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Toxigenic Profile of *Clostridium perfringens* Strains Isolated from Natural Ingredient Laboratory Animal Diets

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Clostridium perfringens **is an anaerobic, gram-positive, spore-forming bacterium that ubiquitously inhabits a wide variety of natural environments including the gastrointestinal tract of humans and animals. *C. perfringens* is an opportunistic enteropathogen capable of producing at least 20 different toxins in various combinations. Strains of *C. perfringens* are currently categorized into 7 toxinotypes (A, B, C, D, E, F, and G) based on the presence or absence of 6 typing-toxins (α, β, epsilon, iota, enterotoxin, and netB). Each toxinotype is associated with specific histotoxic and enteric diseases. Spontaneous enteritis due to *C. perfringens* has been reported in laboratory animals; however, the source of the bacteria was unknown. The Quality Assurance Laboratory (QAL) at the National Institute of Environmental Health Sciences (NIEHS) routinely screens incoming animal feeds for aerobic, enteric pathogens, such as *Salmonella* spp. and *E. coli.* Recently, QAL incorporated anaerobic screening of incoming animal feeds. To date, the lab has isolated numerous *Clostridium* species, including *C. perfringens*, from 23 lots of natural ingredient laboratory animal diets. Published reports of *C. perfringens* isolation from laboratory animal feeds could not be found in the literature. Therefore, we performed a toxin profile screen of our isolated strains of *C. perfringens* using PCR to determine which toxinotypes were present in the laboratory animal diets. Our results showed that most *C. perfringens* strains we isolated from the laboratory animal feed were toxinotype A with most strains also possessing the theta toxin. Two of the *C. perfringens* strains also possessed the β toxin. Our results demonstrated the presence of *C. perfringens* in nonsterile, natural ingredient feeds for laboratory animals which could serve as a source of this opportunistic pathogen.**

Abbreviations: C.perfringens, Clostridium perfringens; GI, gastrointestinal; NE, necrotic enteritis

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Introduction

Clostridium perfringens is a well-known and widely dispersed gram-positive, nonmotile, anaerobic bacterium that ubiquitously inhabits most terrestrial and aquatic environments. Like some other members of the Firmicute phylum, its survivability is greatly enhanced by its ability to produce endospores during periods of environmental stress.[1,14,24,30] *C. perfringens* commonly resides in the gastrointestinal (GI) tract of numerous animal species and has been widely reported in a variety of domesticated animal species (Figure 1).

C. perfringens is an important human and animal pathogen that causes a wide spectrum of diseases.[34] However, not all strains of *C. perfringens* cause disease in animals or humans, and the presence of *C. perfringens* in the intestinal tract usually does not lead to illness. *C. perfringens* typically does not exhibit adherence and invasive properties toward healthy intestinal mucosa, and the development of clinical disease appears to be the result of a complex interaction between host immune status, strain virulence, and other nonspecific factors.[34] Host stresses that lead to abnormal gut microbiota appear to be an important predisposing factor to disease development. Gut microbiota disturbances and *C. perfringens* vulnerability are known to occur from host antibiotic exposure, alterations in feeding regimens, overeating, and dietary changes.[7,22,37] *C. perfringens* is known to produce at least 20 different toxins in varying combinations; these toxins cause a broad range of diseases including necrotic enteritis, gas gangrene, and various enterotoxemia (Figure 1).[22,29,34] The presence or absence of 6 particular toxins ("typing toxins") classifies each strain into 1 of 7 currently recognized toxinotypes (A-G; Figure 1).[29] The 6 typing toxins that cause most of the diseases reported include α, β, epsilon, iota toxins (previously defined as major toxins), enterotoxin, and netB (previously defined as minor toxins). Over the last few years, the *C. perfringens* typing system has been expanded from 5 toxinotypes (A through E) to 7 toxinotypes (A through G). Toxinotype F is a reclassification of enterotoxin-positive (CPE-positive) toxinotype A strains, and toxinotype G strains produce the necrotic enteritis B-like (netB) toxin.[29] The nontyping toxin perfringolysin O (theta) is not a main virulence factor for animal disease but was added to the study because it is thought to have a synergistic action with CPA-mediated and ETX-mediated diseases.[39,41] Table 1 summarizes the mode of action, biologic activity and gene location (plasmid or chromosome) of each toxin of interest within our study.

Enzyme-linked immunosorbent assays (ELISAs) have been used to toxinotype *C. perfringens* strains. ELISAs have been

