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Prevalence of *Clostridium perfringens* Spores in Selected Regions of Saudi Arabia

Saeed S. Banawas[1], Brent A. Kronmiller[2], Mohamed G. Abdelghany[3], Ali A. Alqarawi[4] and Mahfuzur R. Sarker[5,*]
[1]Department of Medical Laboratories, College of Applied Medical Science, and Health and Basic Sciences Research Center, Majmaah University, AlMajmaah, 11952, Saudi Arabia.
[2]Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR, 97331, USA.
[3]Microbiology Department, Faculty of Science, Ain- Shams University, Abbaseya, Cairo, 11566, Egypt.
[4]Department of Clinical Laboratories, College of Applied Medical Science, Hail University, Hail, 2440, Saudi Arabia.
[5]Departments of Biomedical Sciences, and Microbiology, Oregon State University, Corvallis, OR, 97331, USA.

Corresponding author: Mahfuzur R. Sarker (e-mail: sarkerm@oregonstate.edu).

Abstract The prevalence of *Clostridium perfringens* spores in the environment is crucial for the pathogenesis of this bacterium because these dormant spores, upon contact with a suitable host, can return to active growth to cause disease.We evaluated the prevalence of C. *perfringens* spores in the Hail and Qassim regions of Saudi Arabia.Methods: A plating method was used to identify C. *perfringens*. PCR analyses and DNA sequencing were performed for genotypic characterization of the newly isolated C. *perfringens*.25 of 300 collected samples were identified as C. *perfringens* by selecting black colonies on selective media and monitoring b-hemolysis on blood agar plate. PCR analyses showed that all 25 isolates carry a-toxin gene (*plc*), but not the enterotoxin gene (*cpe*), further confirming that these isolates are indeed *cpe*-negative. Genome sequencing found that Saudi isolates are genotypically similar to the American and European isolates; no homologues of toxinotyping genes encoding b-, i-, e- and NetB-toxin were detected in 22 Saudi isolates, with the exception of 3 isolates that carry only e-toxin gene etx. Collectively, our findings suggest that C. *perfringens* are highly prevalent in the Hail and Qassim environment, with 22 (88%) of 25 isolates are type A and remaining 3 (12%) are type D.

Key Words C. *perfringens*, spores, C. *perfringens* enterotoxin, *cpe*, α-toxin, *plc*, food poisoning

1. Introduction

Clostridium perfringens is an anaerobic, gram-positive, spore-forming, enteric bacterial pathogen that causes a wide range of human and animal diseases owing to its prolific toxin-producing capability [1]–[7]. C. *perfringens* can be classified into seven types (A through G), based on the presence of genes encoding six major toxins (α, β, ϵ, τ, C. *perfringens* enterotoxin (*cpe*), and C. *perfringens* necrotic enteritis β-like toxin (NetB)) [1], [8]. *cpe*, the medically important toxin produced by C. *perfringens* type F, is the major virulence factor for C. *perfringens* type F food poisoning (FP) and non-foodborne (NFB) gastrointestinal (GI) diseases [9]. C. *perfringens* type F FP is the third most commonly reported foodborne disease in the United States. The annual cost of illness is estimated to be more than $300 million [10]–[12]. Interestingly, in C. *perfringens* type F isolates, the *cpe*-encoding gene (*cpe*) can be located either on the chromosome or on a plasmid. In general, chromosomal *cpe* isolates are generally linked to FP, whereas plasmid-borne *cpe* isolates are associated with NFB GI diseases [13]–[15]. Nevertheless, some studies have found that plasmid-borne *cpe* isolates can also be causative agents for C. *perfringens* type F FP [15]–[18].

Numerous studies have attempted to understand why type F isolates carrying a chromosomal *cpe* gene are more likely to be associated with C. *perfringens* FP outbreaks [14], [16], [18], [19]. A survey reported that 1.7% of raw meat, fish, and poultry items sold in retail food stores contain type F isolates carrying chromosomal *cpe* [20]. Interestingly, this survey noted the absence of type F plasmid-borne *cpe* isolates in retail foods. These findings suggest that meat, seafood, and poultry, common food vehicles for C. *perfringens* FP in the United States and Europe can be contaminated with type F chromosomal-*cpe* isolates by the time of retail purchase. However, these survey results do not preclude the possibility of food contamination in food processing environments. C. *perfringens* spores are more resistant to a number of lethal factors than their vegetative forms [15], [21], [22].

Spores, especially spores of FP strain (21), can survive thermal processing and sanitizing treatments employed in the food industry. In addition, they are highly hydrophobic, complicating their removal when they are attached to food contact surfaces [23]–[26]. A potential source of pathogen transmission to food products is the contamination of food contact surfaces during food processing, catering, and in domestic environments [21], [26]. Another possibility is that the food items might become contaminated with type F chromosomal-*cpe* isolates residing in environmental niches, such as the soil or home kitchen surfaces [27], [28]. A study that surveyed different soils and home kitchen surfaces in Pittsburgh, PA, did not detect C. *perfringens* isolates from home kitchens, while most of the soil samples tested positive for this bacterial isolate. The soil isolates were predominantly type A, although types C, D, E and F were also identified. All *cpe*-positive soil isolates were genotyped as type F, harboring *cpe* genes on a plasmid [27].

