



NOTE

Bacteriology

Bacteriological and molecular typing of *Clostridium perfringens* strains isolated in retail beef in Beijing, China

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ABSTRACT. *Clostridium perfringens* is an important zoonotic pathogen. This study was designed to explore the prevalence and toxin types of *C. perfringens* in retail beef collected from Beijing, China. Among 221 beef samples collected, 53 samples were positive for *C. perfringens*, resulting in the average prevalence as 23.98%. By toxin gene-based typing, the most *C. perfringens* strains belong to type A (96.23%, 51/53), only 2 strains were identified as type D. By a multi-locus sequence typing (MLST)-based analysis, a total of 36 sequence types (STs) were detected, and the most STs (n=30) represented just a single strain. These findings suggested that the prevalence of *C. perfringens* in retail beef in Beijing was considerably high and these bacteria displayed extreme diversity in genetics.

KEY WORDS: *Clostridium perfringens*, multi-locus sequence typing, retail beef, toxin type

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Clostridium perfringens is a well-known zoonotic pathogen in the world. It is widely distributed in nature and colonizes in the intestines of various animals, causing necrotizing enteritis, gas gangrene and sudden death [2, 3, 7, 16, 17]. Raw meat or meat products can be easily contaminated by this bacteria during the food processing, and subsequently cause severe illness in consumers [10, 19, 24].

In China, beef consumption displayed a gradual increase in past years, but few studies were conducted to explore how this meat was contaminated by *C. perfringens*. In this study, we conducted an investigation on the prevalence of *C. perfringens* in retail beef sold in Beijing, a metropolis consuming huge amount beef each day. As these beef came from a wide geographic ranges, the data obtained will be helpful to understand the overall prevalence of *C. perfringens* in China.

From 2019 to 2020, we collected a total of 221 fresh beef from 50 supermarkets in Beijing city. In laboratory, about 100 g of each beef sample was cut into small pieces and immersed in 100 ml sterile phosphate buffer saline (PBS) for one hour. The soaked solution was collected and centrifuged at 10,000 rpm for 5 min, then precipitation was transferred to *C. perfringens* selective chromogenic culture media plate (CHROMagar, Paris, France) and incubated in anaerobic jar with AnaeroGen (Oxoid Ltd., Basingstoke, UK) for 48 hr at 37°C. When colonies appeared on plate, 1–3 typical colonies in orange color were transferred to *C. perfringens* agar base (Oxoid Ltd.) supplemented with SFP supplement (Oxoid Ltd.) for pure culture. Bacterial culture on the agar plate was washed off with sterile PBS solution and genomic DNA were extracted for species confirmation by PCR [23]. For each sample, only one isolate was used for following toxin gene-based typing and molecular typing analysis, according to the previously published PCR method [15, 20].

By bacterial culture, 53 samples were identified as positive for *C. perfringens*, leading to overall prevalence as 23.98% (Table 1). The beef marketing in Beijing came from four provinces, including Shandong, Hebei, Inner Mongolia and Shanxi. Relatively higher prevalence was found in the beef produced in Inner Mongolia (21/76, 27.63%), and the lowest prevalence was identified in the beef originated from Hebei province (14/72, 19.44%) (Fig. 1).

By toxin gene-based typing, 53 typical *C. perfringens* strains were characterized into two toxigenic types, A and D (Supplementary Table 1). Type A was the main toxin type comprising of 51 strains (96.23%), which means that the majority *C. perfringens* strains harbor only *cpa* gene (producing alpha toxin). The remaining 2 strains (3.77%) were identified as type D, as

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Table 1. Prevalence of *Clostridium perfringens* in retail beef with different geographic sources

Geographic source	No. of samples collected	No. of samples positive for <i>C. perfringens</i>	Prevalence (%)	No. of type A <i>C. perfringens</i>	No. of type D <i>C. perfringens</i>
Shandong	65	16	24.62	16	0
Hebei	72	14	19.44	14	0
Inner mongolia	76	21	27.63	19	2
Shanxi	8	2	25	2	0
Total	221	53	23.98	51	2