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Toxinotype	Documented Disease States in Mammals	Alpha (CPA)	Beta (CPB)	Epsilon (ETX)	Iota (ITX)	Enterotoxin (CPE)	NetB (NetB)
		Toxins					
A	• Humans: myonecrosis (gas gangrene)[9; 14; 17; 20; 24; 25; 30; 40] • Sheep: yellow lamb disease, necrotic enteritis[39] • Poultry: necrotic enteritis[17; 32; 40] • Piglets: enterocolitis, malignant edema[9; 17; 32]	+	-	-	-	-	-
B	• Humans: no reported cases of disease[20] • Sheep/lamb: dysentery, necrohemorrhagic enteritis, hemorrhagic desentery[14; 17; 39] • Cattle/horses/pigs: enterotoxemia, necrotic enteritis[9; 17; 40] • Goats: enterotoxemia, necrotic enteritis[39]	+	+	+	-	-	-
C	• Humans: necrotic enteritis (pigbel)[13; 17; 20; 24; 25; 30; 40] • Sheep: necrotic enteritis, hemorrhagic dysentery[13; 39] • Calves/kids/foals/piglets: neonatal necrotic enteritis[4;13; 32] • Poultry: necrotic enteritis[9; 40]	+	+	-	-	+/-	-
D	• Humans: no reported cases of disease[20] • Cattle/sheep: enterotoxemia[9;13; 17; 24; 26] • Goats: enterotoxemia, colitis[30; 40] • Sheep: enterotoxemia, [13; 14; 17]	+	-	+	-	+/-	-
E	• Humans: no reported cases of disease[20] • Cattle/sheep: enterotoxemia[9;13; 17; 24; 26] • Rabbits: enterotoxemia[32] • Calves: hemmorrhagic enteritis[32]	+	-	-	+	+/-	-
F	• Humans: diarrhea, CPE-associated nonfoodborne GI disease, gas gangrene[9; 14; 17; 24; 26; 30; 38; 40] • Horses: enteric disease[32]	+	-	-	-	+	-
G	• Humans: no reported cases of disease • Avian: necrotic enteritis[26; 30; 32; 38]	+	-	-	-	-	+

(+) toxin is present in all strains; (-) toxin is absent in all strains; (+/-) toxin can be either present or absent in strains of this toxinotype

Figure 1. Toxinotypes and pathogenicity.

Table 1. Mechanism of action and biologic effects of toxin-encoding genes.*

Toxin	Gene	Mode of Action	Biologic Activity	Gene Location
Alpha (α)	*cpa*	Phospholipase C (plc) And sphingomyelinase	Cytotoxic, hemolytic, necrotic, smooth muscle contraction, enteritis	Chromosome
Beta (β)	*cpb*	Pore-forming activity	Cytotoxic, dermonecrotic, edema, hemorrhagic dysentery, enteritis	Plasmid
Epsilon (ε)	*etx*	Pore-forming activity and alteration of cellularmembrane permeability	Edema of various organs, smooth muscle contraction, dermonecrotic, enterotoxic, blood pressure elevation	Plasmid
Iota (ι)	*iap and ibp*	Ia-mediated ADP-ribosylation of G-actin and pore-forming activity	Disruption of actin cytoskeleton and cell membrane, inhibition of smooth muscle contraction, necrotic	Plasmid
Enterotoxin	*cpe*	Pore-forming activity	Enterotoxic, cytotoxic, erythema, leakage of water and ions, oncosis	Plasmid or Chromosome
Theta (θ)	*pfo*	Perfringoysin and pore-forming activity	Cholesterol-specific hemolysin, tissue destruction, antiinflammatory	Chromosome
NetB	*netB*	Pore-forming activity	Necrotic enteritis of poultry, hemolytic gut lesions	Plasmid

*Table 1 was assembled using previously constructed tables describing the mode of action, biologic activity and location of *C. perfringens* toxin-encoding genes.[9,20,26,29,34,38]

traditionally used to detect α toxin (CPA), β toxin (CPB), epsilon toxin (ETX), and enterotoxin (CPE), but no commercially available ELISA kit has been developed that can reliably detect iota toxin (ITX).[1,14,39] Furthermore, biochemical tests alone cannot distinguish between different *C. perfringens* toxinotypes and may overlook samples not actively producing toxins.[26] Recent *C. perfringens* toxinotyping efforts have used traditional polymerase-chain reactions or quantitative polymerase-chain reactions (PCR or qPCR) to successfully determine the presence of toxin-associated genes.[22]

In 2018, strain isolation and toxinotyping of *C. perfringens* was reported from agricultural animal feeds manufactured in Serbia.[22] To our knowledge, there are no published reports of any *Clostridium* spp. being isolated from natural ingredient, laboratory animal feeds. In this paper, we report the isolation and toxigenic profile of 29 *Clostridium* spp. including *C. perfringens* and 5 other clostridial species (*Clostridium baratii, Clostridium beijerinckii, Clostridium bifermentans, Clostridium butyricum, and Clostridium sordellii*) from 10 different laboratory animal diets, including both open and closed formulations, obtained from 4 different commercial feed manufacturers. These results demonstrate that opportunistic, pathogenic bacteria, such as *C. perfringens,* are present in unsterilized, natural ingredient, laboratory animal diets and could act as a source to colonize the GI tract of laboratory species and cause disease, especially in immunocompromised or biologically stressed animals. The presence of *C. perfringens* in these feeds provides a rationale for feed sterilization before use to avoid the introduction of unwanted, potentially pathogenic organisms that may cause unwanted physiologic effects, disease, or death.

