Two Clostridium perfringens Type E Isolates in France
Laure Diancourt, Jean Sautereau, Alexis Criscuolo, Michel Popoff

To cite this version:
Laure Diancourt, Jean Sautereau, Alexis Criscuolo, Michel Popoff. Two Clostridium perfringens Type E Isolates in France. Toxins, MDPI, 2019, 11 (3), pp.E138. 10.3390/toxins11030138. pasteur-02448780

HAL Id: pasteur-02448780
https://hal-pasteur.archives-ouvertes.fr/pasteur-02448780
Submitted on 22 Jan 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Two *Clostridium perfringens* Type E Isolates in France

Laure Diancourt¹, Jean Sautereau¹, Alexis Criscuolo²,† and Michel R. Popoff³,*,†

¹ CNR Bactéries anaérobes et Botulisme, Institut Pasteur, 75015 Paris, France; laure.diancourt@pasteur.fr (L.D.); jean.sautereau@pasteur.fr (J.S.)
² Hub de Bioinformatique et Biostatistique—C3BI, Institut Pasteur, USR 3756, CNRS, 75015 Paris, France; alexis.criscuolo@pasteur.fr
³ Bacterial Toxins, Institut Pasteur, ERL 6002, 75015 Paris, France
* Correspondence: mpopoff@pasteur.fr
† Authors contributed equally to this work.

Received: 12 February 2019; Accepted: 23 February 2019; Published: 1 March 2019

**Abstract:** *Clostridium perfringens* type E is a less frequently isolated *C. perfringens* type and has not previously been reported in France. We have characterized two recent type E isolates, *C. perfringens* 508.17 from the intestinal content of a calf that died of enterotoxemia, and 515.17 from the stool of a 60-year-old woman, subsequent to food poisoning, which contained the plasmid pCPPB-1 with variant iota toxin and *C. perfringens* enterotoxin genes.

**Keywords:** *Clostridium perfringens*; iota toxin; *C. perfringens* enterotoxin; enterotoxemia; food poisoning

**Key Contribution:** The first two *Clostridium perfringens* type E isolates in France have been characterized by whole genome sequencing and iota toxin activity. These strains also contain alpha toxin, perfringolysin, and variant cpe genes.

1. Introduction

*Clostridium perfringens* is a ubiquitous gram-positive, spore forming anaerobic bacterium which produces different toxins and is responsible for various diseases in man and animals such as gangrene, food poisoning, diarrhea, necrotic enteritis, and enterotoxemia. Based on the production of four toxins (alpha, beta, epsilon, and iota), *C. perfringens* was traditionally divided into five toxin types (A to E) [1]. More recently, the *C. perfringens* nomenclature was expanded to seven types (A to G) by adding *C. perfringens* enterotoxin (CPE) and NetB carrying strains as different toxin types [2].

*C. perfringens* type E is characterized by the production of iota toxin. Iota toxin is a binary toxin consisting of a binding component (Ib) and an enzymatic component (Ia) which enters cells and modifies the actin cytoskeleton by ADP-ribosylation of actin monomers [3,4]. *C. perfringens* type E is a less frequently isolated type from clinical samples. This toxin type has been isolated from cases of diarrhea or hemorrhagic enteritis and sudden death in neonatal calves, mainly in the US, and more rarely in other animals such as chickens, lambs, goats, and cows [5–13]. In France, *C. perfringens* type E isolation has not been previously reported. Here we describe two recent type E isolates, 508.17 and 515.17.

2. Results

2.1. Identification of Two *C. perfringens* Type E and Investigation of Iota Toxin Production

The two *C. perfringens* isolates 508.17 and 515.17 were identified as *C. perfringens* type E by detection of iota toxin genes with routine PCR toxin gene identification of *C. perfringens* [14].
The production of iota toxin in *C. perfringens* 508.17 and 515.17 was investigated by western blotting with specific antibodies against Ia and Ib. The reference type A *C. perfringens* strain ATCC 13124 was used as a control. As shown in Figure 1, both components Ia and Ib were detected in the supernatant of *C. perfringens* 508.17 and 515.17 but not in *C. perfringens* ATCC 13124.