Although *Clostridium* spore-mediated disease outbreaks, such as FP and *Clostridioides difficile* infection (CDI), are common in the USA and Europe [21], [29], no such outbreaks have been systematically documented in Middle Eastern countries, including the Kingdom of Saudi Arabia (KSA). The absence of disease monitoring and control systems implies that the outbreaks normally occur in Saudi Arabia, but have not yet been reported. The KSA has no data on the incidence or prevalence of *Clostridium*-associated diseases, except for a limited study in the Dhahran region [30]. Therefore, the purpose of this study was to determine the prevalence of C. *perfringens* spores in the Hail and Qassim regions of the KSA by: 1) isolating C. *perfringens* bacteria from various samples obtained from soil, food, and hospital floors; 2) evaluating the presence of *plc* (encoding β-toxin) and *cpe* in newly-isolated C. *perfringens* strains using polymerase chain reaction (PCR); and 3) examining genome organization by determining the DNA sequences of the representative Saudi isolates. Our results suggest that C. *perfringens* spores are highly prevalent in the Hail and Qassim regions, KSA.

2. Material and Methods

A. Survey of soil, food, and hospital floors for the presence of C. *perfringens* spores

We selected three hospitals from both Qassim (hospitals A-C) and Hail (hospitals D-F) regions of KSA for our study. We collected soil samples from the hospital surroundings, swab samples from hospital floors, and food samples, such as various raw meats, including ground beef, and chicken supplied to hospitals. Collectively, 300 soil, swab, and food samples were collected from different places.

B. Isolation of C. *perfringens* from soil samples

Soil (1.0 g) was collected in a 15-ml sterile plastic tube and then 2.0 ml sterile tryptone glucose yeast (TGY) broth (3% trypticase [Difco, BD Diagnostic Systems, Sparks, MD, USA], 2% glucose [Sigma-Aldrich, USA], 1% yeast extract [Difco, BD Diagnostic Systems, Sparks, MD, USA], and 0.1% L-cysteine [Sigma-Aldrich, USA]) was added and mixed vigorously. An aliquot (0.1 ml) of suspension was cultured onto TSC (tryptose-sulfite-cycloserine, a relatively selective medium for *Clostridium* isolates) (Millipore, Burlington, MA, USA) agar plates and incubated in anaerobic jars containing GasPak (BD EZ anaerobe container system. Becton, Dickinson and company spark, Maryland USA) at 37 ºC for 24 h. For enrichment, 1.0 ml aliquot of TGY-soil suspension was added to each of two tubes containing 9 ml of sterile TGY. One tube was incubated at 37 ºC overnight (18 h) to grow vegetative cells. The other tube was heat-shocked at 75 ºC for 20 min, then anaerobically incubated at 37 ºC overnight (18 h), allowing spores to germinate and grow. If there was growth in a TGY tube, an aliquot (0.1 ml) of TGY grown culture was plated onto the TSC plate and anaerobically incubated at 37 ºC for 24 h. Three black colonies of the TSC plates were selected and allowed to grow in TGY at 37 ºC for 18 h. These TGY-grown cultures were then streaked onto sheep/horse blood agar plates and anaerobically incubated at 37 ºC for 24-48 h. The culture that produced a clear double zone of α-hemolysis, with the inner zone (complete hemolysis) caused by perfringolysin O and the outer zone (partial hemolysis) caused by β-toxin, was considered as C. *perfringens*. C. *perfringens* cultures were stored as glycerol stock at -80 ºC freezer until used.

C. Isolation from raw meat samples

Twenty-five grams of each raw meat or meat product was suspended in 225 ml 0.1% peptone (Difco, BD Diagnostic Systems, Sparks, MD, USA) and the resultant mixture was homogenized for 1–2 min, at low speed, in a sterile blender jar. The blended solution was then serially diluted from 10-1 to 10-8. Thereafter, 0.1 ml of each dilution was plated onto TSC agar plates and anaerobically incubated at 37 °C. After 24–48 h incubation, colonies showing morphology consistent with *Clostridium* isolates (i.e., black color) were selected for further testing to confirm their identity as C. *perfringens* as described above.

D. Screening for the presence of *plc* and *cpe* genes in C. *perfringens* isolates

Total C. *perfringens* DNA was isolated from the overnight TGY medium cultures, using the Wizard® Genomic DNA Purification Kit (Promega) and then subjected to PCR analysis using primers specific to each of the *cpe* and *plc* genes. The design of the primers was based on the C. *perfringens* strain SM101 genome sequence [8]. These PCR analyses utilized 100 ng template DNA, 25 pmol of each primer, 200 μM deoxynucleoside triphosphates (dNTPs) (Roche), 2.5 mM MgCl2, and 1 U Taq DNA polymerase (Fermentas) in a total volume of 50 μl. The reaction mixture was placed in a thermal cycler (Techne) for an initial period of 2 min at 94 °C, then 35 cycles, each 1 min at 94 °C, 1 min at 47 °C, 1 min at 72 °C, followed by an extension period of 10 min at 72 °C. The presence of a PCR amplified product was examined by

subjecting an aliquot of each PCR sample to agarose (1.0%) gel electrophoresis, followed by ethidium bromide staining and photographing under UV light. The following primers were used to detect toxin genes:

Forward *plc* primer:
(5'-GATGGAAAAATTGATGGAACAGGAACT-3'),
Reverse *plc* primer:
(5'-CATGTAGTAGTCATCATCTGTTCCAGCATC-3'),
Forward *cpe* primer:
(5'-GGAGATGGTTGGTTGGATATTAGGGG-3'), and
Reverse *cpe* primer:
(5'-CTTCCAAGTCACATCTTTCGTCAG-3')

E. DNA extractions, library preps, sequencing

Samples were prepared for sequencing with the Nextera DNA library prep. Twenty-five samples of C. *perfringens* isolates were multiplexed onto a single lane, 51 bp single end HiSeq3000 run, at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University, Corvallis, OR, USA.

