MOLECULAR EPIDEMIOLOGY OF A *BACILLUS CEREUS* OUTBREAK

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Bacillus cereus is an infrequent cause of infections however several outbreaks in vulnerable patients have been described. In January 2011, a massive recall of potentially contaminated alcohol prep pads was initiated after The Children's Hospital, in Aurora, CO, identified a cluster of invasive *B. cereus* infections, which was epidemiologically linked to use of non-sterile, alcohol pads on hospitalized children in October 2010. Investigation of this outbreak included molecular methods to examine relatedness of *B. cereus* isolated from cultures of the implicated prep pads. Shortly after this outbreak was reported, a local healthcare facility in Salt Lake City, UT had observed an unusual increase in respiratory cultures containing *Bacillus cereus.* An extensive epidemiologic investigation was conducted and several *Bacillus cereus* isolates were cultured on a variety of environmental sources but not on respiratory equipment. Through use of molecular typing the outbreak was discovered to be polyclonal in nature and unrelated to contaminated medical equipment, No single factor was identified and after extensive Infection Control interventions targeting environmental hygiene and hand hygiene, *B. cereus* positive culture rates decreased.

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INTRODUCTION

Bacillus cereus is a gram-positive, rod-shaped bacterium that is motile, spore-forming and is considered both aerobic and facultative anaerobic in nature. It is commonly found in soil as a saprophytic organism. Since its recognition as an agent of food poisoning as early as 1955, it has been identified to spread through contamination of a wide variety of foods types, including dairy products, meats, and fried foods such as rice that are prepared and left at room temperature for an extended period of time. *A* unique quality of *B. cereus* is its ability to adapt to harsh environmental conditions when nutrients are scarce. Formation of *B. cereus* endospores enables it to preserve itself until its vegetative state at temperatures around 20 – 40^{o} Celsius.

Comparing 16s rRNA structures, *B. cereus* resembles *B. anthracis* (i.e., anthrax) and *B. thuringiensis* (i.e., insect pathogen), respectively. In addition to its symbiotic characteristics, *B. cereus* is also an opportunistic human pathogen in those who are immunocompromised such as newborns and the elderly. Such conditions may cause self-limiting gastroenteritis, periodontal disease, and systemic infections as meningitis, necrotizing enterocolitis, endocarditis, septicemia, pulmonary abscess, ocular and pyogenic infections. By contrast, *B. anthracis* is a serious human pathogen, which causes potentially fatal disease and is an agent of bioterrorism (1)(2)(10).

B. cereus produces three endotoxins responsible for the virulence factor common to gastroenteritis. The most common factor is hemolysin BL (HLB) followed by nonhemolytic enterotoxin (NHE) and lastly, cytotoxin K. Up until recent years, *B. cereus'* role in the human pathogenicity was largely unknown.

Since *B. cereus* is commonly recovered in environmental samples, they are often considered as being clinically insignificant. In recent years, a study conducted by the Center for Disease Control and Prevention (CDC) estimated the total annual cases and death rate caused by *B. cereus* to be steadily rising from 27,000 to as high as 84,000 in the U.S.(3). Refer to Table 1: Estimates of the Annual Cases and Deaths Caused by *B. cereus* in the U.S.

In October 2010, a child being treated for leukemia at The Children's Hospital (TCH) in Aurora, Colorado, developed clinical sepsis within 24-hours of receiving an implanted vascular device. At the site of the device, the child required extensive surgical debridement, antibiotic treatment, and multiple outpatient visits for wound management. Peripheral blood cultures and wound collections from the site of infection revealed the presence of *B. cereus.* In November 2010, a febrile infant diagnosed with congenital heart failure was

Table 1: Estimates of the Annual Cases and Deaths Caused by *B. cereus* in the U.S.

Agent	Cases	Percent	Deaths	Percent
B. cereus	27,360	0.2	0	0
Total bacterial	4,175,565	30.2	1,297	71.7
Total foodborne	13,814,924	100	1,809	100

admitted to TCH for respiratory distress syndrome. Four days following the placement of a jugular intravenous line, the infant developed a fever and became clinically septic. Peripheral blood cultures later recovered the presence of *B. cereus* (2).

To aid in the identification process of such nosocomial events, it becomes very important for clinical staff to recognize differences between contaminants and outbreaks when sudden increase in bacterial isolates is observed. This is especially important when an outbreak is attributed to organisms commonly considered to be commensals or not clinically significant, such as *B. cereus*. The incidence at TCH prompted further investigations as to the origins of *B. cereus* by incorporating molecular-based DNA fingerprinting (or profiles) for individual strains. Through this very process, TCH was able to attribute the origin of their contamination stemming from the use of nonsterile alcohol prep pads. A thorough investigation into the manufacturing practice of these isopropyl pads resulted in a nation-wide recall of all products with specific lot numbers in January 2011.