The biological activity was tested on Vero cells. The concentration of culture supernatant yielding 50% rounded cells was 156 ng/mL (total protein) for 508.17 and 650 ng/mL (total protein) for 515.17. The level of cytotoxicity was increased four-fold after α-chymotrypsin treatment of the culture supernatants. In comparison, the 50% cytotoxic activity of purified recombinant iota toxin on Vero cells was obtained with 15 ng/mL Ib and 8 ng/mL Ia (not shown).

![Figure 1. Production of iota toxin in *C. perfringens* 508.17 (508), 515.17 (515), and ATCC 13124.](image)

Concentrated supernatants of *C. perfringens* 508.17 (5 µg total protein), *C. perfringens* 515.17 (5 µg total protein), and *C. perfringens* ATCC 13124 (5 µg total protein) as control, as well as purified Ia (1 ng) and Ib (1 ng) were run on a 10% SDS-PAGE and transferred on nitrocellulose. Blots were incubated with specific rabbit serum against Ia and Ib, respectively, and then with goat immunoglobulin against rabbit IgG labeled with peroxidase. The bands at 55 kDa in the western blot with anti-Ib and the double bands in the western blot with anti-Ia resulted from partial proteolytic degradation.

### 2.2. In Vitro Actin ADP-Ribosylation

The enzymatic activity of iota toxin in *C. perfringens* 508.17 and 515.17 culture supernatants was tested by in vitro ADP-ribosylation with muscular and cellular actin. As shown in Figure 2, ADP-ribosylation of both muscular and cellular actin was observed with culture supernatants from *C. perfringens* 508.17 and 515.17, as well as the purified Ia control. The type A *C. perfringens* ATCC 13124 did not show any actin ADP-ribosylation.
Iota toxin Ia and Ib components from 508.17 and 515.17 strains are highly similar (99.73% and 99.83% at the amino acid level), closely similar to those of pCPPB-1 iota toxin components (99.87% similarity), and more distantly related to classical iota toxin (91.2% and 89.7%, respectively) (Table 1). Iota toxin sequences from 508.17 and 515.17 strains show a low relatedness (40–42% identity) to iota toxin variant BEC/CPILE found in contaminated food in Japan [15,16].
**Table 1.** Amino acid (aa) sequence comparison of iota toxin components Ia and Ib from 508.17 and 515.17 strains versus classical iota toxin (X73562) from NCIB10748 and iota variant from pCPPB-1.

| Strain/Sequence | Iota Ia Similarity % (aa) | Iota Ib Similarity % (aa) |
|-----------------|---------------------------|---------------------------|
|                 | 508.17                    | pCPPB-1                   |
| 508.17          | 100.00                    | 100.00                    |
| pCPPB-1         |                            |                           |
| 515.17          | 99.73                     | 99.86                     |
|                 | 100.00                    | 100.00                    |
| X73562          | 91.20                     | 91.33                     |
|                 | 91.19                     | 100.00                    |

The two isolates also contain alpha toxin, perfringolysin, and cpe genes. CPE from 508.17 and 515.17 strains are identical at the amino acid sequence level with variant CPE from pCPPB-1, and differ from classical CPE at 12 positions (Table 2).

**Table 2.** Comparative alignment and amino acid similarity of CPE 508.17 and 515.17 versus pCPPB-1 variant CPE as well as pCF4969 and pJFP838-all classical plasmid CPE. The amino acid (aa) changes between variant and classical CPE are indicated. CPE sequences from 508.17 and 515.17 match with that of pCPPB-1 and diverge at 12 positions from classical CPE.

| Strain/Sequence | Variable aa Positions on cpe | Similarity % |
|-----------------|------------------------------|--------------|
|                 | 18 20 32 172 217 257 276 283 287 313 508.17 515.17 pCPPB-1 pCF4969 |
| 508.17          | L V K A S T R Q E N I A -    |              |
| pCPPB-1         | L V K A S T R Q E N I A 99.07 - |
| pCF4969         | F I N G T S K E Q K V S 96.36 97.3 96.36 - |
| pJFP838-all     | F I N G T S K E Q K V S 96.36 97.3 96.36 100.00 |

