Real-time Screening of Foods Using Repetitive Element PCR Reveals a DNA Marker Characteristic for Enterotoxigenic *Bacillus* Species

Breanna R. Brenneman[1], Kyla L. Adamson[2], Matthew R. Beer[3], Yenling Ho[4], Kiev S. Gracias[5], Chelsea M. Priest[6], Erika N. Biernbaum[7], and John L. McKillip*

Department of Biology, Ball State University, Muncie, IN 47306

[1]Current address: University of Virginia, Charlottesville, VA USA

[2]Current address: Elanco, Clinton, IN USA

[3]Current address: Cooper Genomics, Houston, TX USA

[4]CDC Foundation, Indianapolis, IN USA

[5]Department of Biology, Oakland City University, Oakland City, IN 47660

[6]Current address: Community Hospital, Anderson, IN USA

[7]Current address: Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA, USA

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Abstract

Bacillus cereus is traditionally thought to be the only member of its genus accepted as a pathogen in foods like grains, fruits, vegetables, and milk due to the presence of the nonhemolytic (Nhe) operon. However, many other *Bacillus* spp. may also harbor the Nhe operon and be pathogenic, including not just food-associated gastrointestinal toxicoinfections, but human endophthalmitis as well. Real-time PCR targeted the *nheA* gene in 37 samples obtained from food, soil, and reference cultures by analyzing the standard deviations of melt peaks. Repetitive element PCR was used to compare the banding patterns of each sample against *B. cereus* ATCC 14579 and three *B. thuringiensis* strains to "fingerprint" each isolate. Of the original 43 isolated tested, 37 were Gram-positive rods. The remaining six samples were Gram-positive cocci. Twenty-five of the 37 Gram-positive *Bacillus* spp. were *nheA* positive, while twelve were negative. Many of the *nheA* positive strains were species not previously known to contain Nhe and were capable of causing gastroenteritis in consumers.

Introduction

Bacillus spp. are Gram-positive endospore-forming rods ubiquitous in soil worldwide and are primarily aerobic to facultatively anaerobic saprophytes (3). Over 148 distinct *Bacillus* species have been described. This large number of individual species reflects a high degree of genetic diversity. Taxonomical identification of species within the *Bacillus* genus has changed over time as differentiation methods have improved (17). Currently, *Bacillus* species are divided into two groups - the *B. subtilis* and *B. cereus* divisions. The *B. cereus* group includes *B. cereus, B. mycoides, B. anthracis, B. thuringiensis,* and *B. weihenstephanensis*. These species are also grouped under the name *B. cereus sensu lato* (51). Phylogenetically, *B. cereus* is quite closely related to the entomopathogen *B. thuringiensis* and the human pathogen *B. anthracis*, a fact that has led to vigorous discussion on shared virulence properties, DNA sequence conservation among strains, and prevalence in the environment (19, 39, 48). Along with *B. weihenstephanensis*, these species constitute a single genetic subgroup, a rather arbitrary classification designation that brings into question how a species is even defined in this family of bacteria. For example, it seems clear that at least for many *Bacillaceae* other than *B. cereus*, presence and expression of enterotoxin genes is not uncommon (7, 11, 18, 20); nor is it atypical to identify strains of *B. cereus* lacking detectable enterotoxin genes (5, 48). Moreover, *B. cereus* may harbor virulence genes on plasmids more commonly associated with *B. anthracis* (49). Many pathogenic strains of *Bacillus* spp. are primary isolates from clinical, food, and environmental sources. Naturally, many published studies on the *B. cereus* group mention the difficulty in selecting features for reliable identification of these species. The involvement of species in the *B. cereus* group in foodborne illness, as a leading cause of ocular infections (endophthalmitis), and as an indicator of water quality (4, 25), begs the question of how such a closely related set of species and strains could manifest itself so differently in varied environments. Accordingly, a reassessment of identification strategies is in order as even more reports appear in the literature of *Bacillus* spp. involvement in novel ecological niches.

The search for a reliable DNA-based typing approach for *Bacillaceae* has explored several technologies in recent

Table 1.

Lesaffre Yeast	Smuckers French Vanilla
Seasoning	**Snacks**
Basil	Beef Taco from Taco Bell
Oregano	Food Club Quick Oats
Mustard	Ann's House Healthy Energy Blend Nuts
Nutmeg	Kraft Jet-Puffed Marshmallows
Paprika	Dannon Yogurt
Milk	**Coffee Creamer**
Parmalat 2% Reduced Fat Milk	Coffee Mate
Mix N' Drink Skim Milk	Wholesome Farms
Great Value Evaporated Milk	Great Value
Myenberg Vitamin D Goat Milk	Glenview Farms
Carnation Evaporated Milk	Flavor Right Half and Half
Prairie Farms	

Table 1: Foods screened for the presence of *Bacillus* spp. by food type as described in Materials and Methods.

years, including repetitive element PCR (rep-PCR) (34), next generation sequencing (NGS) of whole genomes to identify polymorphic regions (12), and multilocus sequence typing (MLST) (6, 21, 22), which relies on the PCR-based amplification of 400- 600bp internal fragments of housekeeping genes. However, these conserved gene targets are frequently not adequate to effectively resolve *Bacillus* species or strains for identification. Clearly, a repertoire of both phenotypic and novel genotypic-based methods must be utilized for the ever-increasing number of strains appearing in the literature. This trend reflects a growing interest in this group of bacteria (4). The objective and hypothesis of this research is that one may develop a genotypic screening method to reliably detect enterotoxigenic *Bacillus* spp. from contaminated food without the need for culture-based methods. Use of DNA typing/fingerprinting compared to positive control enterotoxigenic (Nhe-producing) *Bacillus*, we show the potential in rep-PCR as a rapid and high-throughput screening tool for a variety of contaminated foods.