In addition to the outbreak at TCH, there have been other *B. cereus* outbreaks at various healthcare facilities. For instance, a separate facility described an outbreak in an NICU, and related *B. cereus* contamination was linked to respiratory equipment and ventilator airflow sensors. Using genotypic fingerprinting methodologies, a tracheal aspirate collected from a NICU infant suspected to be infected was determined to have a genetic *B. cereus* match to that of a similar strain isolated from a ventilator device (4). Such findings

emphasize the importance of stringent cleaning procedures to avoid potential patient contamination. More recently, a local healthcare facility in Salt Lake City, Utah, began to observe an increase in the number of *B. cereus* positive cultures identified from patient samples. Epidemiological evidence suggested that most cultures originated from a single unit (Unit A) at a specific facility (see Figure 1 and Figure 2). This increase was not observed in any other unit at the same facility or any other facility for which there is a surveillance program. As a result of this unexpected increase, an investigation was focused on clinical, demographic, and surveillance culture data. The aim of this project was to examine strain relatedness of *B. cereus* using molecular-based methodologies with intent to isolate the source of the outbreak.

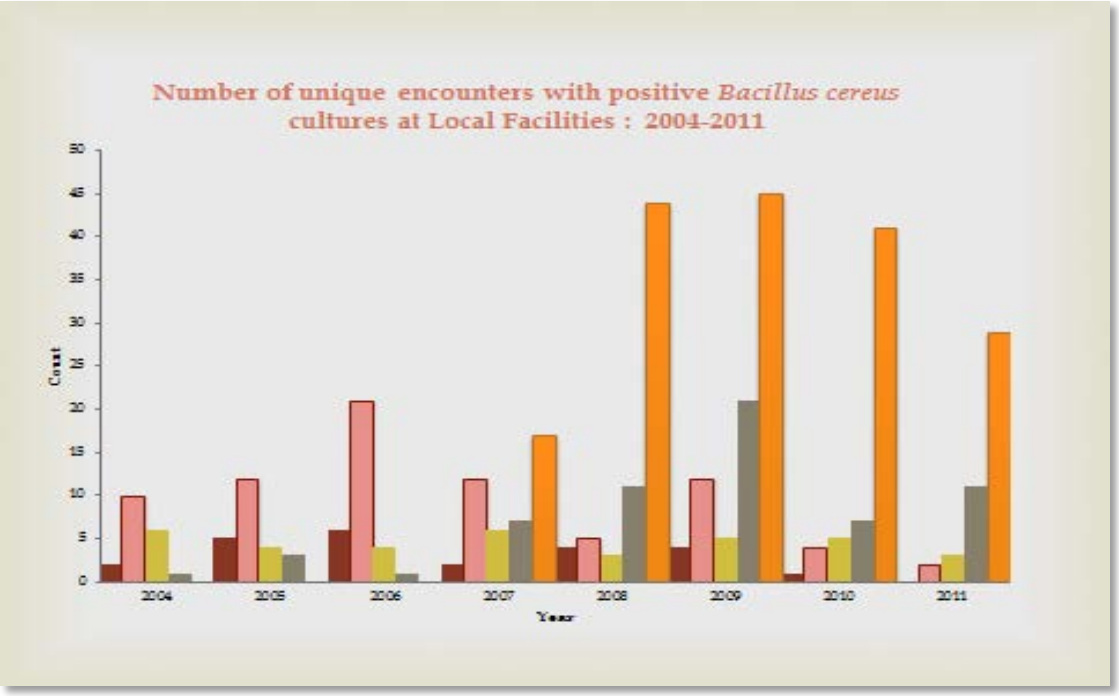

Figure 1: Number of *B. cereus* Positive Cultures at Different Local Healthcare Facilities

