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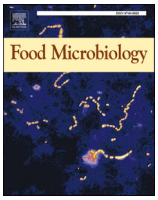

Tracing *Clostridium perfringens* strains from beef processing of slaughter house by pulsed-field gel electrophoresis, and the distribution and toxinotype of isolates in Shaanxi province, China

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ABSTRACT

The purpose of this study was to investigate the distribution and specify the transmission and cross-contamination of *Clostridium perfringens* (*C. perfringens*) in the beef slaughtering and butchering process. The prevalence of 21.2% (150/708) yielded 208 isolates of *C. perfringens*, including 80.8% type A and 19.2% type D, 0.4% (3/708) samples carried both type A and D strains, and 72.5% type D isolates carried both *cpe* and *atyp.cpb2* genes. *C. perfringens* were identified through the whole slaughtering process but no type F (*cpe* and *cpa* isolates) was found. 69 isolates were further analyzed and classified into 28 PFGE genotypes and clade I contained 94.2% isolates and 24 PFGE genotypes, which showed the genetic diversity and epidemic correlation. Our study traced *C. perfringens* contamination along the handling processes and showed a gradually ascending contamination rate during the whole process, revealing widespread cross-contamination from the feces and hides of slaughtered cattle to the carcass in the slaughtering workshop, so as from tools and personnel to meat of the cutting workshops. Strains from different slaughterhouses (regions) have high homology, and type A is the predominant toxinotype. It is necessary to monitor and control several key points of cross-contamination during slaughtering process to reduce a risk of *C. perfringens* infection.

1. Introduction

Clostridium perfringens (*C. perfringens*) is an important anaerobic pathogen causing food-borne gastrointestinal (GI) diseases in humans and animals. An estimated 9.4 million (90% CrI (credible intervals): 6.6 to 12.7 million) cases of foodborne disease occur each year and 10% of food poisoning cases in the United States have been found to be the result of *C. perfringens* which produced the *C. perfringens* enterotoxin (CPE) (Scallan et al., 2011); Foodborne *C. perfringens* is estimated to cause 25 deaths per year (95% CrI 1 to 163) in UK (Holland et al., 2020). Foodborne transmission is the main route for *C. perfringens* (98%, CrI: 84–100) (Vally et al., 2014). Outbreaks primarily involve high protein foods of animal origin such as meat, meat products and dishes, among which, meat and meat products have been reported as the most common food vehicles (Nasr et al., 2007; Miki et al., 2008; Uzal et al., 2014; Ghoneim and Hamza, 2017). The microbial contamination of meat and meat products is affected by raising, slaughtering process, transportation and retails, but contamination at slaughter is the initial stage that affects the subsequent processing and contamination of meat before refrigeration and sale (Sun and Peng, 2012). Contamination of carcasses and meat occurs largely through contamination with the intestinal or fecal contents of the slaughtered animals which serve as an important source to the food supply(Ghoneim and Hamza, 2017).

C.perfringens is a Gram-positive, non-motile, sporulated rod that is ubiquitously present in the environment and normal intestinal flora of humans and animals. It is the causative agent of many histotoxic and enterotoxic diseases in humans and animals (Uzal et al., 2014). *C. perfringens* strains are classified into seven types (A–G) on the basis of their ability to produce the typing toxins, including CPA, CPB, ETX, IA, CPE and NetB (*C. perfringens* necrotic enteritis B-like toxin) (Rood et al., 2018). Identifying *C. perfringens* toxinotypes by the presence of these toxin genes is critical for understanding the pathogenesis and epidemiology. Whilst CPA is produced by all 7 toxinotypes, CPE which is responsible for the *C. perfringens*-mediated human food poisoning and

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antibiotic-associated diarrhea (AAD), is encoded by *cpe* gene produced in type F isolates, although some type C, D and E isolates also encode *cpe* (Rood et al., 2018). The CPB2 toxin may play a role in cases of AAD and sporadic non-foodborne diarrhea (SD) originating from *cpe*-positive *C. perfringens* (Fisher et al., 2005; Lindström et al., 2011). This toxin, exists in a typical and an atypical form, encoded by *consensus cpb2* (*cons. cpb2*) and *atypical cpb2* (*atyp. cpb2*), respectively. The pathogenicity of *atyp. cpb2* containing strains is not clear, while *cons. cpb2* gene is widely found in cattle with enterotoxemia especially in calves (Fohler et al., 2016). For the presence of the *cpb2* and the *cpe* gene, broad differences have been reported for cattle (Bueschel et al., 2003; Gurjar et al., 2008; Kukier and Kwiatek, 2010). In one previous study reporting prevalence of *C. perfringens* isolated from beef produced in cattle slaughtering sites (52.9%) and market retail beef (69.2%), respectively, only 1 isolate was *cpe*-positive *C. perfringens* (Ghoneim and Hamza, 2017), indicating the prevention and control of microorganisms is of crucial importance for the beef slaughterhouse.