F. Bioinformatics: Assembly, Alignment to SM101 and genes, sequence logos of gene regions

Each sample was individually assembled with SPAdes [31]. SPAdes was run with default K-mers and the careful pipeline option to reduce mismatches and short indels. SPAdes assembly output scaffold files were compared against the C. *perfringens* SM101 genome [32], using nucmer [33], delta, and show-coords from MUMer [34]. Each sample was compared to eighteen C. *perfringens* genes as follows: *sleC* (ABG87393.1), *virS* (ABG86783.1), *sigF* (ABG87692.1), *sigG* (ABG86124.1), *sigE* (ABG85707.1), *plc* (ABG86694.1), *cpe* (ABG85760.1), *cspB* (ABG86463.1), *gerKB* (ABG85755.1), *gerKA* (ABG86956.1), *gerKC* (ABG86274.1), *gerAA* (ABG86934.1), *spo0A* (ABG85493.1), *cpb* (WP_003453250.1),

etx (WP_164789292.1), *iap* (WP_003463422.1), *ibp* (BAK40944.1) and *netB* (WP_110003253.1). BlastN [35] was used to identify the general genomic region of the gene in the assembly. When necessary, custom Perl scripts were used to extract and reverse-complement the gene sequence. Promer and show-snps from MUMer were used to identify translated amino acid single nucleotide polymorphisms (SNPs) between the assembled sample and the gene reference sequence. WebLogo [36] was used to evaluate the gene regions across all samples.

3. Results and Discussion

A. Isolation of C. perfringens

Our survey could detect C. *perfringens* isolates from all the targeted locations, i.e., the soil, floor, and food samples from selected hospitals (Table 1). Based on their characteristic, black colonies appearance on TSC plates (Table 1), the isolates were putatively identified as C. *perfringens*. The black colonies on TSC plate are a result of sulfite reduction by C. *perfringens* [4], [37]. The soil samples obtained from all six hospital areas showed characteristic appearance of black colonies on TSC plate, albeit to varying degrees (Table 1). However, floor samples from only two of the six hospitals (Qassim hospital B and C) produced black colonies on TSC plate. Similarly, the food samples from only three of the six hospitals (Qassim hospitals A and B; Hail hospital D) produced the characteristic black colonies on the TSC plates (Table 1). When these isolates were subjected to *Clostridium*-specific biochemical tests, they fermented lactose, produced acid and gas, reduced nitrates to nitrites, and liquified gelatin within 48 h. Collectively, these results demonstrated that our survey successfully isolated *Clostridium* species from Hail and Qassim environments.

	Soil (%)#	Floor (%)	Food (%)
Qassim hospital A	73.3	0	7
Qassim hospital B	45.8	53.3	4.6
Qassim hospital C	37.5	6	0
Hail hospital D	8	0	38.4
Hail hospital E	33.3	0	0
Hail hospital F	25	0	0

Table 1: Isolation of *Clostridium perfringens* from various samples collected from the Hail and Qassim regions

	Soil	Floor	Food
Qassim hospital A	0	0	0
Qassim hospital B	0	0	10
Qassim hospital C	0	0	0
Hail hospital D	0	0	4
Hail hospital E	8	0	3
Hail hospital F	0	0	0
Total	8	0	17

Table 2: Formation of double zones of β-hemolysis on sheep blood agar plates by C. *perfringens* isolates

B. Differentiating C. perfringens from other Clostridium species by monitoring plc and PFO phenotypes on blood agar plates

When newly-isolated *Clostridium* cultures were anaerobically grown on sheep blood agar plates, 25 out of 54 cultures produced a clear double (PFO-mediated inner and the β-toxin-mediated outer) zone of α-hemolysis on the plates (Table 2). These results confirmed that these 25 cultures are C. *perfringens* isolates. However, the remaining 29 cultures, that were identified as *Clostridium* species by TSC plating and biochemical tests, failed to produce α-hemolysis; no inner or outer zone of α-hemolysis was detected on blood agar plates. These results suggest that other *Clostridium* species can be detected in soil, food, and hospital floor samples. However, further studies are required to support this hypothesis.

C. Detection of the plc and cpe genes in C. perfringens isolates by PCR

To confirm the presence of the *plc* gene in our newly-isolated C. *perfringens*, which produced *plc*, we performed PCR analyses, using *plc*-specific primers. As a positive control, *plc* PCR analyses amplified the 900 bp *plc*-specific band from wild-type strains, SM101 and F4969. As expected from

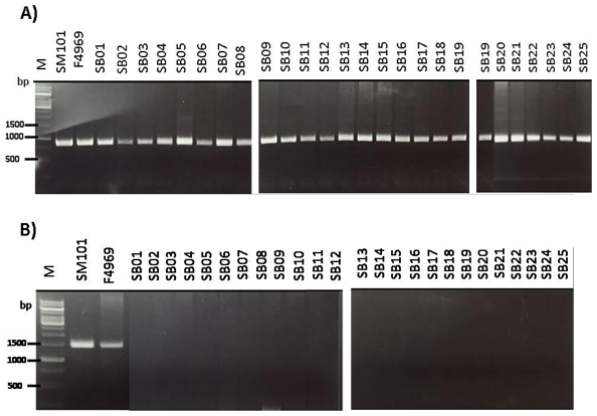

Figure 1: Identification of *plc* and *cpe* genes in *Clostridium perfringens* strains, isolated from Hail and Qassim, KSA. Total DNA, isolated from wild-type (SM101 and F4969) and KSA strains, was subjected to PCR analyses using primers specific for *plc* (A) and *cpe* (B), as described in the Material and Methods. Lane M represents the molecular size marker (base pairs)