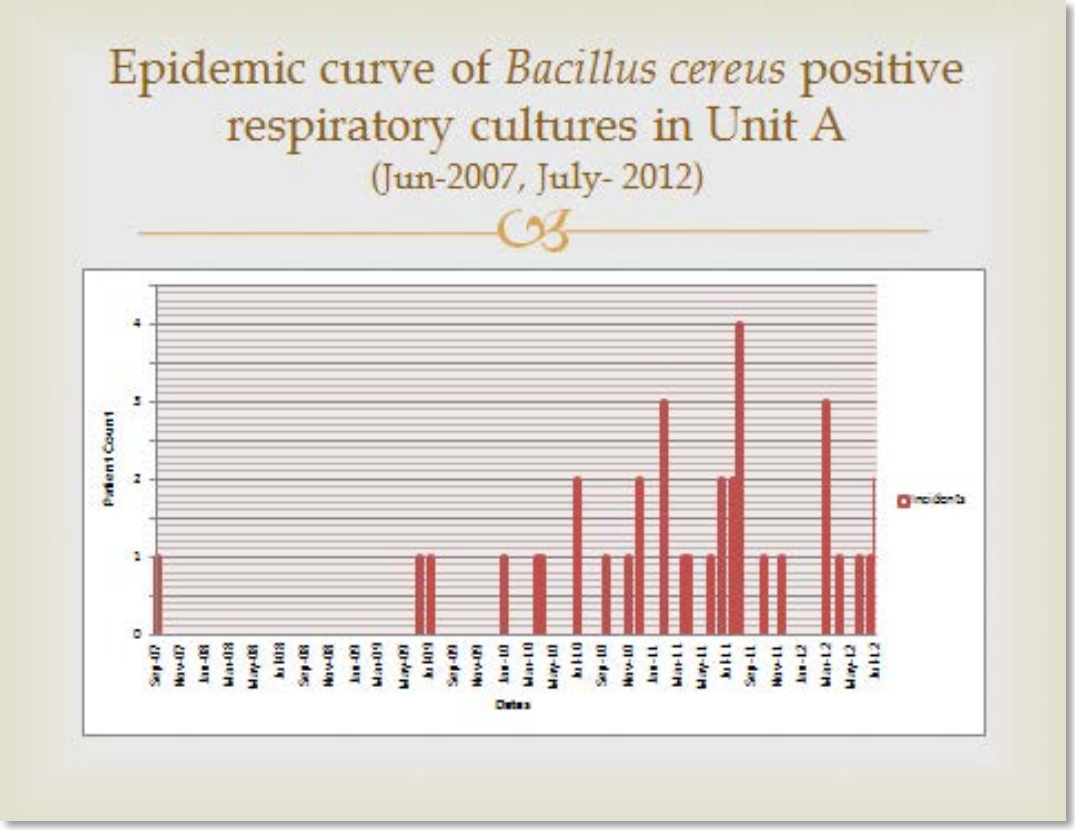

Figure 2: Number of *B. cereus* Positive Cultures in Unit A

TYPING METHODS AND APPLICATION IN HEALTHCARE EPIDEMIOLOGY

Bacterial agents, as compared to parasitic, viral, and fungal, are the most common cause nosocomial infections. The application of strain typing has long since been utilized in epidemiological investigations and can fall into two categories: phenotypic and genotypic methodologies. Examples of common phenotypic expression may be characterized by phage typing, serotyping, and their antibiotic susceptibilities.

Genotype methods are based upon their genetic structure and polymorphisms in DNA restrictions patterns. Molecular-based technologies have become a vital component of outbreak investigations with the main role in tracking the spread nosocomial infections and capable of differentiating endemic from epidemic strains (5). Genotyping methods have become an integral part of clinical and research laboratories. Several methods include restriction endonuclease analysis, multilocus enzyme electrophoresis, multilocus sequence typing, DNA sequencing, repetitive sequence-based PCR (rep-PCR), and pulsed-field gel electrophoresis (PFGE). The most frequent of genotyping methodologies is PFGE and rep-PCR (6).

The rep-PCR method uses primers that target noncoding repetitive sequences along its genome. Its primary function is to distinguish a subset of

bacterial species from other bacteria of similar characteristics by measuring differences between interspersed repeated sequences (4). With added amplification, the different sizes in DNA fragment can be fractionated by electrophoresis to establish a unique DNA fingerprint based on mass and polarity of the amplified fragments. Combining primers from multiple rep-PCR elements results in a higher level of discrimination between species and their strains.

Following a DNA extraction process, the rep-PCR process utilizes a 4-step amplification process consisting of: 1) binding of rep-PCR primers to many specific repetitive sequences throughout the genome; 2) amplification of fragments; 3) electrophoresis separation by mass and charge; and 4) the generation of rep-PCR DNA fingerprint profile.

The detection process is done using a microchip-based consumable that contains a gel dye to which the sample migrates by way of electro kinetic force. As DNA fragments bind to an intercalating dye while fragments are separated, they pass over a laser source, thereby creating a fluorescent intensity graph.

The analysis software has three reference intervals: Similar/Related, Indistinguishable/Identical, and Unrelated. Similar/Related suggests a 95-97% similarity with 1-2 band difference. The Indistinguishable/Identical suggests 97% similarity with no band differences. Finally, the Unrelated suggests <95% similarity and having >2 band difference in its fingerprint pattern (7).

Upon gel analysis, the software creates a cluster analysis (see Figure 3) followed by a dendrogram (see Figure 4), which is a branching diagram that represents the relationships of similarity among a group of entities.