Pulsed field gel electrophoresis (PFGE) technology is widely applied in detection of foodborne pathogenic bacteria and in tracing the source and relationship of the bacteria (Tenover et al., 1995). It is the internationally used method of molecular biology typing (Li et al., 2009; Park et al., 2016), although increasingly being replaced by whole genome sequencing.

In China, random sampling is used to evaluate microbial contamination in beef slaughter processing lines, using detection of aerobic plate count and total coliform counts (Wang et al., 2010), as well as detection of foodborne pathogens including *Salmonella* and *Escherichia coli* (Dong et al., 2019, 2020). However, there are few published data on the prevalence of *C. perfringens* and the transmission routes during cattle slaughtering and meat processing. In this study, samples were collected from three slaughterhouses from central of Shaanxi Province to determine the extent of contamination with and spread of *C.perfringens* during processing. This shows the potential risk posed by *C. perfringens* foodborne disease originating from cattle, and provides scientific data to control the contamination of *C.perfringens*.

2. Material and methods

2.1. Bacteria strains and primers

C. perfringens reference strain CVCC49 (type A, *cpa*, *atyp.cpb2* positive), CVCC54 (type B, *cpa*, *cpb*, *etx* positive) and *C.perfringens* CVCC90 (Type E, *cpa*, *etx*, *cpe* positive) were obtained from the Centre for Veterinary Culture Collection (CVCC) in Beijing, China and saved by food safety and public health laboratory of College of Veterinary Medicine of Northwest A&F University (Yangling, Shaanxi, China). *Salmonella* serotype Braenderup H9812 was kindly provided by Professor Baowei Yang of College of Food Science and Engineering of Northwest A&F University. All primers used in this study are listed in Table 1.

2.2. Sampling and sample preparation

From November 2018 to August 2019, a total of 708 samples from the cattle slaughtering process were collected from 3 slaughter houses comprising 2 small-scale traditional (S2, S3) and 1 larger-scale (S1) with advanced production line equipment located in 3 different districts in Shaanxi Province (Table 2). Feces sampling: Approximately 100 g of dung-lock (dried feces/mud/hair) sample was removed aseptically from the floor where cattle to be slaughtered were waiting in the holding pens, then placed in sterile container. Fecal swabs were obtained using a sterile swab that was inserted 3–4 cm into the rectum and gently rotated and rubbed against the inner wall of the rectum and then put in sterile screw-capped tubes containing 9 mL Fluid thioglycolate (FTG) (Aobox, Beijing, China) medium. Air sampling was conducted by passive exposure (GB18204.3–2013, China) of 5% sheep blood nutrient agar plates (BAP) to air for 5 min–10 min in each of five locations in the slaughterhouses (1 h after the shift started), including air from (i) slaughter workshop, (ii) carcass drip coolers and (iii) the cutting workshop were collected. At each visit to the slaughterhouses, 5–10 carcasses were randomly selected and the same carcass was sampled at 6 steps of the slaughtering/chilling/cutting process and at three different sites on the carcass (Table 2). The sampling sites and methods were modified as described (Sofos et al., 1999; Camargo et al., 2019). Each delimited area was rubbed with a sterile swab moistened with sterile saline (0.85% w/v) against the muscle tissue surface by using two sterile square plastic templates of 100 cm^2 (10 cm × 10 cm) and then put in sterile screw-capped tubes containing 9 mL FTG medium. About 100 g beef meat aseptically removed from each sampling site at each location of the carcass (after the final carcass washing and at the time of dividing into different meat cuts) in the plant by use of forceps and scalpel, and samples were then placed in a sterile container. Rinse water and waste water were also collected 50 mL of each sample into sterilized 50 mL centrifuge tube. Environmental/facilities/equipment swab samples were collected from the slaughter/cutting line and from personnel, with

Table 1
PCR primers used in this study.