2.4. Phylogenetic Analysis

Phylogenetic analysis of the *C. perfringens* core-genome shows that the two strains 508.17 and 515.17 are related but not identical (Figure 3). Both strains are neighbors within a specific clade, emerging between the type A strain MJR7757A (originated from a human host) and type A JFP strains (originated from foal or dog hosts). However, they are distantly related to the classical *C. perfringens* type E strain JGS1987. Of note, the strain 508.17 is characterized by the absence of the toxin gene cpb2, whereas the two type E strains 515.17 and JGS1987 show identical toxin gene content.
Figure 3. Maximum likelihood phylogenetic tree of C. perfringens based on their core genome and presence/absence pattern of 17 toxin genes. Each branch support was assessed by the ultrafast bootstrap approach (only support values <95% are shown). The scale bar represents 0.005 substitutions per nucleotide. The presence/absence of each of the 17 toxin genes is indicated by a black/white square for each of the 115 analyzed genomes. \textit{becAB}: binary enterotoxin of C. perfringens; \textit{colA}: collagenase A; \textit{cpb}: C. perfringens beta toxin; \textit{cpbd}: C. perfringens delta toxins; \textit{cpe}: C. perfringens enterotoxin; \textit{etx}: C. perfringens epsilon toxin; \textit{iap}, \textit{ibp}: C. perfringens iota toxin; \textit{netBEFG}: necrotic enteritis toxins; \textit{pfoA}: perfringolysin; \textit{plc}: C. perfringens alpha toxin; \textit{tpeL}: toxin perfringens large cytotoxin.
3. Discussion

Most C. perfringens type E strains have been found to contain the iota toxin (iap and ibp) genes on a plasmid flanked by the insertion sequence IS1151 and in close proximity to a silent cpe gene [17]. Iota toxin plasmids have mostly a pCPF5603 backbone with insertion of iap and ibp genes within the cpe promoter, thus preventing cpe transcription in addition to nonsense and frame-shift mutations in the open reading frame (ORF) [13]. More recently, four C. perfringens type E strains have been characterized to have iap and ibp genes on a pCPPB-1-related plasmid. pCPPB-1 is a ~67 kbp plasmid containing 72 putative ORFs that are organized in three regions, a putative replication plasmid and transfer region, a toxin region, and a variable region. pCPPB-1 retains the backbone of pCPF4969 that is the classical plasmid containing cpe in C. perfringens type A, but lacks IS elements in the vicinity of toxin genes. In contrast to pCPF5603, pCPPB-1 contains a functional cpe. Indeed, albeit iap and ibp genes are inserted into the cpe promoter region, only the promoter P3 is missing. The two other cpe promoters (P1 and P2) are the major promoters, and are preserved, therefore allowing cpe expression. This is in contrast to the strains with iota toxin plasmid of the pCPF5603 family, which produce no CPE even in sporulation conditions [18]. Iota and cpe genes harbored on pCPPB-1 plasmid show variations with the corresponding genes on classical strains. Indeed, iap and ibp from pCPPB-1 share 87% and 89% identity with the corresponding genes of C. perfringens E NCIB10748 [18,19], respectively, at the nucleotide level. The variant CPE from pCPPB-1 is 96% identical (10 amino acid differences on 319) to classical CPE [18].

Recently, C. perfringens strains isolated from food poisoning outbreaks in Japan were characterized as producing an iota toxin variant called BEC (binary toxin of C. perfringens) or CPILE (Clostridium perfringens iota-like enterotoxin) [15,16]. These strains lack cpe genes and CPE production. The two components BECa/CPILE-a and BECb/CPILE-b share 43% and 41–42% identity with Ia and Ib from the classical C. perfringens type E strain NCIB10748, respectively [15,16]. becA and becB are located on a large size plasmid (pCP-OS1/pCP-TS1, 54,635 bp) for which most parts (69%) are highly similar (92–99% identity) with sequences of the plasmid pCP13 from C. perfringens strain 13 [18]. BEC/CPILE is enterotoxic and induces fluid accumulation in rabbit ileal loop and suckling mice [15,18]. BECa/CPILEa ADP-ribosylates all isoforms of actin monomers and retains a similar structure compared to Ia [20]. Iota toxins from strains 508.17 and 515.17 share the same enzymatic activity profile as BEC/CPILE. Indeed, they also ADP-ribosylate both muscular and cellular actin. However, iota toxins from 508.17 and 515.17 as well as the classical iota toxin retain a low level of identity with BEC/CPILE.