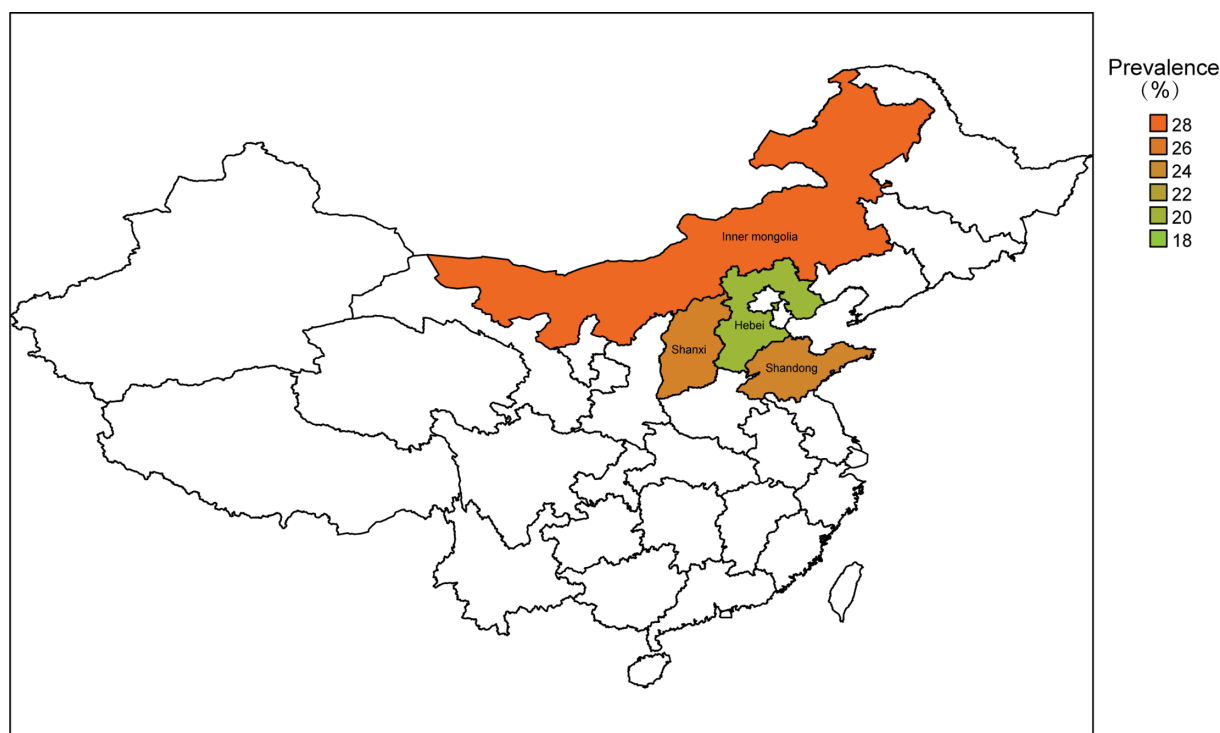


Fig. 1. Producing areas and prevalence of *Clostridium perfringens*-positive beef collected in this study.

cpa and *etx* genes (epsilon toxin) were detected in both strains. The *cpb2* gene, which is responsible for production of beta 2 toxin (CPA2), was detected in 11 strains (20.75%), including two type D strains.

We used the multilocus sequence typing (MLST) scheme described by Jost *et al.* and Chalmers *et al.* to carry out phylogenetic analysis on these *C. perfringens* strains [3, 12]. Briefly, DNA fragments of eight housekeeping genes, including *plc* (*cpa*), *ddlA*, *dut*, *glpK*, *gmk*, *recA*, *sod* and *tpiA*, were amplified and sequenced at Sangon Biotech Co. (Sangon Biotech Co., Ltd., Shanghai, China). Eight sequences of each strain were analyzed and the allele numbers were assigned for different allelic sequences, then the sequence type (ST) was assigned for each strain using BioNumerics 8.0 software (Applied Maths, Keijkstraat, Belgium). Polymorphism of each locus were quantified using the Hunter & Gaston diversity index (HGDI) [9]. The minimum spanning tree (MST) was constructed using the MST method in BioNumerics 8.0 software. Clonal complexes were defined as groups of independent isolates that shared identical alleles at seven or more of the eight loci. The sequences determined for the MLST analysis have been deposited in the GenBank database under accession numbers MZ725945-MZ726368.