Primer	Primer Sequence (5′–3′)	Length/bp	References
*cpa*1	ATGAGCTTCAATTAGGTTCTACT ATCAGCATAAAAATCCTCATT	398	Dong et al. (2013)
*cpa*2	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	325	
cpb	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAG	236	
etx	AGTATCTAATGAAATGTCCATTCC ACTTACTTGTCCTAC	585	
ia	ACTACTCTCAGACAAGACAG TTTCCTTCTATTACTATACG	445	
*atyp. cpb*2	ATTATGTTTAGGAATACAGTTA CAATACCCTTCACCAAATACTC	741	Nowell et al. (2010)
*cons. cpb*2	CAATTGGGGGAGTTTATCCACAA CAATACCCTTCACCAAATACTC	304	
cpe	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233	

Table 2
Sampling details from 3 slaughterhouses.

Process	Category of samples	No. of samples				
		S1 (Jan.)	S1 (Sep.)	S2 (Jan.)	S3 (Mar.)	Total
Slaughtering	Feces	18	6	6	18	48
	Hide	10	6	22	29	67
	after head removal	6	6	4	3	19
	after hide removal	22	15	10	56	103
	after end washing	33	6	22	–	61
	Air	5	5	5	9	24
	waste water	–	16	3	6	25
	rinse water	3	–	3	3	9
	Total	97	60	75	124	356
Chilling	halves carcass	–	–	5	–	5
	Air	2	–	4	–	6
	Total	2	–	9	–	11
Cutting	meat sample	8	15	37	105	165
	operating floor	5	2	12	31	50
	Conveyor	3	–	3	4	10
	tools and personnel	8	20	24	42	94
	Air	3	5	4	10	22
	Total	27	42	80	192	341
Summary		126	102	164	316	708

Note: :Not sampled.

samples including knives, saws, apron, gloves, clothing, conveyor belt and the operating floor, then put in FTG medium same as the other swab samples. All the samples were transported to the lab at 4 °C for processing in the same day.

2.3. Isolation and characterization of C. perfringens

To isolate *C. perfringens*, a 25 g portion of each beef sample was aseptically placed in a sterile stomacher bag containing 225 mL of FTG medium, then homogenized by stomacher for 1–2 min and incubated at 37 °C for 24 h or 48 h. Fecal samples were weighed (Sartorius, Beijing, China) and water samples were pipetted aseptically then added into sterile screw-capped tubes containing 9 mL of FTG medium at a ratio of 1:9 (w/v), well mixed then incubated at 37 °C for 24 h, all the swabs samples inside FTG medium directly incubated at 37 °C. Also placed the air samples' BAP under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) (BugBox, Ruskinn Technology Limited, Bridgend, UK) at 37 °C for 24 h. After the samples were enriched in FTG, one loopful of 24 h enriched culture and suspect characteristic double-zone hemolysis single colony (medium-sized, bright, round) on BAP microscopic examined by Gram's staining, then detected *cpa* gene via PCR, primers used *cpa1* (Table 1) and the length of amplicon is 398 bp. Appropriate negative and positive controls were included in each PCR assay (Fig. 6). Further, the *cpa* positive of enriched sample culture and colonies was streaked onto Trytose Sulfite-Cyloserine (TSC) (Hopebio, Qingdao, China) agar plates and incubated at 37 °C for 1–2 days in an anaerobic condition. Then choose the single typical black colonies on TSC plates and re-streaked onto TSC agar plates, incubated anaerobically at 37 °Cfor 24–36 h to purify the isolates. Further, colonies compatible with *C. perfringens* from TSC agar plates were subcultured in FTG medium, respectively. Gram staining and biochemical tests (lactose fermentation and motility test) were carried out to identify the isolates as described by *C. perfringens* analysis of National Food Safety Standard of PRC (GB4789.13–2012, China). Where available, 1–3 isolates were characterized from each sample. Isolates were stored at −80 °C in Lyophilization medium with 10% powdered skim milk, 7.5% glucose, 10% sucrose and 1% bovine plasma albumin in distilled water until further use.