MATERIALS AND METHODS

Bacillus spp. isolation from soil and food.

Using a previously described method (46), soil was collected (at 4-inch depths) from multiple locations around the Ball State University campus in Muncie, IN. A total of 41 varieties of flavoring/ powder, seasonings, milk, coffee creamers, cheese, snacks, spreads, and drink additives were obtained at local retailers in order to isolate *Bacillus* spp. from these naturally contaminated foods (Table 1). Each sample (5g) was added to 100 mL of brain heart infusion broth (BHIB; BD Diagnostic Systems, Franklin Lakes, NJ). After mixing, the solution was incubated at 32°C while shaking at 160 RPM for 72h. Samples were heat-treated at 80°C for 30 min on a hot plate using a water jacketed vessel and constant shaking, after which the suspension (1 mL) was pipetted onto quadruplicate tryptic soy agar (TSA; Alpha Biosciences, Baltimore, MD) plates and incubated overnight at 37°C. Streak plates were performed from initial growth and incubated at 37°C overnight to obtain pure cultures, confirmed by Gram and endospore staining. Reference strains (Table 2) were obtained from Presque Isle Cultures (Erie, PA USA) and Dr. James Mitchell (Ball State University, Muncie, IN) and subcultured

Table 2.

Strain	Identifying Code	Source
B. subtilis globigii	6201	Presque Isle Cultures
B. stearothermophilus	627	Presque Isle Cultures
Geobacillus stearothermophilus	627A	Presque Isle Cultures
B. sphaericus	633	Presque Isle Cultures
B. megaterium	616	Presque Isle Cultures
B. macerans	626	Presque Isle Cultures
B. pumulis	6222	Presque Isle Cultures
B. brevis	630	Presque Isle Cultures
B. polymyxa	625	Presque Isle Cultures
B. coagulans	6221	Presque Isle Cultures
B. circulans	628	Presque Isle Cultures
B. subtilis	620	Presque Isle Cultures
B. laterosporos	629	Presque Isle Cultures
B. cereus	14579	ATCC
B. thuringiensis kurstaki HD1	33679	Dr. James Mitchell
B. thuringiensis japanensis B23	T23 001	Dr. James Mitchell
B. thuringiensis israelensis	T14 001	Dr. James Mitchell

Table 2: *Bacillus* reference strains used in this study.

onto TSA slants. All cultures were refrigerated at 4°C until DNA extraction and real-time PCR analysis.

DNA Extraction.

Each isolate, including reference strains, was separately cultured in tryptic soy broth (TSB, Weber Scientific, Hamilton, NJ USA) grown aerobically by shaking for 24h as previously described (31). Bacteria were pelleted by centrifugation at 9,000 x g for 3 min at 4°C and the pellets were resuspended in 300µl TE buffer (Amresco, Solon, OH) containing 30 µl of 10% SDS (Promega, Madison, WI), and 20 µl of 20 mg/mL Proteinase K (Ambion, Austin, TX). Following a 37°C 30 min. incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution (Amresco) was added, vortexed, and centrifuged for 10 min. at 10,000 x g and 4°C. The aqueous phase was carefully transferred into clean microcentrifuge tubes and mixed with 0.1 volume of cold 3M sodium acetate (Fisher Scientific, Pittsburgh, PA) and one volume of cold 95% isopropanol (Greenfield Ethanol Co., Brookfield, CT). The microcentrifuge tubes were inverted to mix and centrifuged for 30 min at 16,000 x g at 4°C. The supernatant was discarded, and the dried DNA pellets were quantified spectrophotometrically to assess yield and purity.

Uniplex PCR.

All DNA samples were normalized to a concentration of 1 µg/µL in sterile water. All reactions were performed in triplicate. Real-time PCR was used initially to target the *nheA* gene (32). Primer sequences used for this and other experiments are shown in Table 3.

PCR was performed as previously described (18) with minor modifications. The annealing temperature was changed from 55°C to 52°C to better support annealing of the *nheAF* primer to template DNA, and a melting curve was used to resolve and validate amplicon identity. Each PCR reaction consisted of 1X iQ Sybr Green Supermix (Bio Rad, Hercules, CA), 100 pmol *nheAF* and *nheAR* primers (Integrated DNA Technologies, Coralville, IA), and 0.5 µg of template DNA. Nuclease-free water (Promega) was added for a final volume of 25 µL in 0.2 mL PCR tubes (Corbett Research, Concord, NSW). A positive control was included in each set of reactions, consisting of template DNA from *B. cereus* ATCC 14579, (American Type Culture Collection, Manassas, VA), previously shown to harbor the *nheABC* operon (41).

PCR reactions were performed in a Rotor Gene RG-3000 thermocycler (Corbett Research) using an initial 94°C 120s denaturation step followed by 35 cycles of 94°C for 20s, 52°C for 60s, and 72°C for 60s. A final 72°C 6 min. extension step preceded melting curve analysis (40°C to 95°C in 0.7°C per second increments). Amplicon melting peaks were plotted using Rotor Gene 6 software and melt peak data were exported into Microsoft Excel for analysis. Only melt peaks within 1 standard deviation of the average melt peak of positive control *B. cereus* ATCC 14579 were considered as positive for the presence of *nheA*.