Materials and Methods

***Clostridium* spp. cultivation from animal feed and initial identification.** Twenty-three separate lots of laboratory animal feed were tested from large, commercial, US-based manufacturers. These included several lots of our standard, open-formula NIH-31 rodent feed produced under contract by a commercial source. We also tested 2 lots of the open formula NIH-07 rodent diet from 2 different manufacturers; the NIH-2004 open-formula swine diet, 4 different natural ingredient, closed-formula rodent diets from 2 different manufacturers; and 1 purified, high-fat, rodent diet. For enrichment of *Clostridium* spp., approximately 25 g of each feed sample was aseptically placed into 250 mL of thioglycolate broth and incubated at 35 °C for 24 h. After incubation, each thioglycolate broth bottle was briefly mixed and streaked onto blood agar plates (BAPs) using sterile cotton tipped applicators. BAPs were then incubated at 37 °C (98.6 °F) under anaerobic conditions using a generating system (GasPak EZ, BD Diagnostics, Franklin Lakes, NJ) inside an anaerobic chamber. After 24 to 48 h of incubation, each BAP was examined; suspect colonies were isolated onto fresh BAPs and incubated again at 37 °C (98.6 °F) aerobically and anaerobically. Isolates indicating growth only under anaerobic conditions were archived, and a sample of each isolate was shipped to Charles River Laboratories (Wilmington, MA) for identification using matrix associated laser desorption/ionization time of flight mass spectrometry (MALDI-TOF).

DNA purification and quantification. For each isolate identified as a *Clostridium* spp. by MALDI-TOF, a loopful of colony biomass was placed inside a 2 mL tube prefilled with approximately 1200 mg of acid washed, 100 µm zirconium beads (Ops Diagnostics, Lebanon, NJ) along with the initial reagents recommended by the tissue kit manufacturer (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) for cultured cells. Next, bead tubes were homogenized using a homogenizer (FastPrep-96, MP Biomedicals, LLC, Santa Ana, CA) at max speed for 3 min. After homogenization, tubes were centrifuged at 8,000 × g for 30 s and incubated at 56 °C (132.8 °F) for 30 min. After incubation, tubes were centrifuged at 13,000 × g for 1 min, and each sample's supernatant was then transferred to a new DNA/RNA-free 1.5 mL microcentrifuge tube. The kit's quick-start protocol was then continued from step 3 until purified DNA was eluted into a new DNA/RNA-free 1.5 mL microcentrifuge tube (step 8). Before downstream analysis, purified DNA was quantified fluorometrically DS-11 FX Spectrophotometer/Fluorometer, DeNovix, Wilmington, DE) and an assay kit (dsDNA Broad Range Assay Kit, DeNovix) was used following the manufacturer's instructions. Total genomic DNA was isolated from a subset of test diets DNeasy PowerMax Soil Kit, Qiagen, Hilden, Germany) to assess our ability to identify *C. perfringens* directly from feed via PCR. Five (5.0) g of each diet were added to the kit's 50 mL conical tube along with 0.7 mm garnet beads and 15 mL of the kit's PowerBead and C1 solutions per kit instructions. The tubes were vortexed for 10 min then incubated at 37 °C (98.6 °F) overnight on a shaking tray. The remainder of the kit protocol was followed, and the total genomic DNA was eluted from the Qiagen column using 5.0 mL of C6 solution (elution buffer). The DNA was precipitated with 0.3 M sodium acetate and 10 mL of isopropyl alcohol, washed with 1.5 mL of 75% ethanol, and resuspended in 1.0 mL of DNAse/RNAse-free water. Purified DNA was quantified fluorometrically DS-11 FX Spectrophotometer/Fluorometer, DeNovix) and a broad-range assay kit (dsDNA Broad Range Assay Kit, DeNovix) was used following the manufacturer's instructions.

Sequence verification of cultured isolates. The bacterial identity of each isolate was verified by 16S rRNA gene analysis using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3', M = C/A) and 1492R (5'-GGTTACCTTGTTACGACTT-3').[42] Fifty (50) µl polymerase chain reaction (PCR) mixtures were carried out T100 Thermal Cycler, (Bio-Rad Laboratories, Hercules, CA) using 100 ng of total DNA template along with polymerase reagents (Applied Biosystems, Foster City, CA) according to the kit's suggested protocol. PCR amplifications were performed as follows: Initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with a final 5 min extension period at 72 °C. 16S rRNA gene amplicons were cleansed (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany). Approximately 50 ng of PCR amplicon template was sent to Genewiz (Morrisville, NC) in a premix tube (amplicon + primer) for Sanger sequencing. Sanger sequencing results were trimmed and assembled (CLC Main Workbench 8, Qiagen, Hilden, Germany) using the default settings. Assembled 16S contigs were identified by uploading sequences into the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) using the '16S ribosomal RNA sequences (bacteria and archaea)' database and the top BLAST score result.

***C. perfringens* toxin gene profiling.** To screen isolates for various toxin-associated genes, 50 µl PCR reactions were carried out using 10 ng of sample DNA template and toxin-specific gene primers (Table 2) along with Platinum Taq Polymerase (Invitrogen/ThermoFisher, Carlsbad, CA) in accordance with the manufacturer's guidelines. PCR cycle parameters were as follows: Initial denaturation at 95 °C for 3 min, followed by 35 cycles each of 95 °C for 30 s, 50 °C to 60 °C for 30 s (Table 2; optimal annealing temperature for each primer set), and 72 °C for 1 min with a final 5 min extension period at 72 °C. Upon completion of PCR, 20 µl of product was run on a 1.2% agarose gel containing a 1× concentration of GelGreen Nucleic Acid Stain (Biotium, Fremont, CA). For added assurance, amplicons were sent to Genewiz (Morrisville, NC) for Sanger sequencing, and trimmed results were matched against NCBI's BLAST using the 'nucleotide collection (nr/nt)' database.