2.4. Toxin-genotyping by PCR

All the confirmed and purified frozen isolates from the above step were re-streaked onto TSC plate, incubated anaerobically at 37°Cfor 24–36 h and then typical black single colonies were used as the PCR template. Multiplex PCR assay for targeting the four major toxin genes, *cpa*, *cpb*, *etx*, and *ia* with modification as described by Meer and Songer (1997) and others (Dong et al., 2013), while *cpe* and *cpb2* toxin genes were detected with single PCR reactions (Nowell et al., 2010) for genotyping. Reference strains used as positive control in the multiple PCR reactions were *C. perfringens* CVCC54 and CVCC 90, CVCC49 was used as a control for *atyp.cpb2*, JC15 (*C.perfringens* isolated from chicken meat) for *cons. cpb2* and CVCC90 for *cpe* gene detection. Primers pairs modified and synthesized by GenScript (Nanjing, China) were used as shown in Table 1. The multiplex PCR was performed in a S1000™ thermoCycler (Bio-Rad, USA) described previous (Dong et al., 2013). Each single PCR assay had a final volume of 25 μL, 10 × PCR buffer 2.5 μL, 25 mmol/L $MgCl_2$ 2.0 μL, 2.5 mmol/L dNTPs (Thermo Scientific, USA)1.5 μL, 20 nmol/L of each forward and reverse primer pairs of the following genes (*atyp. cpb2* and *cons. cpb2, cpe*) 0.5 μL, respectively, 1 single colony of isolate as template, step added 1.5 U *Taq* DNA polymerase after the same initial denaturating step as the multiplex PCR. Amplification was obtained with 30 cycles of 94°Cfor 30 s, 48–60 °C for 30 s, 72 °C for 60 s with a 10 min extension at 72 °C. Appropriate negative and positive controls were included in each PCR assay.10 μL aliquot of each amplicon and molecular weight DL 2000 marker (Fermentas, Lithuania) were subjected to electrophoresis in 2% agarose gels (Biowest, Spain) and stained with ethidium bromide, then amplified DNA fragments of specific sizes were visualized under UV illumination (Syngene, Synoptics Group, UK).

2.5. Pulsed field gel electrophoresis

PFGE was performed of the *C. perfringens* isolates according to the procedure described previous (Maslanka et al., 1999) with a few modifications. Strains of *C. perfringens* were inoculated in BHI at 37 °C for 24 h under anaerobic conditions. The OD_{620} was measured and the quantity of each sample was calculated: $1.5/OD_{620} = x$ mL that was used further for DNA preparation. The cultures were centrifuged at 13,000 rpm for 2 min at 4 °C and resuspended in CSB wash buffer (0.1 M Tris-HCl, 0.1 M EDTA, pH 8.0). After the third wash pellets were resuspended in 400 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with adding 10 μL lysozyme (1 mg/mL) (Activity, USA) and 25 μL proteinase K (20 mg/mL) (Takara Bio Inc, China) before being embedded in an equal volume of 1.9% melted SeaKem Gold Agarose (Lonza, Rockland, USA) (kept at 56–60 °C), the mixed samples were allowed to solidify at 4 °C in rectangular moulds. The solid plugs were incubated overnight in 5 mL of CLB (6 mM Tris HCl, 100 mM EDTA, 1 M NaCl, 5 g/L Brij 50, 2 g/L sodium deoxycholate, 5 g/L sodium launylsarcosine) with 10 μL lysozyme and 20 μL proteinase K at 37 °C with shaking at 150 rpm. Plugs were incubated twice in 15 mL ddH_2O at 50 °Cfor 20 min and then rinsed three times with 10 mL TE buffer and then store at 4 °C. The plugs were digested in restriction buffer with 40 units of *Sma* I (Takara Bio Inc, Japan) at waterbath for 30 °C 3 h, then cut to size with a razor blade and each was placed on a tooth of the comb. Electrophoresis was carried out in 1% of SeaKem Gold agarose gels prepared in 0.5 × TBE electrophoresis buffer. The digested DNA was separated by using a CHEF Mapper XA (Bio-Rad, USA) at 14°Cwith included angle was 120° and the run time was 20 h with a voltage of 6 V per cm and a linearly ramped pulse time of 0.5–40 s. The gels were stained with Gelred (Biotium, USA) for 40 min then visualized on the Gel Doc XR (Bio-Rad, USA), the images were saved as TIF files. *Salmonella* serotype Braenderup H9812 (restricted by *Xba* l) as the DNA marker performed by the same procedure. Banding patterns and dendrograms were created by determining the distance matrices and using an unweighted pair group method with arithmetic mean clustering method (UPGMA). Subtyping patterns were analyzed by using BioNumerics software (ver. 7; Applied Maths, USA). Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0%) for the construction of dendrograms. Isolates with <90% similarity according to the dendrogram were clustered as separate genotypes, and with >50% similarity present in the pattern were considered to be epidemiological related (Tenover et al., 1995).