The two French C. perfringens type E isolates have distinct chromosomal genetic backgrounds related but not identical to those of the variant C. perfringens type E [18]. They are distantly related to the classical C. perfringens type E and enterotoxigenic and non-enterotoxigenic type A strains, as well as type A strains with chromosomally located cpe [21,22] (Figure 3). However, the two French isolates have acquired a pCPPB-1 plasmid similar to that found in the variant C. perfringens type E strains isolated from meat products in Japan [18]. The two French isolates 508.17 and 515.17 have distinct origins, from calf and food intoxication in man, respectively. Their relatedness with C. perfringens type E strains reported in Japan raises questions as to whether they share a common source, and what the possible mode of dissemination might be.

4. Materials and Methods

4.1. C. perfringens Isolates

C. perfringens 508.17 was isolated from the intestinal content of a 6-month-old calf that died with an enterotoxemia syndrome in central France. The calf received a vaccination against blue tongue one day before. The strain 515.17 was from the stool of a 60-year-old woman in a nursing home showing a C. perfringens food poisoning. The stool of two other patients of the same food poisoning outbreak yielded classical enterotoxigenic C. perfringens strains with cpe gene located on the chromosome.
4.2. C. perfringens Cultures

*C. perfringens* strains were grown in tryptase-glucose-yeast extract (20 g of Tryptase, 30 g of yeast extract, and 0.5 g of cysteine hydrochloride per liter, pH 7.2) (TGY) [23] under anaerobic conditions at 37 °C overnight. The culture supernatant was concentrated (about 20-fold) by ammonium sulfate precipitation (70% saturation), centrifuged at 10,000 rpm for 10 min, and then dialysis against Tris-HCl 10 mM, pH 7.5. Protein concentration was determined by the Bradford method [24].

4.3. Iota Toxin Production and Purification

Ia and Ib components of iota toxin were produced and purified as previously described [23]. Briefly, *iap* and *ibp* from *C. perfringens* NCIB10748 were cloned into the *Escherichia coli-C. perfringens* shuttle vector pJIR750 yielding pMRP147 and pMRP384 which have been transfected by electroporation into the lecithinase-negative *C. perfringens* strain 667. The recombinant *C. perfringens* strains were grown and the culture supernatants were processed as indicated above. Ia and Ib were purified by DEAE-Sephacel chromatography and gel filtration on Superdex200 as previously described [23]. Rabbit antibodies against purified Ia and Ib were produced and checked as previously described [25,26].

4.4. Western Blotting

*C. perfringens* concentrated culture supernatants Ia and Ib were run on a 10% SDS-PAGE and transferred onto nitrocellulose. After blocking with 5% nonfat dry milk in phosphate-buffered saline, the membranes were washed with Tris-HCl 10 mM (pH 7.5) containing 150 mM NaCl and 0.1% Tween20 (TTS). The membranes were incubated with either rabbit anti-Ia or anti-Ib antibodies (1:3000 concentration) (Jackson Immunoresearch 111-035-006) for 1 h at room temperature. After three washes in TTS, membranes were incubated with horseradish peroxidase goat anti-rabbit immunoglobulins (1:3000) for 1 h at room temperature, and then processed for chemiluminescence with Immobilon Western (Millipore, Guyancourt 78280, France).

4.5. ADP-Ribosylation

For ADP-ribosylation, the following buffer was used: Tris 50 mM (pH 7.5) containing 5 mM MgCl2, 10 mM dithiothreitol, 10 mM thymidine, protease inhibitors (leupeptine 0.1 mM, pepstatin 1 mM, PMSF 2 mM), and biotin-NAD (Trevigen 4670-500-01) 12 µM. In vitro ADP-ribosylation was performed in 20 µl of the above buffer containing either 1 µg muscular (Sigma A-3653) or cellular (Cytoskeleton APHL95) actin, and 1 µL of concentrated *C. perfringens* culture supernatant. After 1 h incubation at 37 °C, the samples were run in SDS-PAGE, transferred onto nitrocellulose, processed with peroxidase streptavidin conjugate (Roche 11-089-153-001) 1:3000 for 1 h, and processed for chemiluminescence as in Section 4.4.