Identification of *C. perfringens* directly from feed. Fifty (50) nanograms of DNA isolated from feed samples were used in a 50 µl total volume PCR reaction using the plc (α toxin) primers listed in Table 2 and Platinum Taq Polymerase. Touchdown PCR

Table 2. Toxin-specific gene primers

Toxin Gene	Oligonucleotide Sequence (5′-3′)	Anneal Temp	Amplicon Size
cpa (α toxin)[21]	GCTAATGTTACTGCCGTTGA	50 °C	approximately 324 bp
	TCTGATACATCGTGTAAG		
plc (α toxin)[24]	CCGTTGATAGCGCAGGACA	54 °C	approximately 219 bp
	CCCAACTATGACTCATGCTAGCA		
cpb (β toxin)[21]	GCGAATATGCTGAATCATCTA	57 °C	approximately 196 bp
	GCAGGAACATTAGTATATCTTC		
etx (ε toxin)[21]	GCGGTGATATCCATCTATTC	54 °C	approximately 655 bp
	CCACTTACTTGTCCTACTAAC		
iap (ι toxin)[5]	AATGCCATATCAAAAAATAA	53 °C	approximately 821 bp
	TTAGCAAATGCACTCATATT		
cpe (enterotoxin)[21]	GGAGATGGTTGGATATTAGG	56 °C	approximately 233 bp
	GGACCAGCAGTTGTAGATA		
pfoA (θ toxin)[12]	ATCCAACCTATGGAAAAGTTTCTGG	60 °C	approximately 532 bp
	CCTCCTAAAACTACTGCTGTGAAGG		
netB (NetB toxin)	GCTGGT*R*[a]CTGGAATAAATGC	56 °C	approximately 384 bp
	TCGCCATTGAGTAGTTTCCC		

R[a] = A/G (nucleotide at this position was modified from Keyburn and colleagues's original primer version of nucleotide "G"

Table 3. *Clostridium* sp. strains isolated from natural ingredient laboratory animal feed

Sample ID	Sample	Feed Manufacturer[a]	Lot[b]	Institute[a]	Species
ATCC 12917	Positive Control Strain - Toxinotype D	NA	NA	ATCC	*C. perfringens*
ATCC 3626	Positive Control Strain - Toxinotype B	NA	NA	ATCC	*C. perfringens*
ATCC 8009	Positive Control Strain - Toxinotype E	NA	NA	ATCC	*C. perfringens*
ATCC 27324	Positive Control Strain - Toxinotype E	NA	NA	ATCC	*C. perfringens*
Uzal Lab Isolate	Positive Control Strain - Toxinotype G	NA	NA	UC Davis	*C. perfringens*
QA1011-17_F	Diet 1 (NIH-31)	1	1	NIEHS	*C. perfringens*
QA3081-14_B	Diet 1 (NIH-31)	1	2	NIEHS	*C. perfringens*
QA1881-14_A	Diet 1 (NIH-31)	1	3	NIEHS	*C. perfringens*
QA4249-17	Diet 2 (Closed Formula)	2	1	NIEHS	*C. perfringens*
QA3578-18_A	Diet 3 (Closed Formula)	1	1	NIEHS	*C. perfringens*
QA1535-17_A	Diet 3 (Closed Formula)	1	2	NIEHS	*C. perfringens*
QA411-18_C	Diet 4 (NIH-07)	1	1	NIEHS	*C. perfringens*
QA1027-18_C	Diet 4 (NIH-07)	3	2	2	*C. perfringens*
QA1025-18_C	Diet 5 (Closed Formula)	2	1	2	*C. perfringens*
QA1026-18_C	Diet 6 (Closed Formula)	2	NA	2	*C. perfringens*
QA1028-18_C	Diet 7 (NIH-2004 - Swine)	3	NA	2	*C. perfringens*
QA407-18_A	Diet 8 (Closed Formula)	2	NA	3	*C. perfringens*
QA408-18_B	Diet 9 (Closed Formula)	2	NA	3	*C. perfringens*
QA409-18_C1	Diet 10 (Closed Formula)	2	1	3	*C. perfringens*
QA1787-16_A	Diet 11 (Purified - High-fat)	4	NA	NIEHS	*C. perfringens*
QA1011-17_B	Diet 1 (NIH-31)	1	1	NIEHS	*C. bifermentans*
QA1011-17_C	Diet 1 (NIH-31)	1	1	NIEHS	*C. butyricum*
QA3081-18_D	Diet 1 (NIH-31)	1	2	NIEHS	*C. butyricum*
QA4071-14_A	Diet 1 (NIH-31)	1	4	NIEHS	*C. bifermentans*
QA1024-18_E	Diet 1 (NIH-31)	1	5	2	*C. baratii*
QA3913-17_B	Diet 2 (Closed Formula)	2	2	NIEHS	*C. beijerinckii*
QA409-18_A	Diet 10 (Closed Formula)	2	1	3	*C. sordellii*
QA1025-18_D	Diet 5 (Closed Formula)	2	1	2	*C. bifermentans*
QA218-16_E	Environmental swab	NA	NA	NIEHS	*C. tertium*

[a]Numbers were assigned to commercial feed manufacturers and institutes in place of their name.
[b]Lot numbers assigned solely to indicate separate lots of a given diet.

cycle parameters were as follows: 95 °C initial denaturation for 3 min; followed by 10 cycles of 95 °C for 30 s, 65 °C for 30 s with a 0.5 °C decrease/cycle, and 72 °C for 30 s; followed by 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and ending with

a single 72 °C extension for 3 min. Twenty µl of each reaction was run on a 2.0% agarose gel containing 1× GelGreen Nucleic Acid Stain (Thomas Scientific, Swedesboro, NJ).