Samples	Contigs	Assembly length	Longest contig	N50
s001	5441	4394834	83758	8892
s002	1140	3695605	91700	27495
s003	1076	3726820	101287	28498
s004	721	3115795	101405	36366
s005	542	3148052	136630	30348
s006	14321	12326586	28624	2726
s007	6419	14213758	264909	12641
s008	1009	3736936	142751	57391
s009	519	3319337	110603	30553
s010	443	3318911	151873	40772
s011	6168	8501859	170643	3915
s012	594	3115402	191201	37292
s013	555	3408298	235785	40516
s014	409	3291632	128382	37491
s015	379	2986986	248334	43576
s016	20168	12912111	73446	974
s017	8044	8489997	110019	2939
s018	27220	22812868	187921	1722
s019	734	3509100	188089	44804
s020	350	3342300	262729	77505
s021	488	3376723	201291	52706
s022	488	3372578	202330	58206
s023	1924	7208676	191597	17641
s024	1727	7238402	160484	22289
s025	337	3309147	192708	63344

Table 3: Assembly of SPAdes yielded de novo assemblies of all Saudi Arabian samples

our blood agar plate phenotypic results, *plc*-specific PCR-amplified bands were obtained from DNA of all 25 surveyed isolates (Figure 1 A). As *cpe* is the main toxin responsible for C. *perfringens* type F FP, we examined whether our isolated C. *perfringens* carry *cpe* gene. Our PCR analyses, using *cpe*-specific primers, amplified the 1500 bp *cpe*-specific band from DNA of the reference strains SM101 and F4969. However, when DNA of our newly-isolated 25 strains were subjected to similar PCR analyses, no *cpe*-specific band was amplified from any DNA sample (Figure 1 B). Kuske et al. [38] had the same result; none of their surveyed C. *perfringens* soil isolates were *cpe*-positive. In contrast, Li et al [27] reported *cpe*-positive soil isolates harboring *cpe* gene on the plasmid. These findings support the possibility that soil may not be the major reservoir for C. *perfringens* chromosomal *cpe* isolates.

D. Comparing C. perfringens nucleotide sequences isolated from the KSA, USA, and Europe

We hypothesized that there might be differences in nucleotide sequence between the American/European and KSA strains, as FP outbreaks in the US and Europe are more frequent than those in the Middle East and KSA. To prove our hypothesis, we compared the whole genome sequence of Saudi 's C. *perfringens* strains with that of the American/European's C. *perfringens* strains. SPAdes yielded de novo assemblies with a wide range of values; number of scaffolds ranging from 337–27220, assembly lengths ranging from 3.1–22.8 kb, and N50 ranging from 974–63344 bp (Table 3). When compared to the C. *perfringens* reference genome, most of the reference genome was found in the assemblies, excluding samples s007 and s008. On average, 87% of the C. *perfringens* genome was assembled in each sample. Samples s007 and s008 did not sequence well; s007 represented 53% of the C. *perfringens* genome and s008 only 9% (Table 4). Thirteen proteins were compared to the assembled samples. As previously mentioned, s007 and s008 were poor-quality assemblies that did not align well with the proteins. Sample s006 also showed a poor alignment with the protein sequences, along with many low-quality errors, due to low coverage in the assembly. Overall, the proteins and assembled contigs were well-matched, with only SNP differences. In the remaining 22 samples, the number of amino acid SNPs ranged from 0–89 (Table 1S), and the average across each gene was aligned to 22 genomes ranging from 0–44.5 SNPs (Table 5). *cpe* (ABG85760.1) was not found in any of the genome assemblies, which confirmed our PCR results (Figure 1B) that none of the 25 samples isolated from different places contained the *cpe* gene. *gerAA* (ABG86934.1) did not align with sample s001. *plc* (ABG86694.1) was aligned to all tested genome samples with average SNPs of 10.95 (Table 5). etx (WP_164789292.1) aligned to samples s019, s021, s022 only. Each KSA sample shared the same single SNP (Table 5). *cpb* (WP_003453250.1), *iap* (WP_003463422.1), ibp (BAK40944.1), and *netB* (WP_110003253.1) were not found when aligned to the KSA isolates.

Based on the data listed in Table 6, proteins, that result from translating nucleotides with significant sequence differences between Saudi Arabian and American standard samples, may give rise to a similar protein pattern for both. For example, *sigE* (ABG85707.1) and *sigF* (ABG87692.1) have significantly different nucleotide sequences, but upon translation, there was no difference in protein patterns between the Saudi Arabian isolates and the American reference sample,

Samples	Assembly size	Percent ID	Contigs in alignment	Percent of SM101 covered by assembly
s001	2633034	96.93%	904	88.95%
s002	2628043	96.74%	374	88.78%
s003	2611250	96.54%	315	88.22%
s004	2557608	96.46%	230	86.40%
s005	2549355	96.58%	246	86.12%
s006	2674583	97.12%	1770	90.35%
s007	1561619	89.82%	1451	52.76%
s008	270015	94.73%	484	9.12%
s009	2567660	96.29%	265	86.74%
s010	2563778	96.31%	207	86.61%
s011	2559681	96.19%	219	86.47%
s012	2556930	96.68%	243	86.38%
s013	2571706	96.41%	212	86.88%
s014	2563279	96.38%	214	86.59%
s015	2482422	95.08%	189	83.86%
s016	2532848	96.48%	459	85.57%
s017	2555097	96.61%	225	86.32%
s018	2528230	96.42%	201	85.41%
s019	2580073	96.55%	234	87.16%
s020	2559669	96.44%	167	86.47%
s021	2533240	96.52%	192	85.58%
s022	2532439	96.56%	178	85.55%
s023	2522041	95.42%	271	85.20%
s024	2521730	95.47%	262	85.19%
s025	2557348	96.47%	158	86.39%