4.6. Cytotoxicity Assay

Vero (African green monkey kidney) cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum at 37 °C and 5% CO2. Cells were grown as confluent monolayers in 96-well plates. Then, the medium was changed to DMEM containing 0.1% bovine serum albumin and the cells were incubated with serial dilutions of *C. perfringens* culture supernatants. Changes in cell morphology characterized by cell rounding were microscopically observed after 18 h incubation.

4.7. Genome Sequencing, Assembly, and Analysis

Genomic DNA from *C. perfringens* strains was extracted and purified as previously described [27]. Whole-genome shotgun sequencing was performed using an Illumina NextSeq 500 sequencer. Libraries were constructed using Nextera XT technology and sequenced using a 2 × 150 nucleotide paired-end
strategy. All reads were preprocessed to remove or correct artefactual or low quality bases. Sequenced reads were assembled using SPAdes (v. 3.11.0, St. Petersburg State University, St. Petersburg, Russian Federation, 2017) [28], and resulting scaffold sequences were annotated using Prokka (v. 1.11, University of Melbourne, Melbourne, Australia, 2014) [29]. Specific toxin genes (i.e., \textit{becAB}, \textit{colA}, \textit{cpb}, \textit{cpb2}, \textit{cpd}, \textit{cpe}, \textit{etx}, \textit{iap}, \textit{ibp}, \textit{netBEFG}, \textit{pfoA}, \textit{plc}, \textit{tpeL}) were searched against genome sequences using BioNumerics (v. 7.6, Applied Maths NV, www.applied-maths.com, Sint-Martens-Latem, Belgium, 2016). In order to identify plasmid sequences within each assembly, the scaffold sequences were reordered against the complete chromosome of \textit{C. perfringens} ATCC 13124 using Contiguator (v. 2.7.4, University of Florence, Florence, Italy, 2014) [30,31], and each non-ordered sequences was used as query to perform a BLAST search against all publicly available \textit{C. perfringens} plasmid sequences (selection criteria: 90% similarity and 70% query coverage).

4.8. Phylogenetic Reconstruction

All publicly available \textit{C. perfringens} genome assemblies were gathered from the NCBI repository (www.ncbi.nlm.nih.gov/genome/genomes/158) in order to build a recombination-purged core-genome using Parsnp (v. 1.1.2, National Biodefense Analysis and Countermeasures Center, Frederick, MD, USA, 2014) [32]. The resulting 1,211,302 aligned core nucleotide characters were analyzed using IQ-TREE (v. 1.6.7.2, University of Vienna, Wien, Austria, 2018) [33] to infer a maximum likelihood phylogenetic tree on 115 taxa with the evolutionary model GTR+F+R10 (automatically selected by IQ-TREE from the data).

4.9. Sequence Accession

The assembled genome sequences of strains 508.17 and 515.17 were deposited in the European Nucleotide Archive and are available under accession numbers UWOV01000001–UWOV01000120 and UWOU01000001–UWOU01000116, respectively.

Author Contributions: Conceptualization, M.R.P. and A.C.; Methodology, L.D., J.S., and M.R.P.; Software, A.C. and L.D.; Writing M.R.P., A.C. and L.D.

Funding: This research received no external funding.

Acknowledgments: We thank the Mutualized Platform of Microbiology (P2M) of Institut Pasteur for genome sequencing.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Petit, L.; Gibert, M.; Popoff, M.R. \textit{Clostridium perfringens}: Toxinotype and genotype. \textit{Trends Microbiol.} \textbf{1999}, \textit{7}, 104–110. [CrossRef]

2. Rood, J.I.; Adams, V.; Lacey, J.; Lytras, D.; McClane, B.A.; Melville, S.B.; Moore, R.J.; Popoff, M.R.; Sarker, M.R.; Songer, J.G.; et al. Expansion of the \textit{Clostridium perfringens} toxin-based typing scheme. \textit{Anaerobe} \textbf{2018}, \textit{53}, 5–10. [CrossRef] [PubMed]