Results

Sequence identification of clostridial isolates. From the 23 separate lots of laboratory animal feed tested, we isolated and identified 7 different species of *Clostridium* (*C. perfringens*, *C. bifermentans*, *C. butyricum*, *C. baratii*, *C. beijerinckii*, *C. sordellii*, and *C. tertium*). Table 3 lists the *Clostridium* isolates selected for our toxinotyping experiment.

Toxin gene profiling of *C. perfringens* isolates. As expected, PCR, gel documentation, and Sanger sequencing verified the presence of toxin genes in all *C. perfringens* isolates, but not in any other species of *Clostridium* isolated from laboratory animal feed (Figure 2). All *C. perfringens* strains isolated from laboratory animal diets possessed the *cpa* gene encoding the α toxin, and 2 of the isolates possessed the *cpb* gene encoding the β toxin. No other typing toxin (epsilon, iota, enterotoxin, and NetB) was found in any *C. perfringens* strain isolated from laboratory animal feed (Figures 2 and 3). Nine of the 15 *C. perfringens* strains tested (60%) had the *pfoA* gene encoding the theta toxin, and 1 strain had the *cpb2* gene encoding the β2 toxin. Two of our 4 positive control *C. perfringens* strains used for PCR validation did not correspond completely with the toxin profile certification provided by the vendor in that the expected toxin genes (epsilon and iota) was not amplifiable using any of our referenced toxin primers (Table 4). Amplification of the enterotoxin gene was possible with only 2 of the 4 referenced primer sets tested in our experiment (Table S1 and Figure S1, Figure S2, Figure S3 and Figure S4).

Identification of *C. perfringens* directly from feed. We attempted to identify *C. perfringens* in animal feed by direct PCR screening for the *plc* gene (α toxin) in total gDNA isolated from a subset of tested feeds. All feeds tested for direct PCR were confirmed to possess *C. perfringens* by culture and isolation. Our initial attempts to amplify the gene target directly from feed using the same PCR parameters used on the purified bacterial isolates were unsuccessful. By using a "touchdown" PCR technique, in which a higher initial annealing temperature (65 °C) is used to minimize nonspecific amplifications, we were able to correctly amplify the expected 219 bp amplicon in 4 of the 7 diets tested by direct PCR screening (Figure 4).

Discussion

Our results established that most of the *C. perfringens* strains that we isolated from natural ingredient animal feed diets were toxinotype A, which is the basic toxinotype for which accumulation of toxin-encoding plasmids yield other distinctive toxinotypes (one exception to this rule is the enterotoxin gene (*cpe*) which is not always plasmid-encoded, but can sometimes be located on the chromosome).[20] Our results indicated that 2 of the *C. perfringens* strains isolated from laboratory animal feed possessed the β toxin gene; this toxin is known to be fatal in mice at low concentrations.[20,38] Although the 2 β toxin amplicons observed in our animal feed isolates by gel documentation were faint (low PCR amplification), repeated gel screening and Sanger sequencing of these amplicons confirmed them as authentic. With the technique we used to cultivate and isolate *C. perfringens* strains from nonsterile natural ingredient, laboratory animal feeds (thioglycollate broth to isolation plates), it is difficult to prove isolated colonies used for this study are of a single strain type and multiple strain types may be present (a fraction of the bacteria possessed a plasmid encoding the β toxin gene). Most of the *C. perfringens* toxin genes are solely plasmid-encoded (for example, *cpb*, *epsilon*, *itx* and *netB*), and strains are capable of transferring toxin-encoding plasmids to neighboring strains of *C. perfringens* which is an important element of its pathologic progression.[20] *C. perfringens* strains can carry up to 3 different toxin plasmids with each plasmid encoding up to 3 different toxins.[20] The nonspecific amplicons observed on the electrophoresis gel (Figure 2) using our referenced netB primers did not reveal any relevant information about the genetic origin by Sanger sequencing and BLAST analysis. Nine of the 15 *C. perfringens* strains tested from laboratory animal feed also possessed the theta toxin. Theta toxin has a synergistic action with CPA which can increase necrotizing and lethal effects on animals.[12,40] Two of our 4 positive controls did not work as expected (Table 4). The toxin profile certification provided by the vendor (ATCC) did not correlate with the toxin profile identified by our PCR analysis of the epsilon (*etx*) and iota (*iap*) genes. For both discrepancies (toxin gene absent in the positive control strain), another positive control sample effectively amplified and confirmed that our referenced gene primers did effectively target the toxin of interest. Multiple referenced primer sets for the *etx* gene[5,41] and *iap* gene[21,29,41] were tested on these positive controls but gene amplification was still unsuccessful (Table S1, Figures S1, S2, S3 and S4). Since the toxin gene missing from each of these positive controls (epsilon and iota) are plasmid-based genes, one possible explanation for the failure to identify them via PCR is that the plasmid responsible for encoding the respective toxin gene was lost due to continual growth on defined media in a laboratory setting outside a GI environment.