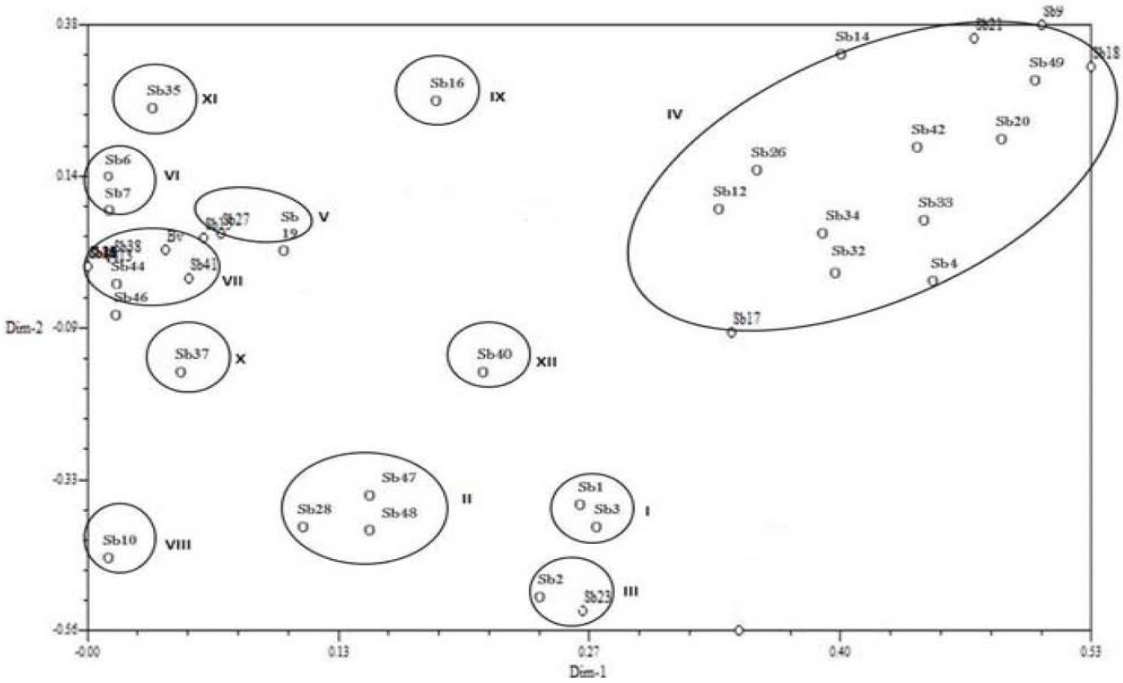

Figure 3: Example of rep-PCR Cluster Analysis

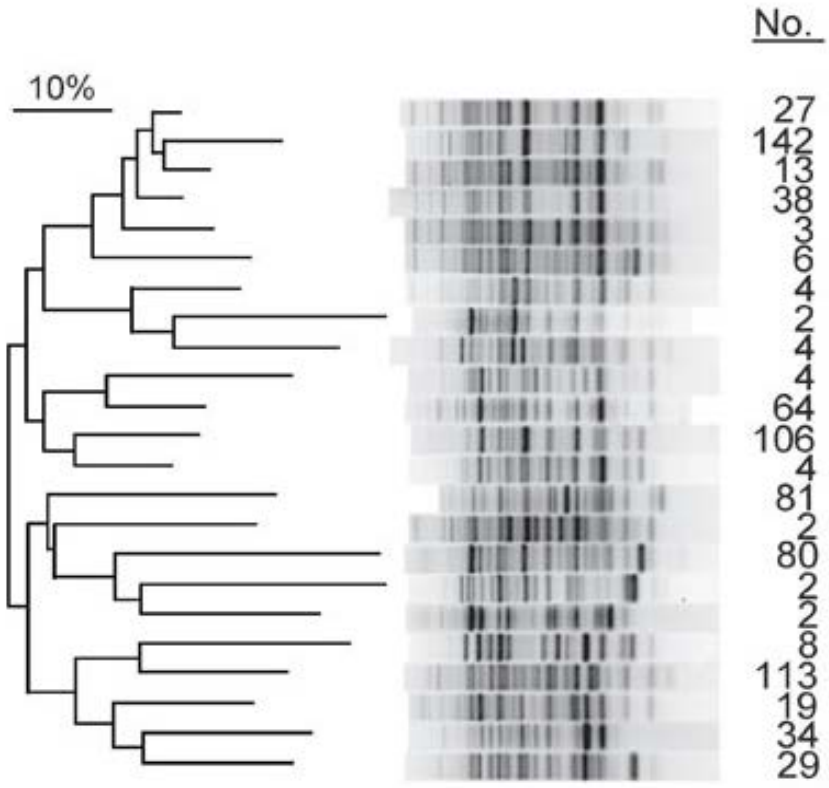

Figure 4: Example of rep-PCR Dendrogram

One of many examples where rep-PCR was utilized for a nosocomial outbreak occurred in 1997 when 29 inpatients at a hospital in Madrid were either colonized or infected with imipenem- and meropenum-resistant *Acinetobacter baumannii* strains (14). The molecular typing result showed that the outbreak was caused by a single imipenem resistant strain (8).

PFGE is the gold standard of molecular typing methods. Bacterial isolates are grown and combined with molten agarose gel then subjected to enzyme lysis and digestion with a restriction enzyme. Samples are then subjected to electrophoresis where the polarity of the current is changed at predominate intervals. PFGE allows large DNA-fragments (10-800 kb) to separate in the agarose. Upon staining with ethidium bromide (or other fluorescent dyes) a digital picture can then be initiated and imported into commercially available software packages for analysis.

