis of this organism and to the intrahost genetic variation identified by our studies.

Many of the autopsy findings in this patient echo several of the gross and histopathologic features described in the inhalational anthrax attack cases occurring in 2001[3,11] and the Sverdlovsk outbreak.[41] For example, the anthrax patients had abundant serosanguinous fluid

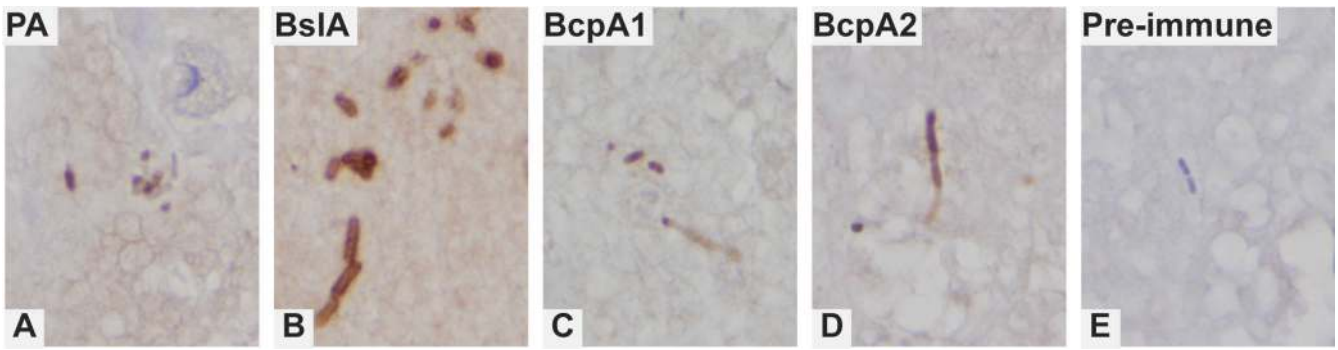

Figure 7. *Immunohistochemistry demonstrates in vivo production of virulence factors protective antigen (A), BslA (B), BcpA1 (C), and BcpA2 (D) in lung tissue obtained at autopsy. No staining was observed in the negative control lung tissue, stained with preimmune rabbit antiserum (E) (immunoperoxidase stain, original magnifications ×100 [A through D]; original magnification ×100 [E]).*

within the pleural cavities, a feature also observed in this patient despite placement of bilateral chest tubes. Similarly, lung sections from the cases in 2001 and our patient had hemorrhage and edema of the pleura and interlobular septa, intra-alveolar edema, bacteria in the pleura, and lack of significant intra-alveolar inflammatory infiltrate. Splenic changes in this Texas patient included congestion and presence of immunoblasts, as was reported in the anthrax cases. Although 2 of the 8 patients (25%) described in the anthrax series had cutaneous lesions,[3] the prominent bullous lesions observed on the right upper extremity observed in our patient appear to be a distinctive feature. Serous fluid obtained from these skin lesions during autopsy showed moderate numbers of gram-positive rods. No growth occurred in culture, perhaps because the patient was treated aggressively with antimicrobial agents to which the isolate was susceptible. The precise molecular etiology of these cutaneous lesions remains unclear.

Very little is known about the molecular pathogenesis of these rare cases of fulminant infection caused by *B cereus* and related highly virulent strains. Two recent publications[20,21] have analyzed contributory bacterial factors in mouse models of infection. Although presumably some of the mouse pathology was caused by in vivo expression of extracellular toxins and other bacterial molecules (likely analogous to anthrax molecular pathogenesis), this issue had not been addressed in human patients. Importantly, our immunohistochemistry studies discovered that PA, BslA, BcpA1, and BcpA2 were made in vivo in this patient (Figure 7). As the elaboration of pili from pilin subunits (BcpA1 and BcpA2) is a feature of *B cereus* strains, but not of *B anthracis*, these findings further corroborated the idea that this patient's infection was not caused by a bona fide *B anthracis* strain. Toxin-expressing *B cereus* and *B anthracis* strains elaborate capsules, which are essential for the pathogenesis of anthrax and inhalation anthrax-like disease. *Bacillus anthracis* makes a capsule composed of poly-γ-D-glutamic acid and can be identified with the antibody specific for poly-γ-D-glutamic acid. Proteins responsible for poly-γ-D-glutamic acid synthesis are encoded by genes on the pXO2 virulence plasmid. In contrast, *B cereus* isolates with pBCXO1 use the *hasACB* operon to synthesize a hyaluronic acid capsule that can be removed from the cell surface by treatment with hyaluronidase. India ink staining of *B cereus* Elc isolates revealed the presence of a capsule (Figure 4). Additional studies are underway to determine the composition of the capsule and to further assess the in vivo gene transcription and virulence factor production by the *B cereus* Elc isolates.