Although we were able to demonstrate *C. perfringens* in laboratory animal diets by direct PCR of total gDNA isolated from feed, our PCR results were not consistent. Lack of consistent PCR amplification among the diets is likely due to the concentration of *C. perfringens* in the diet and/or PCR inhibitors remaining in the gDNA isolated from the feed. Therefore, further analysis using quantitative PCR and spiking experiments would be necessary to determine the sensitivity/detection limit of our assay, and the possible presence of PCR inhibitors in animal diets. As such, we caution against the use of PCR screening of feed samples as the sole method to screen animal feeds for *C. perfringens*.

C. perfringens is ubiquitous in the environment, including in the digestive tract of healthy animals; therefore, analyzing the evolution of pathogenic strains is difficult. In addition, host factors including diet, innate immunity, and normal flora greatly impact colonization by foreign bacteria.[28,31] Because few environmental bacteria are known to carry these types of toxin gene variants, strain adaptation to acquire such toxins is suited for survival in a GI environment.[27] It has been noted that, unlike commensal strains, pathogenic strains of *C. perfringens* isolated from NE or wound infections show rearrangement of chromosomal regions that include hydrolytic enzymes and toxins, which may confer selective advantages for colonizing GI environments.[28,31] Spontaneous disease from *C. perfringens* is rare in laboratory animals but has been reported most often in female mice nursing large litters with older pups (>14 d of age).[8,19,36] The nutritional stress of lactation and an increased intake of high carbohydrate diets may be predisposing factors in these cases, and autoclaving the diet was a way to reduce infections.[8,18] A study demonstrated that C3H/HeJ mice, which have innate immune deficiencies due to absence of the toll-like receptor 4 (Tlr4), were more sensitive to experimental infection with *C. perfringens* as compared with the C3H/HeN strain, which has a normal Tlr4.[23] While disease due to *C. perfringens* has not been

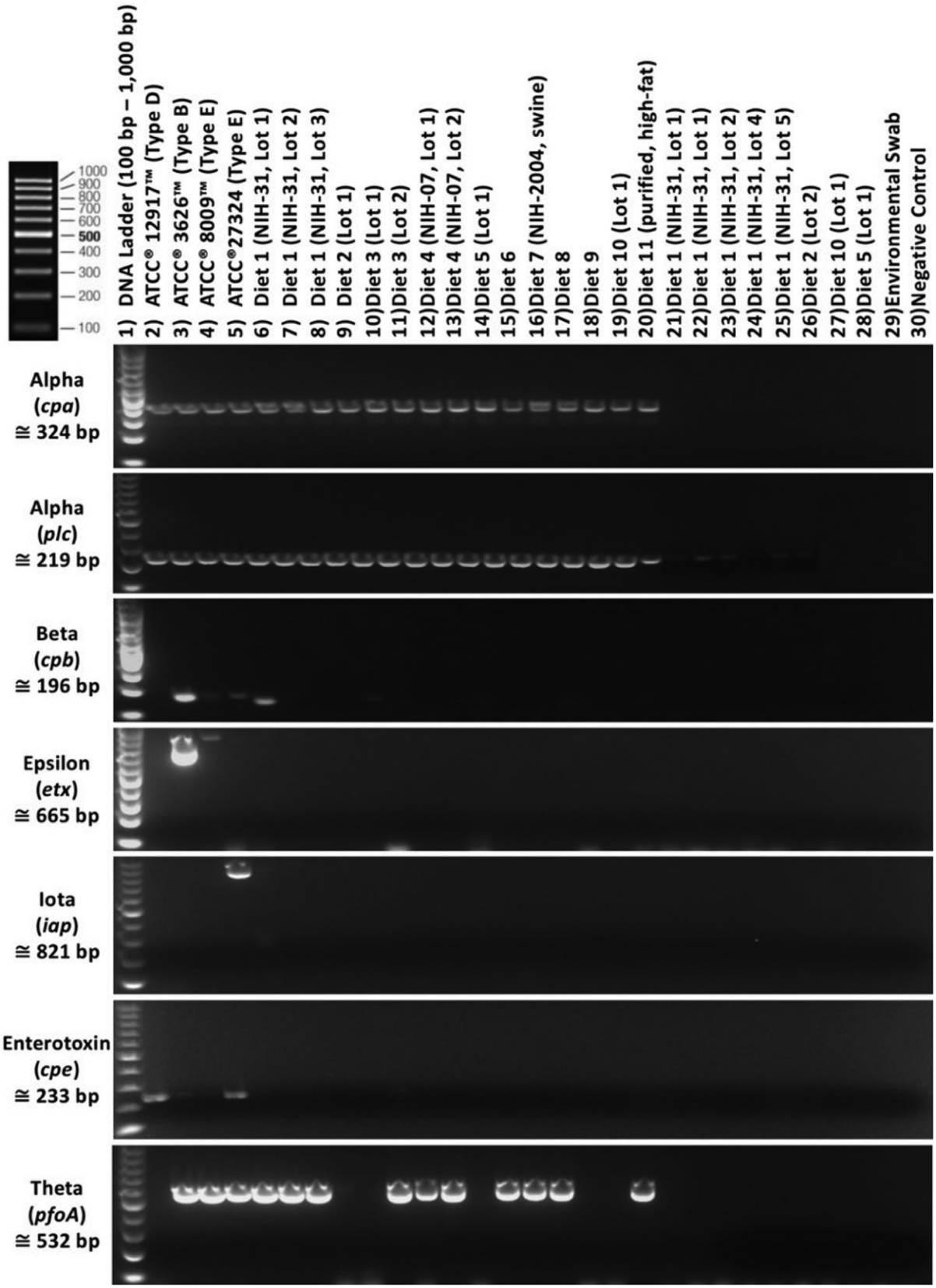

Figure 2. Toxin gene profile of *Clostridium* sp. isolates.

seen in the rodent population at NIEHS, immunocompromised and gnotobiotic animals could be more susceptible to colonization with *C. perfringens* and disease development due to toxins. Therefore, the introduction of *C. perfringens* from laboratory animal feed could pose a serious risk to studies using these types of rodents.