Results from PFGE are applied to a standardized system based on strain relatedness. Bacterial isolates yielding the same pattern are considered to be the same strain. Isolates differing by a genetic event or 1-3 bands are considered closely related. Isolates differing by 4-6 bands representing 2 independent changes are considered possibly related. Six or more band differences, having greater than 3 changes, are considered unrelated.

Advantages of PFGE include being highly discriminatory for analysis of *S. aureus, Pseudomonas aeruginosa, M. avium, E. coli, Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, vancomycin-resistant enterococci, and others.

A limitation of PFGE is its slower turnaround time and its likely inability to be reproducible. One sample analysis could take as long as 3 days to complete (13). Additionally, PFGE control kits are not easily accessible for routine testing. As a result, homebrew controls are a common practice.

MALDI-TOF Mass Spectrometer

The principle behind MALDI-TOF is to ionize a sample using an onboard laser and then proteins are released and accelerated by an electric charge. After passing through an electrode detector, the proteins' "time of flight" is proportional to its mass weight. Proteins are detected with a sensor to create a spectrum that represents the makeup of each sample. In this experiment, the MALDI-TOF was simply used to confirm the presence of *B. cereus* organisms from randomly chosen, cryopreserved subcultures used for fingerprint analysis.

During the course of epidemiologic investigation conducted by Infection Control and hospital epidemiologists, *B. cereus* was isolated from environmental surfaces from Unit A as well as from the hands of a limited number of healthcare workers. Recalled nonsterile alcohol wipes manufactured by the company implicated in the Colorado outbreak had been stocked and used in Unit A until all lots were removed from circulation. These pads were collected and were included in this investigation. This study incorporates the commercially available rep-PCR test system, DiversiLab Strain Typing Kit, manufactured by bioMerieux, Inc. (Durham, NC) for the fingerprint differentiation of *B. cereus*.

OBSERVATION

In 2011, after recognition of an unusually high rate of *B. cereus* positive respiratory cultures in a hospital unit (Unit A), *B. cereus* isolates from this unit were cryopreserved for future investigation. Our “Case Definition” is defined as all infants admitted to Unit A, who had positive cultures for *B. cereus*. Our “Case Discovery” was determined by querying all positive cultures of *B. cereus* from Unit A along with positive cultures across multiple facilities during 2007 through 2012. From 328 identified *B. cereus* patient samples, 36 were specific to Unit A. Of those 36, only 25 samples were readily available for molecular serotyping.

HYPOTHESIS

The origin of our *B. cereus* contamination in Unit A was thought to have originated from several possible locations. Since all cultures were from respiratory specimens, we hypothesized that patients could have been exposed during procedural airway manipulations. A second hypothesis was that patients became colonized from aerosolization. A third hypothesis suggests that there was a widespread environmental contamination transfer of *B. cereus* from healthcare providers to patients. One final hypothesis was colonization of patients following use of contaminated alcohol prep pads that were previously used in Unit A.

OBJECTIVES

The purpose of this project is to use molecular-based methodologies to supplement the ongoing epidemiologic investigation. The objectives specific to this project are to:

1. Culture FDA-recalled nonsterile alcohol prep pads that were initially used in Unit A.
2. Compare the molecular relatedness of *B. cereus* isolates that were cultured from environmental surfaces and healthcare worker hands to clinical isolates collected from the patients.

MATERIALS AND METHODS

Subject Identification

The case definition for this investigation is any neonate (n=36) who was cared for between July 2010 and August 2012 in Unit A and had *B. cereus* isolated from respiratory cultures during hospital admission.

Clinical Samples

B. cereus isolates from respiratory samples were cryopreserved previously in the clinical laboratory for future typing studies. Isolates prior to 2011 were not preserved as at the time the outbreak was not apparent.

Environmental Samples

Random samples of the surrounding environment in Unit A were previously collected from various locations (n=37) such as windowsills, pass through windows, tape boards, vents, ledges, and so forth. Additionally, medical equipment used for intubation and mechanical ventilation was previously cultured. Environmental surfaces and medical devices were collected using moistened swabs with nonbactericidal saline and incubated in Thioglycollate broth for 24-48 hours.

Environmental HEPA filters (n=20) were also sampled for possible contamination. All environmental isolates were cryopreserved at -80^{o} C for future

typing.

To test the hypothesis that aerosolization of *B. cereus* led to widespread environmental contamination, sheep blood agar (SBA) Settle Plates (n=24) were randomly placed throughout Unit A and exposed for a minimum of 1.5 hours and incubated at 36^{o} C for up to 48 hours.

Hand/Finger Impressions

Samples were collected from healthcare providers (n=104), where finger and/or hand impressions were made either pre-hand washing or post-hand washing, then plated on a nutrient agar. The agar was then incubated at 36^{o} C for up to 48 hours.