Table 4: Genome size of C. *perfringens*, and the predicted percentage of Saudi Arabian samples

Genes	Average SNPs	Amt missing Alignments
sleC (ABG85493.1)	4	0
sigE (ABG85707.1)	0	0
gerKB (ABG85755.1)	17.86363636	0
cpe (ABG85760.1)	0	25
sigG (ABG86124.1)	1.136363636	0
gerKC (ABG86274.1)	7	0
cspB (ABG86463.1)	39.13636364	0
plc (ABG86694.1)	10.95454545	0
virS (ABG86783.1)	24.77272727	0
gerAA (ABG86934.1)	3.363636364	1
gerKA (ABG86956.1)	8.590909091	0
sleC (ABG87393.1)	44.45454545	0
sigF (ABG87692.1)	0.227272727	0
etx (WP_164789292.1)	1	22

Table 5: Alignment of C. *perfringens* genes with Saudi samples and missing data, including missing sequences

SM101. Interestingly, all KSA strains, with the exception of sample numbers S23 and S24, had two proteins that differed from the reference sample SM101 in the *sigF* gene. *sigE* in all KSA strains showed no significant difference in amino acid sequence, indicating minimal change in protein function compared with that of SM101. Both nucleotide and protein sequences showed that the protein sequence was not affected by codons changing nucleotides (Table 7). This suggest that the underlying protein (and therefore protein function) is conserved while mutations occur. In other cases, the protein sequence did change, indicating that the amino acids at those positions were not conserved, which probably resulted in altered protein function (Table 6).

To determine the degree of protein conservation between the reference and KSA strains, we ran multiple alignments of a single region for some of the genes in C. *perfringens* that regulate toxin production and sporulation. Sequence logos were used for the multiple alignments of the protein sequences identified in each isolate for the following genes:

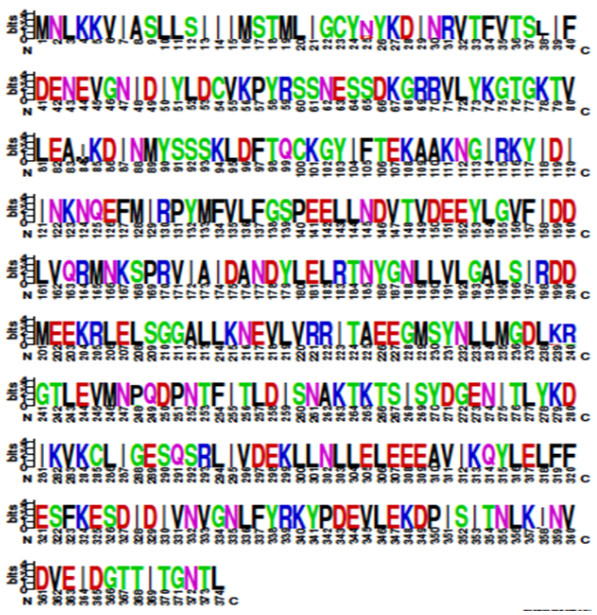

Figure 2: Protein sequence motif represented as a motif logo for *gerKC* (ABG86274)

Figure 3: Protein sequence motif represented as a motif logo for *spo0A* (ABG85493)

gerKC (ABG86274.1) [39], [40], *spo0A* (ABG85493.1) [41], *plc* (ABG86694.1) [42], and *virS* (ABG86783.1) [43]. Sequence logos showed a high sequence similarity between gene versions (Figure 2–5). We found that most Saudi Arabian samples had highly conservative proteins with the C. *perfringens* reference strain SM101, as shown in *gerKC* (ABG86274.1) (Figure 2), *spo0A* (ABG85493.1) (Figure 3), *plc* (ABG86694.1) (Figure 4), and *virS* (ABG86783.1) (Figure 5).

	S 001	S 002	S 003	S 004	S 005	S 006	S 007	S 008	S 009	S 010	S 011	S 012	S 013	S 014	S 015	S 016	S 017	S 018	S 019	S 020	S 021	S 022	S 023	S 024	S 025
sleC (ABG87393.1)	49	46	48	49	48	-10	89	-10	40	40	40	47	40	50	47	51	40	47	40	40	40	40	48	48	40
virS (ABG86783.1)	33	26	28	27	25	-10	-10	-10	30	30	30	28	25	10	0	27	27	25	25	24	25	25	25	25	25
sigF (ABG87692.1)	0	0	0	0	0	0	-10	-10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2	2	0
sigG (ABG86124.1)	1	1	1	1	1	-10	-10	-10	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2	2	1
sigE (ABG85707.1)	0	0	0	0	0	0	-10	-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
plc (ABG86694.1)	13	10	14	11	14	-10	82	-10	11	11	11	11	11	10	11	14	9	11	8	14	8	8	10	10	11
cpe (ABG85760.1)	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10
cspB (ABG86463.1)	0	40	40	50	40	-10	-10	-10	39	39	39	40	41	40	49	40	40	38	41	40	41	41	41	41	41
gerKB (ABG85755.1)	17	15	15	18	15	16	-10	-10	20	20	20	19	17	17	23	17	14	18	17	16	17	17	22	22	17
gerKA (ABG86956.1)	8	8	8	8	8	8	-10	-10	9	9	9	8	9	9	10	9	8	8	9	8	9	9	8	8	10
gerKC (ABG86274.1)	6	6	6	7	6	-10	-10	-10	9	9	9	6	6	6	9	6	6	7	6	6	6	6	10	10	6
gerAA (ABG86934.1)	0	3	3	5	4	3	-10	-10	4	4	4	3	3	3	8	4	4	2	3	4	3	3	2	2	3
spo0A (ABG85493.1)	4	4	4	4	4	4	-10	57	4	4	4	4	4	4	5	4	3	4	4	4	4	4	4	4	4