In addition, because of the bacterium's ubiquitous nature, almost all food sources, whether animal- or plant-based, can

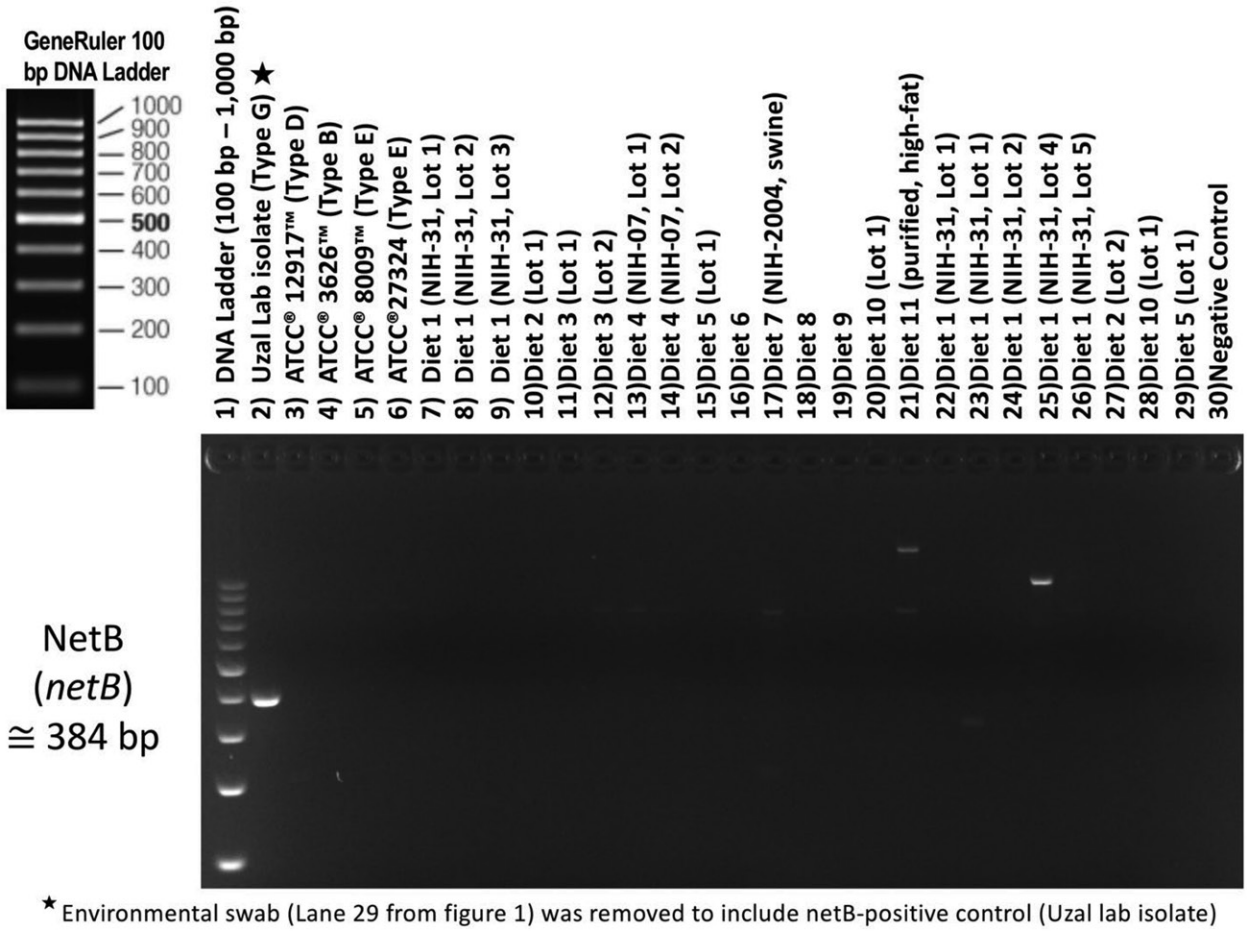

Figure 3. NetB toxin gene profile of *Clostridium* sp. isolates.