Rep-PCR.

DNA templates from all strains analyzed (Table 1) were subjected to repetitive element- PCR (rep-PCR) using a Diversilab kit (Bacterial Barcodes, Athens, GA) specific for fingerprinting *Bacillus* spp. DNA of each sample previously isolated for real-time PCR was re-standardized to 50 ng/µl. Primers for repetitive elements within *Bacillaceae* were included in the Diversilab kit and are shown in Table 3 (24).

All reactions were completed in triplicate and consisted of 18 µl rep-PCR MM1 buffer, 2.5 µl of GeneAmp® 10X PCR Buffer, 2.0 µl of primer mix, and 0.5 µl of Taq DNA Polymerase (5 PRIME, Gaithersburg, MD) and 100 ng of template DNA. Positive kit controls were included with each set of replicates, as were no template controls (NTC). All reactions were performed using a Rotor Gene instrument and consisted of an initial 94°C 2 min. denaturation followed by 35 cycles of 94°C for 30s, 55°C for 30s, and 70°C for 90s. Following a final extension step for 3 min at 70°C, a subset of reactions was subjected to melting curve analysis as described earlier, while others were analyzed using agarose gel electrophoresis. For the latter samples, 5µl of each PCR product was loaded into a 1.5% (w/v) agarose gel (BioExpress, Kaysville, UT) containing 0.625 µg/µl ethidium bromide

(Invitrogen, Carlsbad, CA) (43) and the gel electrophoresed for 1.5h at 70V (constant). The gel was visualized on a Gel Doc XR (Bio-Rad, Hercules, CA) using UV light. The resulting banding patterns were recorded in Microsoft Excel as a virtual gel (Table 4). Banding patterns of *nheA* positive and *nheA* negative were compared against the *B. cereus* reference strain and three *B. thuringiensis* strains, representing additional members of the B. cereus genetic subgroup. Sample bands identical to each reference strain (*B. cereus, B. thuringiensis* var. *kurstaki*, *B. thuringiensis* var. *japanensis,* and *B. thuringiensis* var. *israelensis*) were divided by the number of total bands in each reference strain. The resulting number was multiplied by 100 to determine the percent each sample was identical to *B. cereus, B. thuringiensis* var. *kurstaki, B. thuringiensis* var. *japanensis,* and *B. thuringiensis* var. *israelensis*.

Sequencing of diagnostic rep-PCR product.

The 1,230bp diagnostic band (11) earlier found to be unique to enterotoxigenic *Bacillus* spp. was identified in real-time melting curve plots of *B. cereus* ATCC 14579 and other strains.

RESULTS

Samples

Excluding reference microbes purchased from Presque Isle Cultures, a total of 45 food and soil samples were screened for the presence of *Bacillus* spp. Of these, 21 isolates (48.9%) were found to contain no detectable *Bacillaceae*. Twenty isolates (44.4%) were Gram-positive, spore-forming rods after heat-treatment and subsequent streak-plating on TSA. These included: basil seasoning, nutmeg seasoning, Tazo tea powder, a beef taco from Taco Bell, Lesaffre Yeast, Prairie Farms Whole Milk, Food Club Quick Oats, Ann's House Healthy Energy Blend Nuts, Peter Pan Peanut Butter, Great Value Peanut Butter, Dannon Yogurt, Chevre Fresh Goat Cheese, Saputo Stella Gorgonzola Cheese, Black Creek Extra Sharp Cheddar Cheese, Pilgrim's Choice Blue Stilton Cheese, Cooper Science Building Soil, Lucina Building Soil, Christy Woods Soil, and Ball Gymnasium Soil. An additional six isolates, three of which were isolated from Jiffy Corn Muffin Mix, were Gram-positive cocci. Aside from Jiffy Corn Muffin Mix, Gram-positive cocci were isolated from mustard seasoning and Mix n' Drink Powdered Skim Milk. These Gram-positive cocci accounted for a total of 6.7% of the entire sample pool.

Seventeen *Bacillus* spp. reference strains were purchased from Presque Isle Cultures for subsequent real-time PCR analysis. Overall, a total of 37 samples were either pure-type cultures or Gram-positive rods that were subsequently subjected to DNA extraction in preparation for real-time and rep-PCR.

Real-time PCR

B. cereus ATCC 14579 was used as a positive control to test for the presence of *nheA* and had an average melt peak of

Table 3.

nheA	Primer Sequence 5' → 3'	Position	Accession #	Ref	T_m	G/C
F	TACGCTAAGGAGGGGCA	344 – 360 →	Y19005	9	55.5°C	58.80%
R	GTTTTTATTGCTTCATCGGCT	843 – 823 ←	-	-	51.8°C	38.10%
Direction	**Primer Sequence 5'→ 3'**	**Primer Name**	-	**Ref**	**T_m**	**G/C**
F	ICG ICT TAT CIG GCC TAC	REP 2-I	-	15	57.5°C	50%
R	III ICG ICG ICA TCI GGC	REP 1R-I	-	15	68.3°C	50%

Table 3: Primer sequences, melting temperatures, and guanine and cytosine content for the *nheA* gene used in uniplex PCR and repetitive element (rep-PCR) palindromic sequences in *Bacillus* spp.