2.6. Statistical analysis

Significance of difference among samples from different procedure in the slaughtering process in the same slaughterhouse for the prevalence of *C. perfringens* were analyzed with Tukey test using SPSS statistical software (Ver.21.0 for windows, SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Prevalence of C.perfringens from beef slaughtering process

A total of 708 samples were collected from 3 slaughterhouses (S1–S3), which included 356 samples from slaughter workshop, 11 samples from chilling workshop and 341 samples from the cutting workshop (Table 2). A positive sample prevalence of 21.2% (150/708) yielded 208 isolates of *C. perfringens* (Fig. 1, Fig. 4, Table 4), and the positive percentage of *C. perfringens* from each sample source ranged from 0 to 83.0% (Fig. 4, Table 4). During the beef slaughtering and butchering processes, the prevalence of *C. perfringens* increased gradually, and the *C. perfringens* isolation rate of meat samples from cutting was significantly higher than the rate of carcass samples from chilling

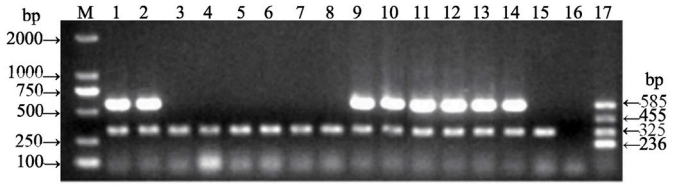

Fig. 1. Results of multiplex PCR detection of partial *C.perfringens* isolates. M.DL 2000 DNA Marker, strips from top to bottom reprsent 2 000 bp,1 000 bp, 750 bp, 500 bp, 250 bp, 100 bp; 16. Negative control (ddH_2O); 17. Positive control, CVCC54 (type B) +CVCC90 (type E) as templates, the amplicons strips from top to bottom represent *etx* (585 bp), *ia* (445 bp), *cpa* (325 bp) and *cpb* (236 bp), respectively; 1–2,9-14: the isolates carried both *cpa* and *etx* genes which were type D strain; 3–8,15: isolates only carried *cpa* gene which were type A strain.

workshop and slaughtering workshop ($p < 0.05$) (Fig. 4, Table 4). At the slaughtering step, the average isolation rate of *C. perfringens* from feces and hide are 22.9% and 14.9%, respectively, both higher than that of samples after head and hide removal (10.5% and 11.7%, respectively). After carcass exposure and ending washing, the isolation rate of the carcass sample doubled and rose to around 23.0%, showing a clear contamination of the meat at this step. Accompany with that, the *C. perfringens* isolation rates from air and waste water are relatively high (33.3% and 40.0%, respectively), indicating air polluted by feces and/or hide in the workshop could be an important source of *C. perfringens* contamination at the slaughtering step (Fig. 4, Table 4).

At the chilling step, the prevalence of *C. perfringens* of halves carcass was slightly decreased to 20.0% but without statistical difference in comparison to carcasses after ending washing before entry into the cooler (Fig. 4, Table 4). At the meat cutting step, the prevalence of *C. perfringens* of meat samples was significantly higher than swabs from the operating table/conveyor/tool and personnel/air ($p < 0.05$) while there was no statistical difference within the later groups ($p > 0.05$) (Fig. 4, Table 4), suggesting the beef can be cross-contaminated with *C. perfringens* between carcasses mediated by the operating table/conveyor/tool and personnel in the meat cutting workshop.