Among 53 *C. perfringens* strains, MLST typing identified 36 unique STs (ST1-36) (Supplementary Table 1). The ST14 contained the largest number of strains (n=8), 5 of them were originated from Inner Mongolia and 3 from Hebei province. The other frequently identified sequence type was ST32, containing 7 strains universally originated from Shandong province. Four STs (ST1, 16, 20 and 28) were comprised of just a couple of strains, while the remaining 30 STs were identified as singleton. High diversity was observed in the eight loci, with the HGDI value arranging from 0.660 to 0.921 (Table 2). The average number of alleles for all loci was 17. The loci *plc* and *dut* contained the largest number of alleles (n=22), while the minimum alleles was observed in *recA* and *gmk* (n=12). Unlike other loci, where the trimmed sequence from all the strains show an identical length, two different length fragments were detected in the *dut* gene (441 bp and 444 bp), with 3 alleles possessing a 3-bp internal insertion.

MST analysis clustered 14 of 36 STs into 6 clonal complex (CC) subtypes, CC1 to CC6 (Fig. 2), the strains contained in the same CC were considered to be more closely related in genetics than other strains. The main subtype CC1 contained four STs

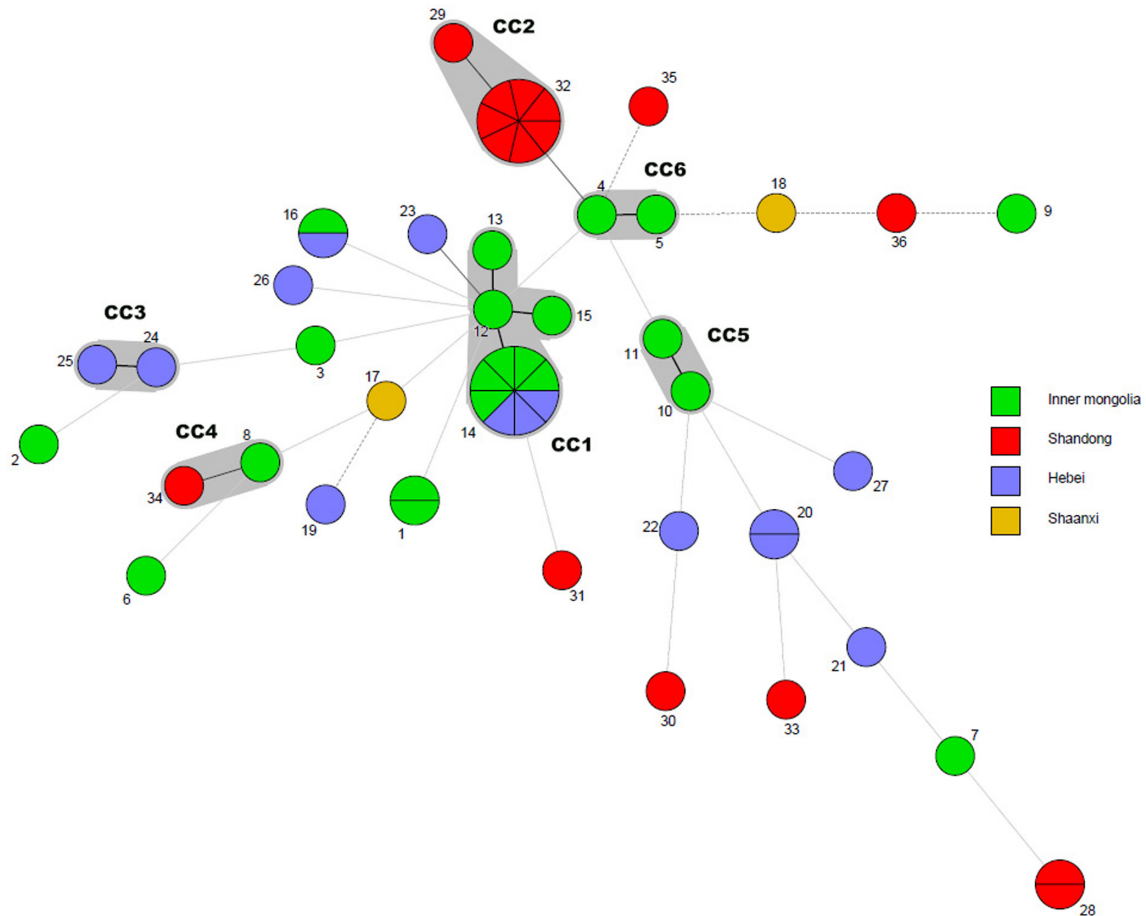


Fig. 2. Minimal spanning tree constructed with the 53 *Clostridium perfringens* strains originated from retail beef in Beijing (2019–2020). Each circle represents a specific sequence type (ST), the strains sharing the same ST were included in the same circle. Different colors represent different geographic origins of these *C. perfringens* strains. The length between circles represents the genetic distance. The circles in shadow parts represents a clonal complex (CC), defined as group of independent strains that share identical alleles at seven or more of the eight loci. A total of six CCs (CC1–CC6) were identified among these group of strains. The strains representing ST 4 and 5 belong to type D *C. perfringens*. The 11 strains representing single ST respectively (including ST 2, 3, 4–7, 17, 18, 20, 21 and 26) harbors *cpb2* in their genomes.

Table 2. Genes, number of alleles and Hunter & Gaston diversity index (HGDI) values of *Clostridium perfringens* strains obtained in Beijing (2019–2020)