3. Stiles, B.G.; Pradhan, K.; Fleming, J.M.; Samy, R.P.; Barth, H.; Popoff, M.R. \textit{Clostridium} and \textit{Bacillus} binary enterotoxins: Bad for the bowels, and eukaryotic being. \textit{Toxins} \textbf{2014}, \textit{6}, 2626–2656. [CrossRef] [PubMed]

4. Barth, H.; Aktories, K.; Popoff, M.R.; Stiles, B.G. Binary bacterial toxins: Biochemistry, biology, and applications of common \textit{Clostridium} and \textit{Bacillus} proteins. \textit{Microbiol. Mol. Biol. Rev.} \textbf{2004}, \textit{68}, 373–402. [CrossRef] [PubMed]

5. Songer, J.G. Clostridial diseases in domestic animals. In \textit{Handbook on Clostridia}; Dürre, P., Ed.; CRC Press, Taylor and Francis Group: Boca Raton, FL, USA, 2005; pp. 527–542.

6. Songer, J.G.; Miskimmins, D.W. \textit{Clostridium perfringens} type E enteritis in calves: Two cases and a brief review of the literature. \textit{Anaerobe} \textbf{2004}, \textit{10}, 239–242. [CrossRef] [PubMed]

7. Uzal, F.A.; Songer, J.G. Diagnosis of \textit{Clostridium perfringens} intestinal infections in sheep and goats. \textit{J. Vet. Diagn. Investig.} \textbf{2008}, \textit{20}, 253–265. [CrossRef] [PubMed]
8. Keokilwe, L.; Olivier, A.; Burger, W.P.; Joubert, H.; Venter, E.H.; Morar-Leather, D. Bacterial enteritis in ostrich (Struthio Camelus) chicks in the Western Cape Province, South Africa. Poul. Sci. 2015, 94, 1177–1183. [CrossRef] [PubMed]

9. Kim, H.Y.; Byun, J.W.; Roh, I.S.; Bae, Y.C.; Lee, M.H.; Kim, B.; Songer, J.G.; Jung, B.Y. First isolation of Clostridium perfringens type E from a goat with diarrhea. Anaerobe 2013, 22, 141–143. [CrossRef] [PubMed]

10. Redondo, L.M.; Farber, M.; Venzano, A.; Jost, B.H.; Parma, Y.R.; Fernandez-Miyakawa, M.E. Sudden death syndrome in adult cows associated with Clostridium perfringens type E. Anaerobe 2013, 20, 1–4. [CrossRef] [PubMed]

11. Songer, J.G. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 1996, 9, 216–234. [CrossRef] [PubMed]

12. Ferrarezi, M.C.; Cardoso, T.C.; Dutra, I.S. Genotyping of Clostridium perfringens isolated from calves with neonatal diarrhea. Anaerobe 2008, 14, 328–331. [CrossRef] [PubMed]

13. Billington, S.J.; Wieckowski, E.U.; Sarker, M.R.; Bueschel, D.; Songer, J.G.; McClane, B.A. Clostridium perfringens type E animal enteritis isolates with highly conserved, silent enterotoxin gene sequences. Infect. Immun. 1998, 66, 4531–4536. [PubMed]

14. Popoff, M.R. Detection of toxigenic Clostridia. In PCR Detection of Microbial Pathogens; Sachse, K., Frey, J., Eds.; Humana Press: Totowa, NJ, USA, 2002; Volume 216, pp. 137–152.

15. Irikura, D.; Momma, C.; Suzuki, Y.; Nakama, A.; Kai, A.; Fukui-Miyazaki, A.; Horiguchi, Y.; Yoshinari, T.; Sugita-Konishi, Y.; Kamata, Y. Identification and Characterization of a New Enterotoxin Produced by Clostridium perfringens Isolated from Food Poisoning Outbreaks. PLoS ONE 2015, 10, e0138183. [CrossRef] [PubMed]

16. Yonogi, S.; Matsuda, S.; Kawai, T.; Yoda, T.; Harada, T.; Kumeda, Y.; Gotoh, K.; Hiyoshi, H.; Nakamura, S.; Kodama, T.; et al. BEC, a novel enterotoxin of Clostridium perfringens found in human clinical isolates from acute gastroenteritis outbreaks. Infect. Immun. 2014, 82, 2390–2399. [CrossRef] [PubMed]