Alcohol Prep Pads

Nonsterile alcohol prep pads (n=18) from recalled lots were broken down into 2 components and tested separately. Each pad contains a dry section (the outer layer) and a wet section (central portion of the 2x2 pad) or inner layer. The external surface of the alcohol prep pad was sampled using a sterile cotton tip swab moistened with thioglycollate (or BBL LIM) broth. The swab was then immersed into thioglycollate broth and incubated at 36^{o} C for up to 48 hours.

The external surface of the alcohol packaging was then disinfected using 10% bleach or DNase wipes and placed in a negative pressure biological safety cabinet where they were allowed to air dry with the UV light turned on for 10 minutes. After fully drying, the packaging was grasped with sterile forceps and cut open with a pair of sterile scissors. Finally, the alcohol pads were then

carefully removed and placed on separate sterile petri dishes. The alcohol pads were placed into a laminar flow hood and allowed to dry at room temperature for up to 24 hours. Care was taken to ensure that plates were not completely sealed while drying.

After 24 hours, each dried alcohol wipe was placed into thioglycollate broth and incubated at room temperature for 7 days. The broth was examined for growth every 24 hours. Samples from broth with positive growth were sub-cultured onto an SBA plates and streaked for colony isolation.

Control Material

Control strains were *B. cereus* isolates obtained from patients in units other than Unit A at the same facility.

Sample Collection

Clinical respiratory samples (n=25) from patients in Unit A were collected in a sterile respiratory transport container, then given to the clinical laboratory where conventional microbiological methods were utilized to identify any positive growth. Samples were also cryopreserved for future testing.

Repetitive Sequence-base PCR

Bacterial isolates were cryopreserved in a milk-based nutrient and stored at -80^{o} C. Samples were later retrieved, thawed, and plated onto SBA agar and incubated at 37^{o} C in a CO_2 incubator for 24 hours. A 10 uL loop full of a colony was extracted using an Ultra Clean Microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA.) as directed from the product insert. The

extracted DNA was amplified using a DiversiLab Bacillus DNA fingerprinting kit (bioMerieux, Inc.), which includes rep-PCR master mix 1, GeneAMP 10x PCR buffer, Primers Mix, AmpliTaq DNA polymerase, and kit-specific positive and negative controls in accordance with the manufacturer's product insert. 18 uL of rep-PCR MM1, 2 uL Primer Mix, 0.5 uLs of AmpliTaq DNA Polymerase, and 2.5 uL of GeneAmp 10X PCR buffer (Applied BioSystems, Foster City, CA) were combined to achieve a total of 23 uL of master mix per reaction. Next, 2 uL of DNA template was added. Reaction tube(s) were sealed and thermal cycler preheated at 94^{o} C. Thermal cycling parameters were set as follows: Initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds; extension at 70°C for 90 seconds; and the final extension at 70°C for 30 seconds; hold at 4^{o} C upon completion. Following amplification, the product was stored at 20° C until DiversiLab detection could be performed. Analysis of rep-PCR product was implemented using a DiversiLab system in which the amplified fragments of various sizes and intensities are separated and detected using a microfluidics Lab-chip. All individual chips were loaded with factory-designed ladder, positive and negative control, and individual designated samples. The chip was loaded according to DiversiLab's Operator's Manual (7)(16).

There are three possible options listed within the DiversiLab software in order to generate comparisons between graphs and similarity percentiles; Pearson Correlation (PC), The Kullback-Leibler (KL), and the Extended Jaccard (XJ) method. Each take into account the presence/absence and intensity of

peaks in generated electropherograms; however, each are weighted differently with PC placing more weight on peak intensity, KL on the presence or absence of peaks and XJ. The Kullback-Leibler (KL) method option was chosen for its presence and absences of peaks more than peak intensities (7).

MICROBIAL RESULTS

Clinical Samples

Suspect respiratory samples (n=36) were confirmed positive for *B. cereus* species using conventional microbiological methodologies.

Environmental Samples

From 37 randomly collected environmental sources from Unit A, *B. cereus* was recovered from 5 locations using conventional microbiological methodologies. These locations were identified as: windowsill, ledge, vent, pass-through window, and a tape board that is used to prepare tape to secure the endotracheal tubes.

B. cereus was recovered from one out of 24 settle plates. Four out of 20 HEPA filters yielded *B. cereus*, while 6 out of the same 20 yielded *B. thuringiensis*.

Hand/Finger Impressions

A total of 104 randomly collected hand and/or finger impressions from healthcare workers were collected, of which 8 cultures yielded Bacillus species. From these 8 culture positives, 3 were identified as *B. cereus*, while the remaining 5 were *B. thuringiensis*.

Alcohol Prep Pads

There was no *B. cereus* organism recovered from any of the 18 alcohol pads processed and cultured.

MALDI-TOF Assay

Fourteen previously tested *B. cereus* cultures were randomly tested onboard the MALDI-TOF system to confirm the presence of *B. cereus*. All 14 matched to their previous culture results.