81.96°C over three runs. As shown in Table 5, fourteen test samples (37.84% = green highlighted) were consistently within 1 standard deviation (SD) of the positive control over three separate real- time PCR runs (SD determined using SigmaStat for Windows). Standard deviations of samples positive all of three replications for *nheA* are included in Table 6, part B. These samples included reference strains *B. macerans, B. brevis, B. cereus, B. thuringiensis var. kurstaki, B thuringiensis var. japanensis,* and *B. thuringiensis var. israelensis*. Food samples consistently within one SD of the positive control originated from Prairie Farms Whole Milk, Ball Gymnasium soil, Lucina Hall soil, Cooper Science soil, Christy Woods soil, basil seasoning powder, "Clean" Peter Pan Peanut Butter, and Great Value Peanut Butter. Five samples (13.51% = yellow highlighted had two melt peaks within 1 SD of the positive control strain. These included the pure strain *B. laterosporos* and food samples from a beef soft taco from Taco Bell, nutmeg powder, Chevre Fresh Goat Cheese, and Saputo Stella Gorgonzola Cheese.

Table 4.

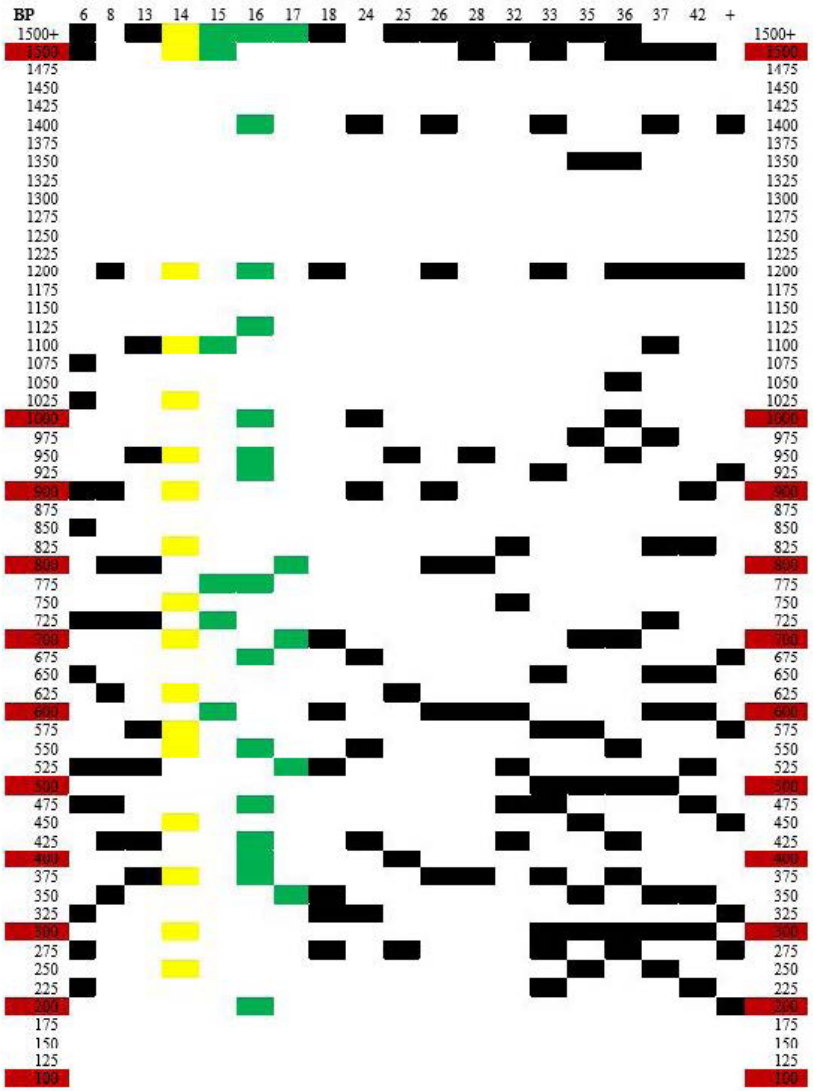

Table 4: Banding patterns of all *nheA* positive samples, excluding samples 9, 10, 22, 27, 31, 39, and 43. Red cells represent the length in base pairs of each DNA ladder band. Yellow cells represent the column of positive control *B. cereus* ATCC 14579. Green cells represent banding of *B. thuringiensis var. kurstaki* (#15), *B. thuringiensis var. japanensis* (#16), and *B. thuringiensis var. israelensis* (#17).

Table 5.

#	Sample	#	Sample
1	*B. subtilis globigii*	23	Jiffy Cornbread Cream Colony
2	*B. stearothermophilus*	24	Ball Gym Soil
3	*Geobacillus stearothermophilus*	25	Lucina Building Soil
4	*B. spaericus*	26	Basil Seasoning Powder
5	*B. megaterium*	27	Taco Bell Soft Beef Taco
6	*B. macerans*	28	Cooper Science Soil
7	*B. pumulis*	29	Mustard Seasoning Powder
8	*B. brevis*	30	Jiffy Cornbread Translucent Colony
9	*B. polymyxa*	31	Nutmeg Powder Seasoning
10	*B. coagulans*	32	Christy Woods Soil
11	*B. circulans*	33	Chevre Fresh Goat Cheese
12	*B. subtilis*	34	Black Creek Sharp Cheddar Cheese
13	*B. laterosporos*	35	Saputo Stella Gorgonzola Cheese
14	*B. cereus*	36	Pilgrim's Choice Blue Stilton Cheese
15	*B. thuringiensis kurstaki*	37	"Clean" Peter Pan Peanut Butter
16	*B. thuringiensis japanensis*	38	Tazo Tea Powder
17	*B. thuringiensis israelensis*	39	Ann's House Energy Blend Nuts
18	Prairie Farms Whole Milk	40	Dannon Yogurt
19	Jiffy Cornbread Yellow Colony	41	Lesaffre Yeast Isolate
20	Mix N' Drink Powdered Milk	42	Great Value Peanut Butter
21	Nesquick	43	*Salmonella*-Contaminated Peter Pan Peanut Butter
22	Food Club Quick Oats		
	= 3 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak		
	= 2 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak		
	= 1 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak		
	= 0 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak		
	= Not Gram-positive rods, but isolated after heat-treatment		