Gene	Length (bp)	No. of alleles	HGDI
<i>ddlA</i>	429	18	0.854
<i>dut</i>	441 or 444	22	0.921
<i>glpK</i>	574	20	0.894
<i>gmK</i>	475	12	0.845
<i>plc</i>	544	22	0.794
<i>recA</i>	475	12	0.66
<i>sod</i>	478	17	0.697
<i>tpi</i>	451	13	0.844

(consisting of 11 strains), while the other subtypes (CC2–CC6) just contained two STs. In phylogenetic tree, there was no clear evidence that strains with the same geographic source likely clustered together. The two type D strains (S05 and S06) showed the closest genetic relationship and formed the CC6 in MST. While, the 11 strains harboring *cpb2* gene almost evenly distributed across the MST tree (Supplementary Table 1, Fig. 2).

According to the data revealed in this study, the prevalence of *C. perfringens* in beef was around 24% in north area of China. This data is higher than the findings obtained in other countries. For example, in Seoul, Korea, the prevalence of *C. perfringens* in retail beef was below 16.67% [11]. While in Lahore city of Pakistan, prevalence of this organism in beef was no more than 1% [13]. In these beef-derived *C. perfringens*, type A was the most frequently observed toxin type. Although less frequently observed, *etx*-positive *C. perfringens* strains should be paid more attention as it can cause

severe respiratory and neurologic signs in cattle [6]. On the other hand, *cpb2* positive strains was also be found in apparently healthy animals [1, 21, 22], and its role in enteric disease of domestic animals and human needs to be further investigated.

Molecular typing schemes like MLST have been developed with the aim to associate *C. perfringens* strains to a specific disease presentation, toxin pattern or a specific host [3, 4, 8, 12, 18, 25]. However, in this study, no evidence was provided that a specific CC or ST could be highly associated with any toxin type, host sources or collection date. The lack of relevance between the phylogenetic features and biological properties may be due to the extremely open genome and genetic diversity observed in *C.*

perfringens species [5, 14]. In the future, more conservative housing keeping genes even the whole genome based sequence should be used for correlation analysis between phylogenetic features and biological properties.

CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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REFERENCES

- Allaart, J. G., van Asten, A. J., Vernooij, J. C. and Gröne, A. 2014. Beta2 toxin is not involved in in vitro cell cytotoxicity caused by human and porcine cpb2-harboring *Clostridium perfringens*. *Vet. Microbiol.* **171**: 132–138. [Medline] [CrossRef]
- Athira, C. K., Milton, A. A. P., Reddy, A., Mekhemadhom Rajendrakumar, A., Abhishek., Verma, M. R., Kumar, A., Nagaleekar, V. K. and Agarwal, R. K. 2018. Diversity of toxin-genotypes among *Clostridium perfringens* isolated from healthy and diarrheic neonatal cattle and buffalo calves. *Anaerobe* **49**: 99–102. [Medline] [CrossRef]
- Chalmers, G., Bruce, H. L., Hunter, D. B., Parreira, V. R., Kulkarni, R. R., Jiang, Y. F., Prescott, J. F. and Boerlin, P. 2008. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. *J. Clin. Microbiol.* **46**: 3957–3964. [Medline] [CrossRef]
- Deguchi, A., Miyamoto, K., Kuwahara, T., Miki, Y., Kaneko, I., Li, J., McClane, B. A. and Akimoto, S. 2009. Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. *PLoS One* **4**: e5598. [Medline] [CrossRef]
- Feng, Y., Fan, X., Zhu, L., Yang, X., Liu, Y., Gao, S., Jin, X., Liu, D., Ding, J., Guo, Y. and Hu, Y. 2020. Phylogenetic and genomic analysis reveals high genomic openness and genetic diversity of *Clostridium perfringens*. *Microb. Genom.* **6**: 10. [Medline]
- Filho, E. J., Carvalho, A. U., Assis, R. A., Lobato, F. F., Rachid, M. A., Carvalho, A. A., Ferreira, P. M., Nascimento, R. A., Fernandes, A. A., Vidal, J. E. and Uzal, F. A. 2009. Clinicopathologic features of experimental *Clostridium perfringens* type D enterotoxemia in cattle. *Vet. Pathol.* **46**: 1213–1220. [Medline] [CrossRef]
- Fohler, S., Klein, G., Hoedemaker, M., Scheu, T., Seyboldt, C., Campe, A., Jensen, K. C. and Abdulmawjood, A. 2016. Diversity of *Clostridium perfringens* toxin-genotypes from dairy farms. *BMC Microbiol.* **16**: 199. [Medline] [CrossRef]
- Hibberd, M. C., Neumann, A. P., Rehberger, T. G. and Siragusa, G. R. 2011. Multilocus sequence typing subtypes of poultry *Clostridium perfringens* isolates demonstrate disease niche partitioning. *J. Clin. Microbiol.* **49**: 1556–1567. [Medline] [CrossRef]
- Hunter, P. R. and Gaston, M. A. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* **26**: 2465–2466. [Medline] [CrossRef]
- Jang, Y. S., Kim, D. H., Bae, D., Kim, S. H., Kim, H., Moon, J. S., Song, K. Y., Chon, J. W. and Seo, K. H. 2020. Prevalence, toxin-typing, and antimicrobial susceptibility of *Clostridium perfringens* from retail meats in Seoul, Korea. *Anaerobe* **64**: 102235. [Medline] [CrossRef]
- Jeong, D., Kim, D. H., Kang, I. B., Chon, J. W., Kim, H., Om, A. S., Lee, J. Y., Moon, J. S., Oh, D. H. and Seo, K. H. 2017. Prevalence and toxin type of *Clostridium perfringens* in beef from four different types of meat markets in Seoul, Korea. *Food Sci. Biotechnol.* **26**: 545–548. [Medline] [CrossRef]
- Jost, B. H., Trinh, H. T. and Songer, J. G. 2006. Clonal relationships among *Clostridium perfringens* of porcine origin as determined by multilocus sequence typing. *Vet. Microbiol.* **116**: 158–165. [Medline] [CrossRef]