MOLECULAR RESULTS

Clinical Samples

Using the rep-PCR methodology, 25 of the known 36 culture positive samples were analyzed using the DiversiLab Strain Typing Kit. Nine of the 36 samples were not readily available for testing and therefore were excluded from this study. The majority of the positive cultures collected and tested within a 4-month period were determined to be unrelated. We observed a cluster of 6 clinical samples having a related pattern and 2 additional patients were observed to have identical strains (see Table 2).

Environmental Samples

From the environmental swabs (n=81), 14 that were confirmed culture positive for *B. cereus* were then tested using rep-PCR. Nine of 14 isolates were determined to have identical strain patterns, while the remaining 5 isolates were unrelated strains.

Hand/Finger Impressions

From the 104 randomly collected hand and/or finger impressions from healthcare workers, 3 were determined to have positive *B. cereus*, while the remaining 5 isolates were *Bacillus species* (see Table 2). The DiversiLab results did not have any matching patterns or groups of clustering (see Figure 5 and

Table 2: Patient/Environment DiversiLab Results

		Culture Results	DiversiLab Results			
	n	*B. cereus* Positive	Number Tested	Identical Patterns	Related Patterns	Unrelated Patterns
Patient Isolates	36	36	25	2	6	19
Environmental Isolates	81	14	14	9	0	5
HCW	104	8*	8	N/A	N/A	N/A
Patients compared to Environment	N/A	N/A	N/A	0	0	All
Patients compared to HCWs	N/A	N/A	N/A	0	0	All

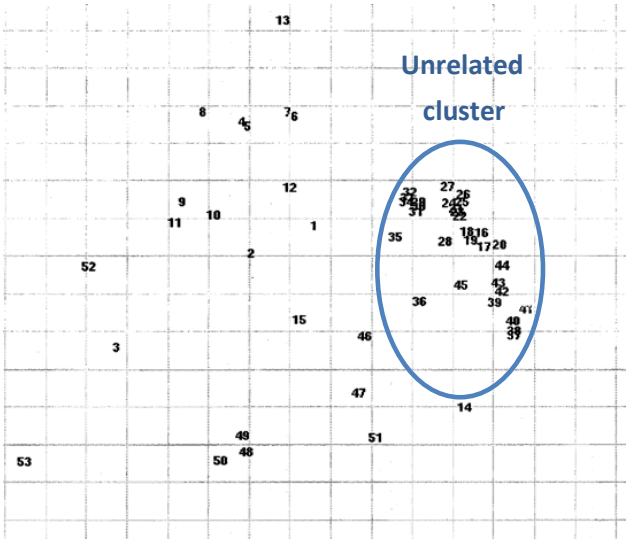

Figure 5: Species Classification - Scatterplot

Figure 6). This suggests there was no relatedness between healthcare-to-patient isolates.

Alcohol Prep Pads

There were no *B. cereus* organisms recovered from any of the 18 alcohol pads, suggesting the alcohol prep pads were unrelated to transmission of *B. cereus* in Unit A.