Table 5: Samples were positive or negative for the presence of *nheA* 3/3, 2/3, 1/3, or 0/3 times. Green samples indicate positive detection of *nheA* in real-time PCR over three separate runs. Yellow samples indicate *nheA* positive samples in two of three real-time PCR runs. Orange samples indicate *nheA* positive samples in one of three real-time PCR runs. Red samples indicate negative *nheA* detection in real-time PCR.

Six samples (16.22% = orange highlighted) had one melt peak within 1 SD of the positive control train. These included pure strains *B. polymyxa* and *B. coagulans*. In addition, food samples with only 1 of 3 melt peaks consistent with *B. cereus* positive control were from Food Club Quick Oats, Pilgrim's Choice Blue Stilton Cheese, Ann's House Energy Blend Nuts, and a jar of Peter Pan Peanut Butter involved in a food recall that may have contained *Salmonella*.

Twelve of the 37 samples (32.43% = red highlighted) had either no melt peaks or melt peaks greater or less than one SD of the positive control strain. These included: pure strains *B. subtilis globigii, B. stearothermophilus, Geobacillus stearothermophilus, B. spaericus, B. megaterium, G. pumulis, B. circulans,* and *B. subtilis*. Foods negative for nheA included Black Creek Extra Sharp Cheddar, Tazo Tea powder, Dannon Yogurt, and Lesaffre Yeast.

As shown in Table 6, part A, there were 4 of 16 food samples that tested positive for *nheA* for three melt peaks. Additionally, 4 food samples displayed two positive melt peaks, while 4 more displayed one melt peak. Four food samples displayed zero melt peaks. All soil samples were positive for *nheA* with three melt peaks. Six reference strains displayed three positive melt peaks for *nheA*, while only one strain had two melt peaks consistent with the positive control. Two strains had one melt peak in line with the positive control, while 8 strains were completely negative for the presence of *nheA*.

After real-time PCR, SD were calculated for sample melt peaks to compare against *B. cereus* ATCC 14579. Samples 6, 8, 14, 15, 16, 17, 18, 24, 25, 26, 28, 32, 37, and 42 resulted in standard deviations < 1 when compared against the 82°C average positive control melt peak. Any sample with a SD < 1 indicated a positive detection for the *nheA* gene. Data are shown in Table 6, part B.

Table 6.

A.

# *nheA* +	Total Food	% Food	Total Soil	% Soil	Total Ref.	% Ref.
3/3	4	25	4	100	6	35.29
2/3	4	25	0	0	1	5.88
1/3	4	25	0	0	2	11.76
0/3	4	25	0	0	8	47.06
	16	**100**	**4**	**100**	**17**	**100**

B.

Sample	**6**	**8**	**14**	**15**	**16**	**17**	**18**
S.D.	0.27	0.29	0.18	0.22	0.23	0.12	0.62
Sample	**24**	**25**	**26**	**28**	**32**	**37**	**42**
S.D.	0.38	0.27	0.32	0.34	0.29	0.27	0.30

Table 6: A: Number of samples with *nheA* positive melt peaks three, two, one, and zero times in divisions of food, soil, and reference strains. **B:** Samples with corresponding SDs < 1 when compared against the positive control strain *B. cereus* ATCC 14579 during real-time PCR.

range of the DNA ladder, as shown in Table 4. These bands corresponded to lengths of 1500 bp, 1200 bp, 1100 bp, 1025 bp, 950 bp, 900 bp, 825 bp, 750 bp, 700 bp, 625 bp, 575 bp, 550 bp, 450 bp, 375 bp, 300 bp, and 250 bp. Table 7 includes all banding patterns for all *nheA* positive samples. All other banding patterns were compared against *B. cereus* reference strain (#14) and three *B. thuringiensis* spp. reference strains (#15-17). All samples were compared against *B. cereus, B. thuringiensis var. kurstaki, B. thuringiensis var. japonensis,* and *B. thuringiensis var. israelensis*. The resulting percent identities of the banding patterns to each reference strain of each sample are recorded in Table 7.

Rep-PCR

Repetitive element PCR was utilized on *B. cereus* ATCC 14579, which was labeled as sample 14 for real-time and rep-PCR. This strain was subsequently used as the standard against which all other *nheA* positive samples in rep-PCR were compared. Sample 14 displayed 16 bands within the

B. cereus (sample #14)

Samples 17 and 25 were 6% identical to the banding pattern of sample 14. Samples 6, 15, 18, 24, and 32 were 13% identical to sample 14. Samples 8, 13, 26, and 28 were 19% identical to sample 14 banding. Samples 16 and 42 were 25% identical to sample 14, while samples 33 and 35 were 31% identical. Samples 36 and 37 were 44% identical to sample 14. No samples were more than 44% identical to sample 14.

Nine *nheA* negative samples were analyzed using rep-PCR, and include samples 1-5, 7, 9, 11, and 12 (data not shown). Samples 2, 3, 5, and 7 were 6% identical to the banding

pattern of sample 14. Samples 1, 4, and 12 were 19% identical to sample 14. Sample 11 was 44% identical to the banding pattern from sample 14.