The *C. perfringens* isolation rate of air samples from slaughtering workshop was extremely different with samples from chilling and cutting ($p = 0$), implying that the contamination was unrelated to the air in chilling and cutting workshop because of the low temperature inside. There was no statistical difference among the *C. perfringens* prevalence of air from slaughtering workshop and feces, hide surface ($p > 0.05$) (Fig. 4, Table 4), which indicated the *C. perfringens* contamination in slaughtering workshop may result from the hide and rectum contents during the hide removal process. The prevalence of *C. perfringens* of samples collected in September from S1 was significantly higher than samples collected in January and March from all 3 slaughterhouses ($p < 0.05$) (Fig. 4, Table 4). Unlike in the slaughtering workshops, no statistical difference among the positive rate of cutting workshop from the 3 slaughterhouses was observed. It seems that the contamination of *C. perfringens* is related to the environmental temperature. Surprisingly, there was no significant difference between the isolation rate of *C. perfringens* from traditional mode (S2 and S3) and that of large-scale with advanced production line equipment slaughtering and processing mode (S1) although the isolation rate of *C. perfringens* from cutting workshop of S2 is slightly higher than S1.

3.2. Toxin-genotyping of C. perfringens isolates

All 208 *C. perfringens* isolates were confirmed by colony multiplex PCR combined with single PCR for *cpe* gene (Fig. 1 and 3), and the results demonstrated that all isolates possessed the *cpa* gene, among which 80.8% (168/208) isolates were identified as type A and the rest 19.2% (40/208) possessing both *cpa* and *etx* genes were identified as type D (Table 3). In the collected samples, only 0.4% (3/708) carried both type A and D strains. According the results of PCR detecting *cpb2 atyp.* and *cons.*, in total 143 isolates carried *atyp*. *cpb2* gene. Among that, 110 of 168 (65.5%) type A isolates carried both the *cpa* and *atyp*. *cpb2* gene (type Aβ2a) (Fig. 2, Tables 3 and 5) and the 33 out of 40 (82.5%) type D isolates carried *atyp*. *cpb2* gene (type Dβ2a) (Tables 3 and 5). Totally 29 out of 208 (13.9%) isolates carried *cpe* inside type D (type Dβ2ae) (Table 3). Besides, 13 isolates from 4.2% (7/165) meat samples carried *cpe,* however, no type F *C. perfringens* and no *cons. cpb2* was found in all those isolates (Figure not attached).

3.3. PFGE typing results

The dendrogram generated from the obtained PFGE patterns, virtual gel images and profiles of each isolate were summarized in Fig. 5. The

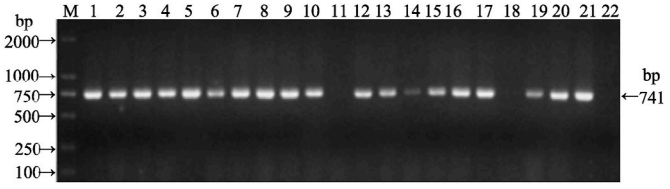

Fig. 2. PCR results of *aty.cpb2* gene detection of partial *C.perfringens* isolates. M.DL 2000 DNA Marker; 22.Negative control (ddH_2O); 21.Positive control, CVCC49 single colony as template and the target band is 741 bp; 1–10,12-17,19–20: all *atyp.cpb2* positive isolates; 11,18: *atyp.cpb2* negative isolates.

Table 3
Toxin-genotypes of *C.perfringens* isolates from 3 slaughterhouses.

Source	Process	*C. perfringens types*					
		A n= (%)	Aβ2a n= (%)	D n= (%)	Dβ2a n= (%)	Dβ2ae n= (%)	Total n= (%)
S1	Slaughtering	11 (16.2)	35 (51.5)	4(5.9)	2 (2.9)	16 (23.5)	68 (100.0)
	chilling	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Cutting	5 (13.9)	16 (44.4)	3(8.3)	0 (0.0)	12 (33.3)	36(100.0)
	Total	16 (15.4)	51 (49.0)	7 (6.7)	2 (1.9)	28 (26.9)	104 (100.0)
S2	Slaughtering	5 (41.7)	7 (58.3)	0 (0.0)	0 (0.0)	0 (0.0)	12(100.0)
	chilling	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1(100.0)
	Cutting	8 (30.8)	18 (69.2)	0 (0.0)	0 (0.0)	0 (0.0)	26 (100.0)
	Total	13 (33.3)	26 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	39 (100.0)
S3	Slaughtering	8 (53.3)	7 (46.7)	0 (0.0)	0 (0.0)	0 (0.0)	15 (100.0)
	chilling	–	–	–	–	–	–
	Cutting	21 (42.0)	26 (52.0)	0 (0.0)	2 (4.0)	1 (2.0)	50(100.0)
	Total	29 (44.6)	33 (50.8)	0 (0.0)	2 (3.1)	1 (1.5)	65 (100.0)
Summary		58 (27.9)	110 (52.9)	7 (3.4)	4 (1.9)	29 (13.9)	208 (100.0)