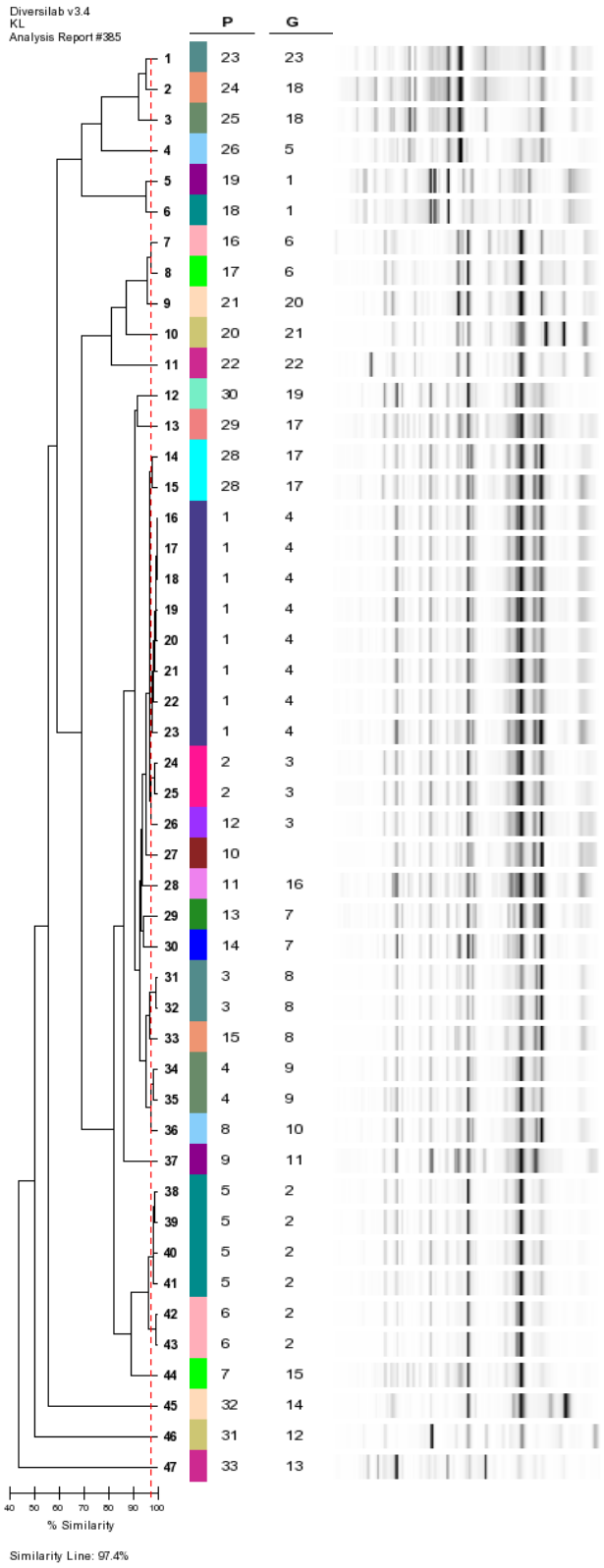

Figure 6: Unit A, *B. cereus* Final Dendrogram. A dendrogram is a branching diagram that represents the relationships of similarity among a group of entities.

DISCUSSION

Infection Control professionals at a local facility identified an increased rate of *B. cereus* positive respiratory cultures in a single hospital unit. The unusual feature was that the organism was exclusively isolated from respiratory samples. In light of a previous outbreak in which the respiratory tract was involved and medical equipment was implicated (3), the initial efforts were focused on identifying contaminated medical equipment. Because *B. cereus* is commonly found in the environment, environmental surfaces were also examined for contamination. Testing of respiratory equipment used for endotracheal intubation and mechanical ventilation were all negative; however, surfaces were indeed found to be colonized. Molecular data failed to link the nonclinical samples to the patients; therefore, we conclude that there was not a common source for the increased rate.

Interestingly, molecular data revealed a single clone proven to be identical by molecular typing in 6 patients over a 4-month period. Except for the cluster, colonization was polyclonal in nature with no relation between clinical and environmental samples. Colonization was not linked to any healthcare worker, nor environmental samples, but was likely a result of transient hand carriage. Hence, we concluded that the primary route of patient acquisition was from endemic transmission unrelated to a common vector.

Although there was a lack of evidence that a contaminated medical device played a role in the increased rates *of B. cereus*, we found the organism on different environmental surfaces and on the hands of healthcare workers. Molecular data did not link these to clinical samples; however, the contaminated surfaces and healthcare worker fingers support the hypothesis of environmental contamination and transmission through transient hand carriage. Therefore, Infection Control efforts focused on improving environmental hygiene and reinforcing the importance of hand hygiene was the main intervention, which led to a reduced rate of clinical cultures positive for *B. cereus*.

In light of the recent outbreak in TCH, concerns existed for involvement of contaminated alcohol prep pads (3). Unlike TCH, we did not identify any contaminated alcohol prep pads from recalled lots previously used in Unit A. Our investigation determined them to be unrelated to the increased rate of *B. cereus* positive cultures in our facility. This is not surprising as our cases centered around respiratory tract colonization whereas the outbreak at TCH was related to invasive infections at anatomic sites where prep pads were used.

Choosing an appropriate molecular-based method depends on the epidemiological context in which the method is to be utilized. One methodology may be more ideal than others depending on specific circumstances and challenges that present themselves. Though traditional typing methods based on phenotype have been used for many years, genotype methodologies have been utilized more recently for their ability to use genetic components where DNA fingerprinting (or strain typing) are specifically utilized to examine the relatedness

of isolates. This is especially important when investigating nosocomial infections or epidemic outbreaks. By use of these genotypic methods, the sensitivity of these assays is a major asset when investigating outbreaks of infection.

Advantages of rep-PCR methods include increased ability to detect and/or prevent outbreaks, semi-automated, PCR-based, web-based library access, may be performed at lower costs, faster analytics, increased sensitivities, higher throughput, and some have the ability to standardize and digitize gel images.

Disadvantages include the need to manually inspect electropherograms and virtual gels to confirm the similarity calculated by the software.

We had initially evaluated PFGE results from samples forwarded to a reference laboratory, but had chosen not to pursue further PFGE testing since results were returned inconclusive, methods were not standardized and adding new isolates to already typed samples was cumbersome.

We chose to use the DiversiLab Strain Typing kit to distinguish subsets of bacterial species differing from other bacteria of the same species by some minor fingerprint difference. The main reason we chose this method was that the system was readily available in our epidemiology lab, was semi-automated and allowed us to perform additional typing as new clinical isolates and environmental isolates were discovered. We found that rep-PCR or DiversiLab Strain Typing kit was highly applicable and a more robust method for routine subtyping in hospital epidemiological investigations. Given the ability to generate a library of characterized strains with fingerprint data that is stored on a cloud-based server,

this method allows for relatively rapid and convenient analysis of isolates at any time point, which is important if problems persist or recur.

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