- Khan, M., Nazir, J., Anjum, A. A., Ahmad, M. U., Nawaz, M. and Shabbir, M. Z. 2015. Toxinotyping and antimicrobial susceptibility of enterotoxigenic *Clostridium perfringens* isolates from mutton, beef and chicken meat. *J. Food Sci. Technol.* **52**: 5323–5328. [Medline] [CrossRef]
- Lacey, J. A., Johansen, P. A., Lyras, D. and Moore, R. J. 2016. Genomic diversity of necrotic enteritis-associated strains of *Clostridium perfringens*: a review. *Avian Pathol.* **45**: 302–307. [Medline] [CrossRef]
- Lebrun, M., Filée, P., Mousset, B., Desmecht, D., Galleni, M., Mainil, J. G. and Linden, A. 2007. The expression of *Clostridium perfringens* consensus beta2 toxin is associated with bovine enterotoxaemia syndrome. *Vet. Microbiol.* **120**: 151–157. [Medline] [CrossRef]
- Liu, Y., Xiu, L., Miao, Z. and Wang, H. 2020. Occurrence and multilocus sequence typing of *Clostridium perfringens* isolated from retail duck products in Tai'an region, China. *Anaerobe* **62**: 102102. [Medline] [CrossRef]
- Milton, A. A. P., Sanjukta, R., Gogoi, A. P., Momin, K. M., Priya, G. B., Das, S., Ghatak, S., Sen, A. and Kandpal, B. K. 2020. Prevalence, molecular typing and antibiotic resistance of *Clostridium perfringens* in free range ducks in Northeast India. *Anaerobe* **64**: 102242. [Medline] [CrossRef]
- Nakano, V., Ignacio, A., Llanco, L., Bueris, V., Sircili, M. P. and Avila-Campos, M. J. 2017. Multilocus sequence typing analyses of *Clostridium perfringens* type A strains harboring tpeL and netB genes. *Anaerobe* **44**: 99–105. [Medline] [CrossRef]
- Regan, S. B., Anwar, Z., Mirafior, P., Williams, L. B., Shetty, S., Sepulveda, J., Moreh, J., Bogdanov, S., Haigh, S., Lustig, A., Gaehde, S., Vartanian, A., Rubin, N. and Linden, J. R. 2018. Identification of epsilon toxin-producing *Clostridium perfringens* strains in American retail food. *Anaerobe* **54**: 124–127. [Medline] [CrossRef]
- Rood, J. I., Adams, V., Lacey, J., Lyras, D., McClane, B. A., Melville, S. B., Moore, R. J., Popoff, M. R., Sarker, M. R., Songer, J. G., Uzal, F. A. and Van Immerseel, F. 2018. Expansion of the *Clostridium perfringens* toxin-based typing scheme. *Anaerobe* **53**: 5–10. [Medline] [CrossRef]
- Uzal, F. A., Vidal, J. E., McClane, B. A. and Gurjar, A. A. 2010. *Clostridium Perfringens* Toxins Involved in Mammalian Veterinary Diseases. *Open Toxicology J.* **2**: 24–42. [Medline] [CrossRef]
- van Asten, A. J., Nikolou, G. N. and Gröne, A. 2010. The occurrence of cpb2-toxigenic *Clostridium perfringens* and the possible role of the beta2-toxin in enteric disease of domestic animals, wild animals and humans. *Vet. J.* **183**: 135–140. [Medline] [CrossRef]
- Wang, R. F., Cao, W. W., Franklin, W., Campbell, W. and Cerniglia, C. E. 1994. A 16S rDNA-based PCR method for rapid and specific detection of *Clostridium perfringens* in food. *Mol. Cell. Probes* **8**: 131–137. [Medline] [CrossRef]
- Wen, Q. and McClane, B. A. 2004. Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Appl. Environ. Microbiol.* **70**: 2685–2691. [Medline] [CrossRef]
- Xiao, Y., Wagendorp, A., Moezelaar, R., Abee, T. and 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. *Appl. Environ. Microbiol.* **78**: 7060–7068. [Medline] [CrossRef]