***B. thuringiensis var. kurstaki* (sample #15)**

When compared against *B. thuringiensis var. kurstaki*, samples 17, 24, 25, and 35 shared no identical banding. Samples 8, 16, 18, 26, 32, 33, and 36 were 20% identical to B. thuringiensis var. kurstaki. Samples 6, 13, 14, 28, and 42 were 40% identical, while sample 37 was 80% identical to *B. thuringiensis var. kurstaki*.

***B. thuringiensis var. japanensis* (sample #16)**

Samples 6, 17, and 35 shared no identical banding with *B. thuringiensis var. japanensis*, while samples 15 and 18 were 8% identical. Samples 28 and 32 were 17% identical to *B. thuringiensis var. japanensis*, but samples 8, 13, 26, and 37 were 25% identical. Samples 14 and 42 were 33% identical to *B. thuringiensis var. japanensis*. Samples 24 and 33 were 42% identical to *B. thuringiensis var. japanensis* banding, while sample 36 was 50% identical.

Table 7.

Compared against #14																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	2	3	3	16	2	4	1	2	2	1	3	3	2	5	5	7	7	4
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #14	13	19	19	100	13	25	6	13	13	6	19	19	13	31	31	44	44	25
Compared against #15																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	2	1	2	2	5	1	0	1	0	0	1	2	1	1	0	1	4	2
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #15	40	20	40	40	100	20	0	20	0	0	20	40	20	20	0	20	80	40
Compared against #16																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	0	3	3	4	1	12	0	1	5	1	3	2	2	5	0	6	3	4
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #16	0	25	25	33	8	100	0	8	42	8	25	17	17	42	0	50	25	33
Compared against #17																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	1	3	2	1	0	0	4	3	0	0	1	1	1	1	2	1	1	2
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #17	25	75	50	25	0	0	100	75	0	0	25	25	25	25	50	25	25	50
	= Between 40% and 99% identical to yellow reference strain																	
	= Between 30% and 39% identical to yellow reference strain																	
	= Between 20% and 29% identical to yellow reference strain																	
	= Sample all others were compared against																	

Table 7: Comparison of all rep-PCR banding patterns with *B. cereus* (sample #14), *B. thuringiensis var. kurstaki* (sample #15), *B. thuringiensis var. japanensis* (sample #16), and *B. thuringiensis var. israelensis* (sample #17). Yellow cells denote the specific sample all other banding patterns were compared against. Blue cells represent banding patterns 20% to 29% identical to each yellow reference strain. Green cells represent banding patterns 30% to 39% identical to each yellow reference strain. Red cells represent banding patterns identical banding that was 40% and above to each yellow reference strain. No samples were more than 44% identical to *B. cereus*. Sample 37 was 80% identical to *B. thuringiensis var. kurstaki*. Sample 36 was 50% identical to *B. thuringiensis var. japonensis*. Samples 13, 35, and 42 were 50% identical to *B. thuringiensis var. israelensis*, while samples 8 and 18 were 75% identical.

***B. thuringiensis var. israelensis* (sample #17)**

When compared against *B. thuringiensis var. israelensis*, samples 15, 16, 24, and 25 were 0% identical. Samples 6, 14, 26, 28, 32, 33, 36, and 37 were 25% identical to *B. thuringiensis var. israelensis*. Samples 13, 35, and 42 were 50% identical to *B. thuringiensis var. israelensis*, while samples 8 and 18 were 75% identical.

As shown in Table 5, samples 9, 10, 22, 39, and 43 had one *nheA* positive amplicon during real-time PCR. Samples 27 and 31 had two *nheA* positive amplicons late in analysis after initially appearing to only contain one positive melt peak. Consequently, these samples were not subjected to rep-PCR, as the real-time results were inconsistent. Rep-PCR efforts were instead directed at samples that had either three melt peaks or two melt peaks early in analysis within 1 SD of the positive control.

DISCUSSION

The debate over the ideal method for identification of *Bacillus* isolates has raged for over 50 years (42). Recent public awareness of potential bioterrorism using the anthrax toxin produced by *B. anthracis* has led government agencies to fund multiple studies aimed at rapidly differentiating *B. anthracis* from other closely related *Bacillus* species, such as *B. cereus* and *B. thuringiensis*. *B. anthracis* produces the anthrax toxin encoded by two plasmid-based operons, pXO1 and pXO2 (3, 17, 42). The anthrax toxin primarily kills herbivore mammals but can also kill humans (42, 48). Not to be underestimated, *B. cereus* can cause severe food poisoning through its production of emetic and diarrheal toxins (3, 19). While heavily used as an insecticidal agent in crops with its Cry crystalline toxins, *B. thuringiensis* has also recently been demonstrated to cause food poisoning symptoms in humans similar to *B. cereus* (3, 42). Ironically, species like *Bacillus coagulans*, which was found to contain *nheA* at least once in this study, are readily used as probiotics in human health (30). It should be noted that the *nhe* genes have been among the most common reference virulence genes targeted in PCR-based assays performed in foods, including dairy foods (37). NheA/*nheA* has thus been widely accepted as an indicator of virulence potential in *Bacillus* spp. *senso lato*.