17. Li, J.; Miyamoto, K.; McClane, B.A. Comparison of virulence plasmids among Clostridium perfringens type E isolates. Infect. Immun. 2007, 75, 1811–1819. [CrossRef] [PubMed]

18. Miyamoto, K.; Yumine, N.; Mimura, K.; Nagahama, M.; Li, J.; McClane, B.A.; Akimoto, S. Identification of novel Clostridium perfringens type E isolates that carry an iota toxin plasmid with a functional enterotoxin gene. PLoS ONE 2011, 6, e20376. [CrossRef] [PubMed]

19. Perelle, S.; Gibert, M.; Boquet, P.; Popoff, M.R. Characterization of Clostridium perfringens iota-toxin genes and expression in Escherichia coli. Infect. Immun. 1993, 61, 5147–5156. [PubMed]

20. Toniti, W.; Yoshida, T.; Tsurumura, T.; Irikura, D.; Momma, C.; Kamata, Y.; Tsuge, H. Crystal structure and structure-based mutagenesis of actin-specific ADP-ribosylating toxin CPILE-a as novel enterotoxin. PLoS ONE 2017, 12, e0171278. [CrossRef] [PubMed]

21. Deguchi, A.; Miyamoto, K.; Kuwahara, T.; Miki, Y.; Kaneko, I.; Li, J.; McClane, B.A.; Akimoto, S. Genetic characterization of type A enterotoxigenic Clostridium perfringens strains. PLoS ONE 2009, 4, e5598. [CrossRef] [PubMed]

22. Xiao, Y.; Wagendorp, A.; Moezelaar, R.; Abee, T.; Wells-Bennik, M.H. A wide variety of Clostridium perfringens type A food-borne isolates that carry a chromosomal cpe gene belong to one multilocus sequence typing cluster. Appl. Environ. Microbiol. 2012, 78, 7060–7068. [CrossRef] [PubMed]

23. Gibert, M.; Petit, L.; Raffestin, S.; Okabe, A.; Popoff, M.R. Clostridium perfringens iota-toxin requires activation of both binding and enzymatic components for cytopathic activity. Infect. Immun. 2000, 68, 3848–3853. [CrossRef] [PubMed]

24. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976, 72, 248–254. [CrossRef]

25. Popoff, M.R.; Milward, F.W.; Bancillon, B.; Boquet, P. Purification of the Clostridium spiroforme binary toxin and activity of the toxin on HEp-2 cells. Infect. Immun. 1989, 57, 2462–2469. [PubMed]

26. Perelle, S.; Scalzo, S.; Kochi, S.; Mock, M.; Popoff, M.R. Immunological and functional comparison between Clostridium perfringens iota toxin, C. spiroforme toxin, and anthrax toxins. FEMS Microbiol. Lett. 1997, 146, 117–121. [CrossRef] [PubMed]

27. Mazuet, C.; Leguey, C.; Sautereau, J.; Ma, L.; Bouchier, C.; Bouvet, P.; Popoff, M.R. Diversity of Group I and II Clostridium botulinum strains from France including recently Identified subtypes. Genome Biol. Evol. 2016, 8, 1643–1660. [CrossRef] [PubMed]
28. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A New genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 2012, 19, 455–477. [CrossRef] [PubMed]

29. Seeman, T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 2014, 30, 2068–2069. [CrossRef] [PubMed]

30. Galardini, M.; Biondi, E.G.; Bazzicalupo, M.; Mengoni, A. CONTIGuator: A bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol. Med.* 2011, 6, 11. [CrossRef] [PubMed]

31. Shimizu, T.; Ohtani, K.; Hirakawa, H.; Ohshima, K.; Yamashita, A.; Shiba, T.; Ogasawara, N.; Hattori, M.; Kuhara, S.; Hayashi, H. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* 2002, 99, 996–1001. [CrossRef] [PubMed]

32. Treangen, T.J.; Ondov, B.D.; Koren, S.; Phillippy, A.M. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* 2014, 15, 524. [CrossRef] [PubMed]

33. Nguyen, L.T.; Schmidt, H.A.; von Haeseler, A.; Minh, B.Q. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* 2015, 32, 268–274. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).