Table 6: Alignments of translated nucleotide sequences of Saudi Arabian isolates showing possibilities of proteins with conserved and non-conserved amino acid sequences, suggesting the possibility of differences in functionality

	S 001	S 002	S 003	S 004	S 005	S 006	S 007	S 008	S 009	S 010	S 011	S 012	S 013	S 014	S 015	S 016	S 017	S 018	S 019	S 020	S 021	S 022	S 023	S 024	S 025
sleC (ABG87393.1)	121	118	120	121	122	NH	153	NH	95	95	95	119	95	119	112	118	95	118	95	97	95	95	117	117	95
virS (ABG86783.1)	74	64	68	67	68	NH	NH	NH	71	71	71	68	62	44	122	70	68	67	62	65	62	62	68	68	62
sigF (ABG87692.1)	10	10	11	8	11	11	NH	NH	11	11	11	10	11	11	27	11	11	9	11	11	11	11	18	18	11
sigG (ABG86124.1)	6	6	8	7	6	NH	NH	NH	5	5	5	6	7	6	13	6	8	6	6	6	6	6	12	12	6
sigE (ABG85707.1)	10	11	11	10	12	11	NH	NH	12	12	12	12	12	12	28	12	10	12	12	12	12	12	10	10	12
plc (ABG86694.1)	26	23	27	28	27	NH	0	NH	24	24	24	22	25	27	45	28	26	29	25	27	25	25	28	28	25
cpe (ABG85760.1)	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH
cspB (ABG86463.1)	NH	137	137	167	136	NH	NH	NH	132	132	132	134	135	132	164	137	134	134	135	135	135	135	151	151	135
gerKB (ABG85755.1)	38	30	30	42	30	33	NH	NH	60	60	60	42	38	38	62	38	29	42	38	38	38	38	66	66	38
gerKA (ABG86956.1)	51	57	57	49	52	51	NH	NH	75	75	75	52	50	50	77	50	54	51	50	49	50	50	55	55	51
gerKC (ABG86274.1)	24	23	23	27	25	NH	NH	NH	51	51	51	25	23	24	49	23	23	28	23	24	23	23	56	56	23
gerAA (ABG86934.1)	NH	39	37	29	25	32	NH	NH	28	28	28	37	24	26	72	42	35	21	26	32	26	26	41	41	24
spo0A (ABG85493.1)	9	11	10	10	9	11	NH	53	10	10	10	8	10	10	17	9	8	10	10	9	10	10	16	16	10

Table 7: Nucleotide sequence alignments of Saudi Arabian isolates showing the recorded similarities and differences

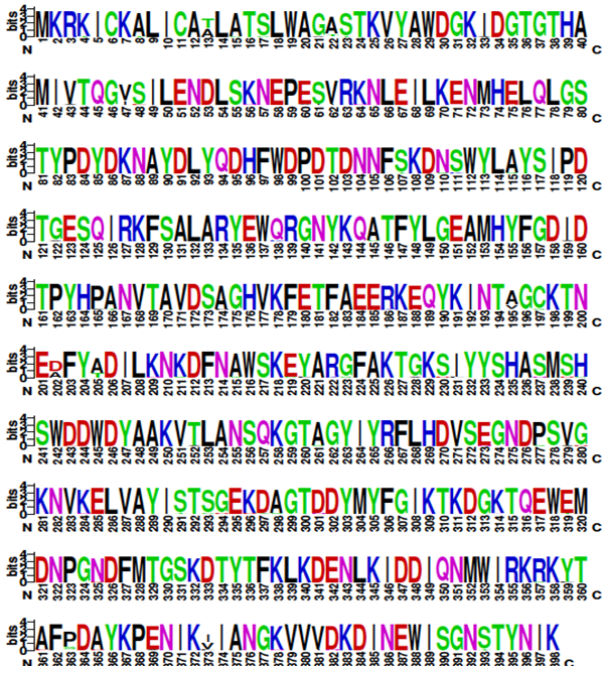

Figure 4: Protein sequence motif represented as a motif logo for *plc* (ABG86694)

4. Conclusion

Our study successfully surveyed soil and hospital environmental samples from the Hail and Qassim areas and isolated 25 C. *perfringens* isolates. All these isolates were *plc* and β-toxin positive. However, none of the isolates were *cpe*-positive. Furthermore, genome-sequencing analyses showed