These strains were originally differentiated into species at a time when biologists did not possess the molecular tools to delve deeper than biochemical tests and phenotypical observations (3, 17, 42). While this strategy worked well for other genera, 16S rRNA analysis of differences among *B. cereus, B. thuringiensis, and B. anthracis* have shown these species to have a nucleotide sequence difference of < 1% (48). Thus, the emerging "holy grail" of *Bacillus* research would be to accurately differentiate these species. Recent advances in molecular biology have allowed scientists to scrutinize the genetic properties of these three "species" (42). After exhaustive studies using DNA-DNA hybridization, 16S and 23S rRNA comparative analyses, multilocus sequence typing (MLST), fluorescent amplified fragment length polymorphism analysis, rep-PCR, and small nucleotide polymorphism (SNP) analyses, scientists have been unable to reliably differentiate these three *Bacillus* species.

While many methods have been pursued, most results have suggested that *B. cereus, B. thuringiensis,* and *B. anthracis* should be considered the same species due to highly conserved nucleoidal genetic sequences (3, 17, 39, 43, 48). Due to the easily identifiable symptoms of *B. anthracis* and *B. cereus*, there is recent concern among biologists that the *"B. anthracis"* species may in fact be an oversampled subset of *B. cereus* (42). Other scientists speculate that *B. anthracis* may have only recently evolved to the point to be considered distinct from *B. cereus* (20). Either way, a separate study confirmed that enough of a difference exists between the genome of *B. anthracis* when compared against *B. cereus* or *B. thuringiensis* to consider *B. anthracis* as identifiable using pulsed-field gel electrophoresis (50).

Of the 45 total food and soil samples in this study, 20 *Bacillus* isolates were obtained (44.4%). Twenty-one samples (48.9%) were not found to contain *Bacillus* isolates. Three isolates were plated from Jiffy Corn Muffin Mix along with two other samples for a total of 6.7% after heat-treatment but were Gram-positive cocci. Because this research examined *Bacillus* spp., any non-Gram-positive rod specimens were not analyzed further.

Bacillus spp. are ubiquitous in nature and form endospores that readily transfer to foods (3, 16, 17). Initially for the *Bacillus* isolation approach, nutrient rich BHIB incubation overnight at 32°C did not allow for endospore formation. Endospores optimally form when the bacteria are stressed and require 1 to 2 days for full development (3, 36). While most samples had already been screened for *Bacillus* presence, the remaining few were instead shaken for three days at the same conditions to allow sufficient time for endospore formation. Consequently, endospores were better isolated after this change. It is likely that *Bacillus* spp. endospores were present in many samples that lacked detectable *Bacillus* isolates initially, like Nestle Nesquik, given their general ubiquity (17). These samples were then subjected to real-time PCR analysis.

There are three *nhe* genes that are encoded on the *nheABC* operon (3) and have been shown to remain conserved as a cluster during genetic recombination (19). It can reasonably be assumed that the presence of the most proximal subunit of *nhe* indicates the presence of the other two genes. In the literature, all genes encoding the Nhe and Hbl enterotoxins have been readily located downstream in both *B. cereus* and *B. thuringiensis* (35). Of 616 *Bacillus* isolates tested, none were found to harbor only a single or two of the genes for each operon.

Over three separate real-time PCR runs, all 4 soil samples had three melt peaks within 1 SD of *nheA* positive *B. cereus* ATCC 14579. Thus, they were also positive for the presence of the *nheA* gene. By extension, these strains were also positive for the presence of the *nheABC* operon and could be considered pathogenic. Samples with three melt peaks consistent with the *B. cereus* positive control also resulted in standard deviations much less than 1, as shown in Table 6B. These melt peaks were extremely similar to each other and to the positive control, meaning that the amplified product was, in fact, *nheA*.

Of 16 total food isolates, four displayed three *nheA* positive melt peaks, while four displayed two *nheA* positive melt peaks. Additionally, 4 food isolates displayed only one *nheA* positive melt peak, while four were found to contain no identifiable *nheA* genes. Over three real-time PCR runs, samples with three melt peaks within 1 SD of the *nheA* positive control strain were also considered positive for the presence of *nheA*. Samples with two of three melt peaks within 1 SD of the positive control strain were also considered to be positive for the presence of *nheA*, even with an erroneous third melt peak. While real-time PCR is an accurate assay for gene detection, it is still sensitive to pipette error as well as PCR inhibitors (29). Thus, it is likely that user error prevented a third melt peak within 1 SD of the positive control.

Samples with one of three melt peaks within 1 SD of the positive control were treated as potentially positive for the presence of *nheA*. However, further research of these strains needs to be performed for a definitive answer. One positive melt peak was not determined to be strong enough evidence to ignore two negative results.

After real-time PCR analysis, reference cultures *B. macerans, B. brevis, B. cereus, B. thuringiensis var. kurstaki, B. thuringiensis var. japanensis,* and *B. thuringiensis var. israelensis* also displayed three melt peaks within 1 SD of *nheA* positive *B. cereus* over three runs. Additionally, *B. laterosporos* displayed two of three total melt peaks consistent with the positive control strain, and by extension contained the *nheABC* operon. The *B. thuringiensis* and *B. cereus* sample results were expected and confirm earlier work indicating that both are pathogenic (33, 39, 48). While the reference strains *B. circulans* and *B. megaterium* were not positive for the presence of *nheA* in this study, they were found to harbor each Hbl gene in a separate study (44). It is very possible these strains contained a polymorphic version of *nheA*.

To the best of our knowledge these samples, minus *B. cereus* and *B. thuringiensis*, are novel findings that are not usually associated with food pathogenicity (3, 16, 17, 18). However, it is an unsurprising find that *Bacillus* isolates harboring the *nheA* gene were identified in food, at least in *B. thuringiensis* and *B. cereus*. *B. cereus* and *B. thuringiensis* are arguably the same species (3) and have been demonstrated to be pathogenic in food (18). There is a general consensus among biologists that most, if not all, *Bacillus* isolates undergo horizontal gene transfer (17).