A confluence of circumstances aided the rapid and efficient response to this event. We believe it is instructive to share several items that may be useful to consider when future analogous clinical situations occur. First, the patient was admitted to our hospital during the period when a very large outbreak of *Escherichia coli* O104:H4 hemorrhagic colitis and fatal hemolytic uremic syndrome was occurring in Germany and elsewhere in Europe.[42,43] This *E coli* public health problem had led several members of our pathology department to discuss how we might respond if one of our 5 system hospitals were the epicenter of a similar infection outbreak. Fortuitously, this discussion had occurred less than 24 hours before we became aware of this patient's circumstances. Thus, many aspects of a laboratory and other response plan, including the genomic analysis strategy, had been considered very recently. A second factor that assisted the efficiency of our response was that one of us (J.M.M.) serves as the chair of the Department of Pathology and Laboratory Medicine and has expertise in clinical microbiology and a strong interest and background in genome-scale analysis of microbial pathogens.[29,30] These factors, coupled with the institutional availability of a next-generation sequencing instrument and in-house bioinformatics expertise,[29,30] meant that we had the capacity to rapidly sequence and analyze the genome of the causative organism. Thus, the strain analysis component of the response proceeded efficiently in our Houston facility. That is, there was no need to outsource the genomic analysis (such as was necessary in the recent European *E coli* O104:H4 outbreak), thereby saving critical time. Importantly, although not available at our institution, new types of next-generation sequencing[39,44] would have further decreased the time required for rapid genome analyses, and thus, would have been especially useful. Increased speed of genetic analysis is of special importance in cases like this involving potential bioterrorism or other events with considerable public-health implications occurring in large hospitals in major metropolitan areas.

The initial hints that the patient was infected with an unusually virulent *Bacillus* occurred during the microscopic examination of the BAL and microbiology laboratory workup of the gram-positive organism recovered from all of the patient's specimens. The striking abundance of gram-positive bacilli and corresponding paucity of expected, acute inflammation in the antemortem BAL led the pathology resident and attending cytopathologist to suspect something badly awry in this patient. Simultaneously, a medical technologist in the clinical microbiology laboratory raised concern about the pure and luxuriant growth of an organism presumptively identified as *B cereus* in all specimens. Within 24 hours, the same resident performed the autopsy. Knowledge of the BAL results and preliminary microbiology data, combined with the highly unusual clinical presentation and rapid demise, prompted the resident to conduct an extensive review of the patient's medical record. All available clinicians, consultants, nurses, and microbiology personnel were contacted to obtain additional clinical details and impressions. Inasmuch as some of the autopsy findings were similar to inhalational anthrax cases and the patient was a welder, this crucial information informed our search for, and subsequent discovery of, a small number of similar cases reported in the literature.

Part of the response to the event included a discussion of whether to offer antimicrobial agent prophylaxis to close contacts. To our knowledge, there has been no report of secondary transmission to hospital staff or family in patients infected with these *B cereus* anthrax-like organisms. However, given the fulminant course of the patient, the existence of so few comparable cases, the unknown likelihood of transmission, and the comparatively low risk associated with antimicrobial prophylaxis, it was ultimately decided to offer prophylaxis to anyone intimately involved with the patient who may have been exposed to his respiratory secretions. To date, no cases of secondary transmission have occurred.

One additional key point assisted the efficiency of our response to this patient's infection. We concluded early

in the investigation that it was important to engage experienced anthrax investigators in collaborative studies. All local and national investigators we contacted generously assisted our studies. Because of the concentration of expertise, we opted to collaborate with investigators at the Great Lakes Regional Center of Excellence for Biodefense and Emerging Infectious Diseases, administratively based at the University of Chicago, Illinois. A request for collaborative interaction was made, very rapidly approved, and an investigative strategy was developed within hours. This center has particular extensive expertise with *B anthracis* and unusually virulent *B cereus*, and thus, was a logical choice for providing specialized assistance. Arrangements were made to exchange strains and serologic reagents, a process that greatly assisted the efficient response to this event.

CONCLUSION

One of us (J.M.M.) recently proposed the inception of a third training track in pathology termed *genomic pathology*,[45] designed to complement the traditional anatomic and clinical pathology tracks. As the introgression of genome-scale analyses proceeds rapidly and inexorably into contemporary patient care and pathology practice, the career opportunities for this new type of trainee will increase considerably, and new patient-care niches will be created. We believe cases such as this highlight the need for, and potential utility of, a cadre of pathologists trained and facile in genomic pathology.

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