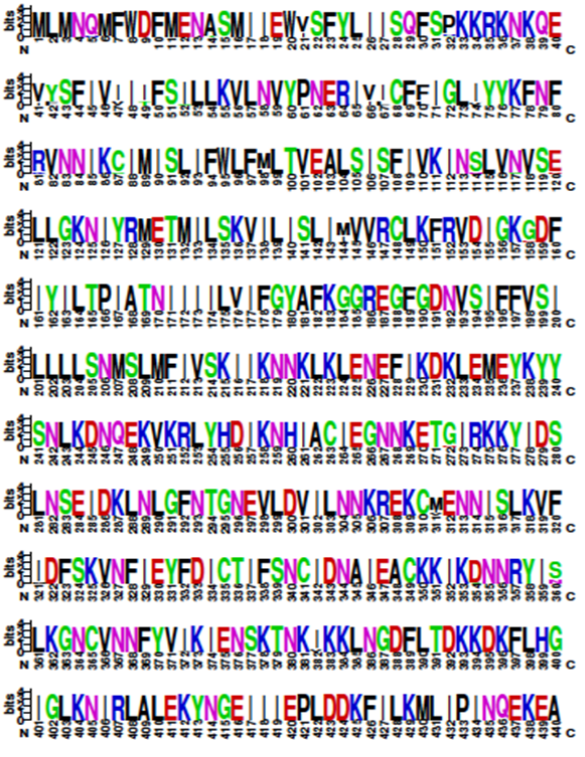

Figure 5: Protein sequence motif represented as a motif logo for *virS* (ABG86783)

that Saudi C. *perfringens* isolates are genotypically similar to the American and European isolates. However, some proteins between isolates might be functionally different, which should be investigated. Interestingly, among six toxinotyping gene alignments, *plc* aligned to all 25, and etx to only 3, Saudi C. *perfringens* genomes. However, *cpe*, *cpb*, iap, ibp and netB did not align to any of the 25 Saudi isolates. Collectively, our findings suggest that C. *perfringens* are highly prevalent in the Hail and Qassim environment, with 88% isolates are type A (*plc*-positive) and 12% are type D (*plc*-and etx-positive). The absence of C. *perfringens* type F (*cpe*-positive) in Hail and Qassim area might be a reason for limited reports on C. *perfringens* type F FP outbreaks in KSA compared to USA. Future surveys using samples from more KSA states are needed to confirm this hypothesis.

5. Abbreviations

CDI, *Clostridioides difficile* infection; *cpe*, *Clostridium perfringens* enterotoxin; NetB, *Clostridium perfringens* necrotic enteritis B-like toxin; NFB, non-foodborne; PFO, perfringolysin O; SNPs, single nucleotide polymorphisms; TGY, trypticase-glucose-yeast extract; TSC, tryptose-sulfite-cycloserine

Data Availability

C. *perfringens* sequences were deposited into GenBank under BioProject PRJNA954388.

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Author Contributions

SB,MJ,AQ,BK and MS: Conceptualization, Methodology, Software SB,MJ,AQ,BK and MS: Data curation, Writing-Original draft preparation. SB,MJ: Visualization, Investigation. MS: Supervision.: BK: Software, Validation.: SB,MJ,BK nad MS: Writing- Reviewing and Editing.

Conflict of interest

The authors declare no conflict of interests. All authors read and approved final version of the paper.

References

[1] McClane, B. A., Robertson, S. L., & Li, J. (2013). *Clostridium perfringens*. In M. P. Doyle & R. Buchanan (Eds.), *Food Microbiology: Fundamentals and Frontiers* (4th ed., pp. 465–486). ASM Press.

[2] Theoret, J. R., McClane, B. A., Uzal, F. A., Songer, J. G., Prescott, J. F., & Popoff, M. R. (2016). Toxins of *Clostridium perfringens*. *Clostridial Diseases of Animals, 45*.

[3] Freedman, J. C., Shrestha, A., & McClane, B. A. (2016). *Clostridium perfringens* enterotoxin: action, genetics, and translational applications. *Toxins (Basel), 8*(3), 73.

[4] Uzal, F. A., Freedman, J. C., Shrestha, A., Theoret, J. R., Garcia, J., Awad, M. M., et al. (2014). Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. *Future Microbiol., 9*, 361–377.

[5] McClane, B., Uzal, F. A., Miyakawa, M. F., Lyerly, D., & Wilkins, T. (2004). The enterotoxic clostridia. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The Prokaryotes* (pp. 698–752). Springer.

[6] Asha, N. J., & Wilcox, M. H. (2002). Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhoea. *Journal of Medical Microbiology, 51*, 891–894.

[7] Borriello, S. P., Larson, H. E., Welch, A. R., Barclay, F., Stringer, M. F., & Bartholomew, B. A. (1984). Enterotoxigenic *Clostridium perfringens*: a possible cause of antibiotic-associated diarrhoea. *Lancet, 1*, 305–307.

[8] Rood, J. I., Adams, V., Lacey, J., Lyras, D., McClane, B. A., Melville, S. B., ... & Van Immerseel, F. (2018). Expansion of the *Clostridium perfringens* toxin-based typing scheme. *Anaerobe, 53*, 5–10.

[9] Sarker, M. R., Carman, R. J., & McClane, B. A. (1999). Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive C. *perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Molecular Microbiology, 33*, 946–958.

[10] Hoffmann, S., Batz, M. B., & Morris, J. G. (2012). Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *Journal of Food Protection, 75*, 1292–1302.

[11] Lindström, M., Heikinheimo, A., Lahti, P., & Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology, 28*, 192–198.

[12] Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., ... & Griffin, P. M. (2022). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases, 17*, 7–15.

[13] Xiao, Y., Wagendorp, A., Moezelaar, R., Abee, T., & Wells-Bennik, M. H. (2012). A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal *cpe* gene belong to one multilocus sequence typing cluster. *Applied and Environmental Microbiology, 78*, 7060–7068.

[14] Collie, R. E., & McClane, B. A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology, 36*, 30–36.

[15] Sarker, M. R., Shivers, R. P., Sparks, S. G., Juneja, V. K., & McClane, B. A. (2000). Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. *Applied and Environmental Microbiology, 66*, 3234–3240.