One study determined that of the *B. cereus* and *B. thuringiensis* isolates obtained from rice, 84.3% and 100% of them produced the *Nhe* enterotoxin, respectively (1). Sixty-one percent and 100% of these same isolates produced the Hbl enterotoxin, respectively. A separate study found that of 136 *B. cereus* isolates obtained from milk, over half were toxic against HeLa cells (10). Additionally, 73.2% were toxic against HEL cells. A third study noted that of emetic strains identified, 77.5% of *B. cereus* strains also produced *Nhe* (27). Yet another study found that the *nheABC* operon was present in every *B. thuringiensis* strain tested (35). The presence of the *nheABC* operon does not necessarily indicate a virulent strain, but has a very high likelihood of expressing these genes in a host environment or in food under permissive conditions (3, 41). Thus, future work to determine the pathogenicity of *nheA* positive samples would include the use of a Tecra VIA immunoassay kit to detect enterotoxin proteins (3, 17, 18, 27). Without this step, the virulence of *nheA* positive samples cannot be definitively determined.

These data suggest that at least 8 of the 16 isolates from food were positive for the presence of the *nheABC* operon. An additional four food isolates may also be enterotoxigenic, meaning that there is a 75% chance of any food isolate consumed being potentially enterotoxigenic. Additionally, three reference strains were identified that have not been previously known to harbor enterotoxigenic genes. A large degree of genetic variation exists in nhe sequences among *Bacillus* spp. (18), giving rise to false negative results in PCR-based detection assays. Strains negative for *nheA* in real-time PCR have been found to produce the enterotoxin *Nhe* as determined using a Tecra VIA kit. It is very possible that some of the *nheA* negative strains from real-time PCR may still be enterotoxigenic due to polymorphism (15).

After real-time PCR analysis, it was necessary to determine how similar the unidentified *Bacillus* food and soil isolates were to the reference strains *B. cereus, B. thuringiensis var. kurstaki, B. thuringiensis var. japanensis,* and *B. thuringiensis var. israelensis* using rep- PCR. If banding patterns of the unidentified isolates were very similar to rep-PCR banding patterns of reference strains, then this research would not have identified new strains harboring enterotoxigenic genes.

Within *Bacillus*, most virulence factors are encoded on plasmids (42), which have been demonstrated to readily transfer between differing species (3, 19). Indeed, a recent study indicated that the virulence genes associated with *B. cereus* infection undergo frequent rearrangement both within the bacterial nucleoid and between species (26). Thus, a better method than traditional biochemical tests to detect pathogenic *Bacillus* strains is to screen for virulence operons present in plasmids or in nucleoidal DNA (28, 42). *Bacillus* genomes that have been sequenced display a high level of genetic synteny in their gene order. Two genes that encode for bacterial ribosomes, 16S and 23S rDNA, contain genetic sequences that are < 1% different when compared between *B. cereus, B. thuringiensis,* and *B. anthracis* (48). A dissimilarity of 3% between 16S or 23S rDNA sequences is the minimal "cut off " between two strains to be considered as distinct species. Additionally, the *gyrB* gene sequence shared among these species is very homologous (38). Because these genes are shared among different species within the *Bacillus* genus, they cannot be used to differentiate species (42, 48). However, 16S and 23S rRNA can be used to differentiate between different strains of *B. anthracis* (13). Ultimately, the many attempts at differentiating *B. cereus, B. thuringiensis,* and *B. anthracis* have led to complete genomic sequencing of 16 strains of these three species (42). This large data pool has allowed *Bacillus* to serve as a good model for genetic conservation and to allow thorough study of virulence gene transfer. Additionally, the abundance of sequencing information on *Bacillus* genomes has allowed scientists to statistically differentiate sequencing error from actual polymorphisms.

Interestingly, there are a number of mechanisms that facilitate the movement of genes between different members of the *Bacillus* genus. One such mechanism is through the natural action of bacteriophage. After lysing its host cell, the bacteriophage will insert its genes into *Bacillus* genomes. While normally either lytic or lysogenic, it is possible for prophage to undergo random mutation, which renders it unable to enter the lysogenic cycle. In this way, genes from one species of bacteria can be transferred to *Bacillus spp*. As previously mentioned, *Bacillus* operons may be on conjugative plasmids. Additionally, *Bacillus* spp. are naturally competent, allowing these microbes to naturally take up random DNA in their vicinity (19).

The virulence genes for *Nhe* are present in more strains of *Bacillus* than is currently accepted within the scientific community. This research identified several "species" of *Bacillus* that were not previously known to harbor the *Nhe* enterotoxigenic operon. Given that a debate is currently underway about the very identity of *B. cereus* and other strains, it is improper for food safety experts to screen food products only for *B. cereus*. Phenotypic-based classification techniques have failed to accurately differentiate *Bacillus* species. Additionally, no molecular-based approach can accurately differentiate *Bacillus* (42). The bottom line is the determination of species within *Bacillus* does not even matter when concerned with food safety. Molecular techniques should instead screen for virulence determinants in microbes instead of identifying said microbes (47). Since endospore formation enables *Bacillus* spp. to be ubiquitous in the environment and on food, all foods should be examined in this way (3, 16, 36). This is the only true way to determine whether food products are safe for human consumption.

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