Note: "-": Not sampled. type A = cpa^+; type Aβ2a = cpa^++$cpb2aty^+$; type D = cpa^++etx^+;
type Dβ2a = cpa^++etx^++$cpb2aty^+$; type Dβ2ae = cpa^++etx^++$cpb2aty^+$+cpe^+.

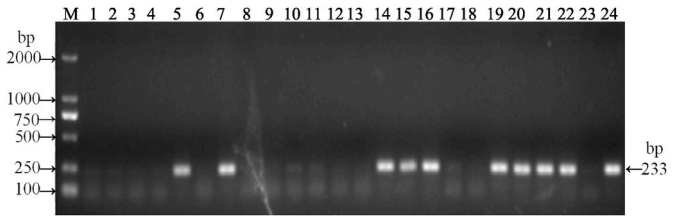

Fig. 3. PCR results of *cpe* gene detection of partial *C.perfringens* isolates. M.DL 2000 DNA Marker; 23.Negative control (ddH_2O); 24.Positive control, CVCC90 (type E) as template and the target band is 233 bp; 1–4,6,8–13,17-18: all *cpe* negative isolates; 5,7, 14–16,19-22 al l *cpe* positive isolates.

DNA fragments of those isolates were separated to 11-21well-distributed bands of 30–700 kb, which provided good discrimination between different isolates (Figs. 5 and 7). The similarity of all 69 *C. perfringens* isolates ranged from 59.9% to 100%, based on which, the isolates were divided into four clades (I to IV) with a similarity higher than 75.9%. Clade I contains 94.2% (65/69) of the isolates, while clade II to IV only contains 5.8% (4/69) of the isolates. Isolates with the similarity greater than 90% were classified into one PFGE genotype (PT), and all isolates were divided into 28 different PFGE genotype (PT1-PT28) (Fig. 5). In 10 different clusters (PT6-1, …PT19-3), isolates with 100% similarity were identified, revealing that those isolates in the same cluster were the same clone. The *C. perfringens* strains from the 3 different slaughterhouses (S1, S2 and S3) were then divided into 9, 13 and 17 PFGE genotypes, respectively. Among them, except PT9, PT20 and PT23 had type D isolates, the rest were all type A. When the PFGE type was the same, the same toxinotype with 97.1% probability.

At the slaughtering stage, identical isolates from different processes in the same slaughterhouse, such as BT1903144 from knife and BH1903123 from hide in PT23 from S2, BFS190239 from fecal sample and BH190289 from hide in PT10 from S1 (Fig. 5), indicated the feces could contaminate the hide further to the carcass during the hide removal of the slaughtering process as well as the possibility of mutual contamination among tools, carcass surfaces and air in slaughtering workshops. At the cutting stage, PT19 including 14 strains with similarity≥95.1% were all isolated from S2 with type A (most of the isolates are Aβ2a), which was the preponderant PFGE type. Among this, PT19-1, PT19-2 and PT19-3 were with 100% similarity, all isolated from meat samples except BT190356, BT190385 and BT190384 from tools from cutting workshop. Similar results were observed in the PT6-1 and PT7-1 from S2, PT12-1 from S1(Fig. 5), these data all together demonstrated the cross-contamination between tools (conveyor belt, operation table, knives) or personnel (gloves, apron et al.) and beef, as well as beef to beef in the meat cutting process.

Two same isolates BA1903149 from air in slaughtering workshop and BM190314 from meat sample in cutting workshop in PT9-1 were identified from S2 (Fig. 5), indicating the contaminated bacteria can be transmitted from the slaughtering site to the cutting workshop. Interestingly, isolates within some clusters, such as in the PT7-2, PT10-1, PT10-2 and PT12-1 clusters, had 100% similarity (Fig. 5), respectively, but they were isolated from different stages of slaughtering processes in different slaughterhouses, between which the nearest linear distance is 104 km, and the longest linear distance is 374 km. Cattles slaughtered at S1 were transported from Gansu Province about 800 km far away, but the cattle slaughtered at S2 and S3 were mainly raised by the surrounding farmers without *trans*-regional transport, and accordingly, most of *C. perfringens* type D (92.5%) (Table 3) were isolated from S1. The fact that some *C. perfringens* from different regions share the same PFGE genotype, besides, both the prevalence and the toxinotype of isolates were related with the geographical position, indicated the possibility of horizontal transmission existed in different areas of northwest of China or the high frequency of common clonal types in these areas.