[16] Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P. E., Canard, B., & Cole, S. T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology, 15*, 639–647.

[17] Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-*cpe* or IS1470-like-*cpe*) as a common cause of food poisoning. *Journal of Clinical Microbiology, 46*, 371–373.

[18] Tanaka, D., Isobe, J., Hosorogi, S., Kimata, K., Shimizu, M., Katori, K., ... & Nakamura, S. (2003). An outbreak of food-borne gastroenteritis caused by *Clostridium perfringens* carrying the *cpe* gene on a plasmid. *Japanese Journal of Infectious Diseases, 56*(3), 137–139.

[19] Grass, J. E., Gould, L. H., & Mahon, B. E. (2013). Epidemiology of foodborne disease outbreaks caused by *Clostridium perfringens*, United States, 1998–2010. *Foodborne Pathogens and Disease, 10*, 131–136.

[20] Wen, Q., & McClane, B. A. (2004). Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Applied and Environmental Microbiology, 70*, 2685–2691.

[21] Talukdar, P. K., Udompijitkul, P., Hossain, A., & Sarker, M. R. (2017). Inactivation strategies for *Clostridium perfringens* spores and vegetative cells. *Applied and Environmental Microbiology, 83*, e02731-16.

[22] Li, J., & McClane, B. A. (2006). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology, 72*, 4561–4568.

[23] Bae, Y. M., & Lee, S. Y. (2012). Inhibitory effects of UV treatment and a combination of UV and dry heat against pathogens on stainless steel and polypropylene surfaces. *Journal of Food Science, 77*, M61–M64.

[24] Alzubeidi, Y. S., Udompijitkul, P., Talukdar, P. K., & Sarker, M. R. (2018). Inactivation of *Clostridium perfringens* spores adhered onto stainless steel surface by agents used in a clean-in-place procedure. *International Journal of Food Microbiology, 277*, 26–33.

[25] Udompijitkul, P., Alnoman, M., Paredes-Sabja, D., & Sarker, M. R. (2013). Inactivation strategy for *Clostridium perfringens* spores adhered to food contact surfaces. *Food Microbiology, 34*, 328–336.

[26] Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology, 85*, 227–236.

[27] Li, J., Sayeed, S., & McClane, B. A. (2007). Prevalence of enterotoxigenic *Clostridium perfringens* Isolates in Pittsburgh (Pennsylvania) area soils and home kitchens. *Applied and Environmental Microbiology, 73*, 7218–7224.

[28] Matches, J. R., Liston, J., & Curran, D. (1974). *Clostridium perfringens* in the environment. *Applied Microbiology, 28*, 655–660.

[29] Li, J., Paredes-Sabja, D., Sarker, M. R., & McClane, B. A. (2016). *Clostridium perfringens* sporulation and sporulation-associated toxin production. *Microbiology Spectrum, 4*, 331-347.

[30] Al-Tawfiq, J. A., & Abed, M. S. (2010). *Clostridium* difficile-associated disease among patients in Dhahran, Saudi Arabia. *Travel Medicine and Infectious Disease, 8*, 373–376.

[31] Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., ... & Pevzner, P. A. (2013). Assembling genomes and mini-metagenomes from highly chimeric reads. *Lecture Notes in Computer Science. Annual International Conference on Research in Computational Molecular Biology, Springer, 17*, 158–170.

[32] Myers, G. S., Rasko, D. A., Cheung, J. K., Ravel, J., Seshadri, R., DeBoy, R. T., ... & Paulsen, I. T. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Research, 16*, 1031–1040.

[33] Delcher, A. L., Phillippy, A., Carlton, J., & Salzberg, S. L. (2002). Fast algorithms for large-scale genome alignment and comparison. *Nucleic Acids Research, 30*, 2478–2483.

[34] Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., & Salzberg, S. L. (2004). Versatile and open software for comparing large genomes. *Genome Biology, 5*, R12.

[35] Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403–410.

[36] Crooks, G. E., Hon, G., Chandonia, J. M., & Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Research, 14*, 1188–1190.

[37] McDonnell, J. L. (1986). Toxins of *Clostridium perfringens* type A, B, C, D, and E. In F. Dorner & J. Drews (Eds.), *Pharmacology of Bacterial Toxins* (pp. 477–517). Pergamon Press, Oxford.

[38] Kuske, C. R., Barns, S. M., Grow, C. C., Merrill, L., & Dunbar, J. (2006). Environmental survey for four pathogenic bacteria and closely related species using phylogenetic and functional genes. *Journal of Forensic Sciences, 51*, 548–558.

[39] Paredes-Sabja, D., Torres, J. A., Setlow, P., & Sarker, M. R. (2008). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology, 190*, 1190–1201.

[40] Banawas, S., Paredes-Sabja, D., Korza, G., Li, Y., Hao, B., Setlow, P., & Sarker, M. R. (2013). The *Clostridium perfringens* germinant receptor protein *gerKC* is located in the spore inner membrane and is crucial for spore germination. *Journal of Bacteriology, 195*, 5084–5091.

[41] Huang, I. H., Waters, M., Grau, R. R., & Sarker, M. R. (2004). Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiology Letters, 233*, 233–240.

[42] McClane, B. A., & Rood, J. I. (2001). Clostridial toxins involved in human enteric and histotoxic infections. In *Clostridia Biotechnology and Medical Applications* (pp. 169–209).

[43] Shimizu, T., Yaguchi, H., Ohtani, K., Banu, S., & Hayashi, H. (2002). *Clostridial VirR/virS* regulon involves a regulatory RNA molecule for expression of toxins. *Molecular Microbiology, 43*, 257–265.