Table 4. Toxin profile of *C. perfringens* strains isolated from natural diets

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Strain Source	DNA Ladder	ATCC® 12917™	ATCC® 3626™	ATCC® 8009™	ATCC® 27324™	Diet 1	Diet 1	Diet 1	Diet 2	Diet 3	Diet 3	Diet 4	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11
Toxin Gene	*cpa*	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱
	plc	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱	✱
	cpb		✱		✱	✱				✱										
	etx	✱	✱																	
	iap			✱	✱															
	cpe	✱			✱															
	cpb2		✱		✱							✱								
	pfoA		✱	✱	✱	✱	✱	✱			✱	✱	✱		✱	✱	✱			✱
	netB																			
Toxinotype		F	B	A	E	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A
		Detected					Not Detected (Excpected)								Detected with 2 of 4 primer sets					

Toxin genes verified to be present by PCR are highlighted in green. Toxin genes documented to be present in positive control strain (ATCC®) but nonamplifiable by PCR using referenced primer sets are highlighted in red. Toxin genes documented to be present in control strain but only amplifiable using some of the referenced primer sets are highlighted in orange.

potentially be contaminated with *C. perfringens*.[15] Microbial screening for *C. perfringens* has limited utility because positive results are common and indicate very little unless extremely high numbers of *C. perfringens* are enumerated.[22] Most sterilization processes, including ionizing radiation, do well at destroying vegetative cells, but do not always effectively eliminate their

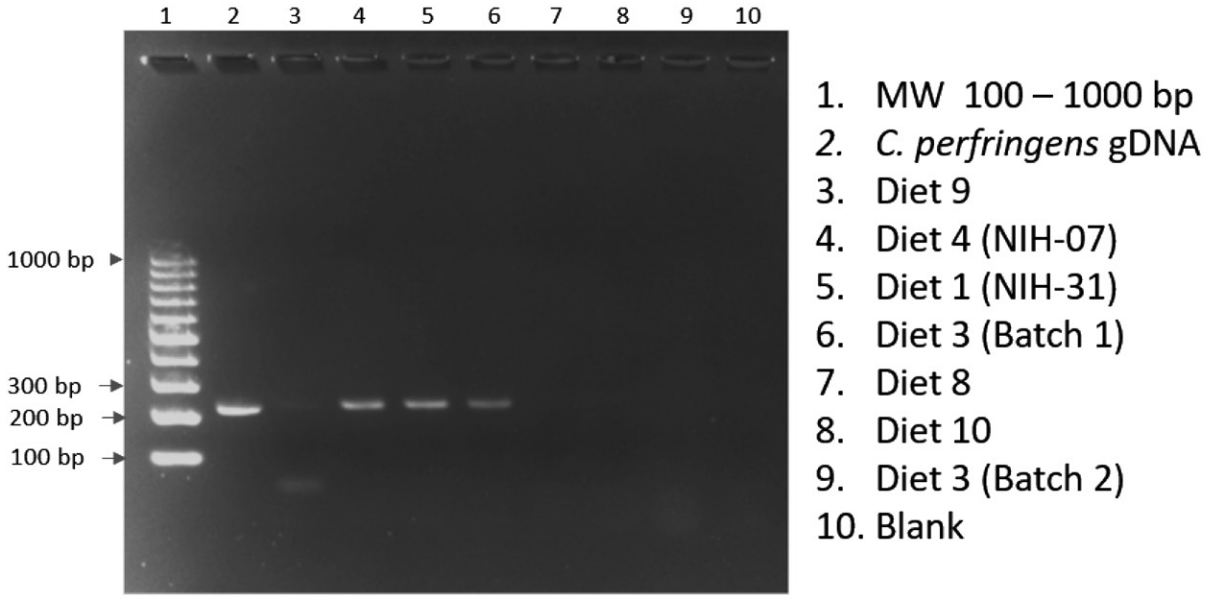

Figure 4. Direct feed analysis for C. perfringens (α toxin – plc gene).

spores.[10] For this reason, it may be beneficial to couple irradiation with microbial screening when using nonautoclavable diets. Surviving spores in sterilized feed can germinate and multiply rapidly.[6,10,35] Proper heating is the most reliable method of spore inactivation, but the required temperature and time is dependent on feed properties such as pH, water content, and fat content.[6] We test our facility's feed autoclaves weekly using VERIFY dual-species self-contained biologic indicators (STERIS Life Sciences; Mentor, OH) which contains spores from *Geobacillus stearothermophilus* and *Bacillus atrophaeus*. Our facility's vivarium houses over 50,000 rodents, and we have not documented an animal clinical case or sentinel necropsy that has demonstrated the presence of disease associated with *C. perfringens* by in-sourced or out-sourced diagnostic testing.

Because the isolation of *C. perfringens* from laboratory animal diets has not been previously reported, we wanted to develop our own capabilities to rapidly screen incoming diets for *C. perfringens* and effectively evaluate their toxigenic profile via PCR-based assays. Based on our findings, *C. perfringens* appears to be a common contaminant of laboratory animal feeds. Almost all environmental isolates identified exclusively fall into the toxinotype A category. The presence of *C. perfringens* in these laboratory animal diets provides a rationale for feed sterilization before use, and we strongly recommend proper feed sterilization and, if deemed necessary, microbial screening prior to use to prevent exposure of animals to potentially pathogenic strains of *C. perfringens* that can lead to disease, death or subclinical alterations in physiology that can affect research outcomes.

Supplementary Materials

Figure S1. ATCC's PCR validation (assay 2 and 3) of toxin genes *etx*, *iap* (*iA*) and *cpe.*

Figure S2. Validation of *etx* gene primer sets against positive controls.

Figure S3. Validation of *iap (iA)* gene primer sets against positive controls.

Figure S4. Validation of *cpe* gene primer sets against positive controls.

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