4. Discussion

During the cattle slaughtering and butchering process, contamination can occur via slaughter facility and carcass handling. Based on these key procedures, regulations and guidelines are in placed to ensure the quality and safety of the beef distributed to human consumption (Camargo et al., 2019). Studies on the types of *C. perfringens* at different steps of beef slaughtering process as well as their toxin genes investigated within this study are rare. This is the first time that tracing *C. perfringens* along the slaughtering and processing lines has been reported and *C. perfringens* was found at all steps in the beef slaughtering and processing. Our research provides data regarding the major sources of contamination and points of cross-contamination from farm to retail. Moreover, beef meat is regarded as the major source of *C. perfringens* for

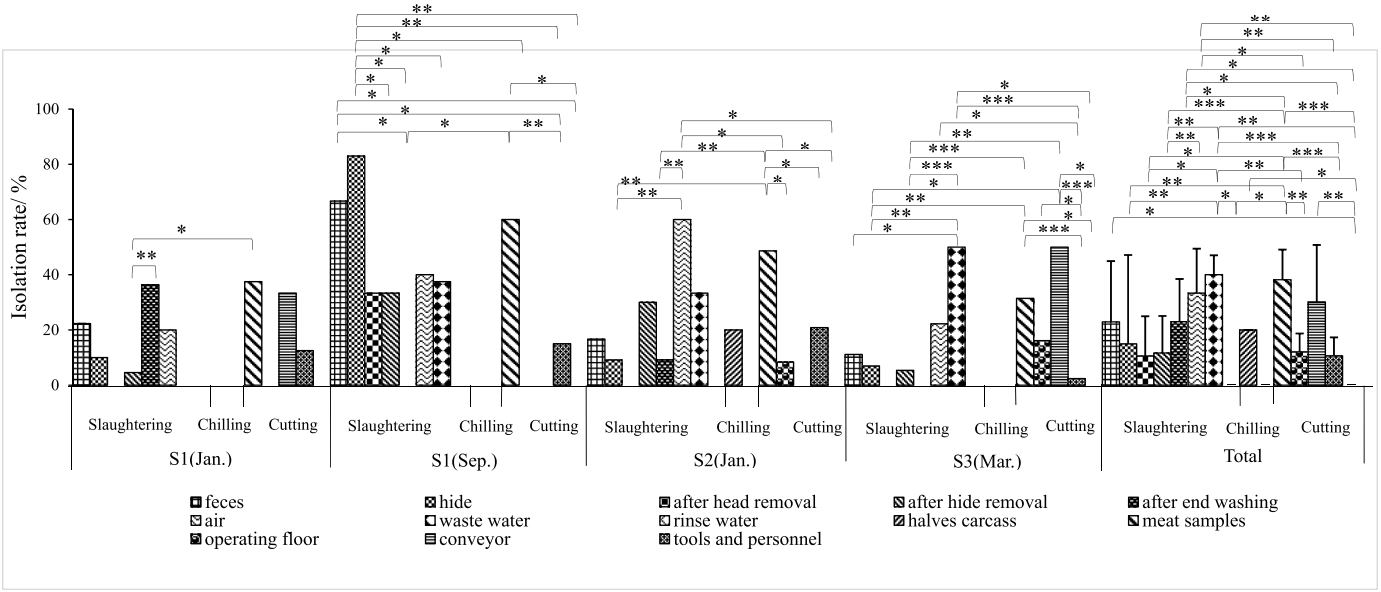

Fig. 4. Isolation rate of *C.perfringens* from the whole slaughtering and butchering process of each slaughterhouse. Note: * indicates there is statistics significant difference between the column ($p < 0.05$); ** indicates highly significant difference ($p < 0.01$); *** indicates extremely significant difference ($p < 0.001$). The column without bar of halves carcass at chilling step in column of Total since samples only collected from S2(Jan.).

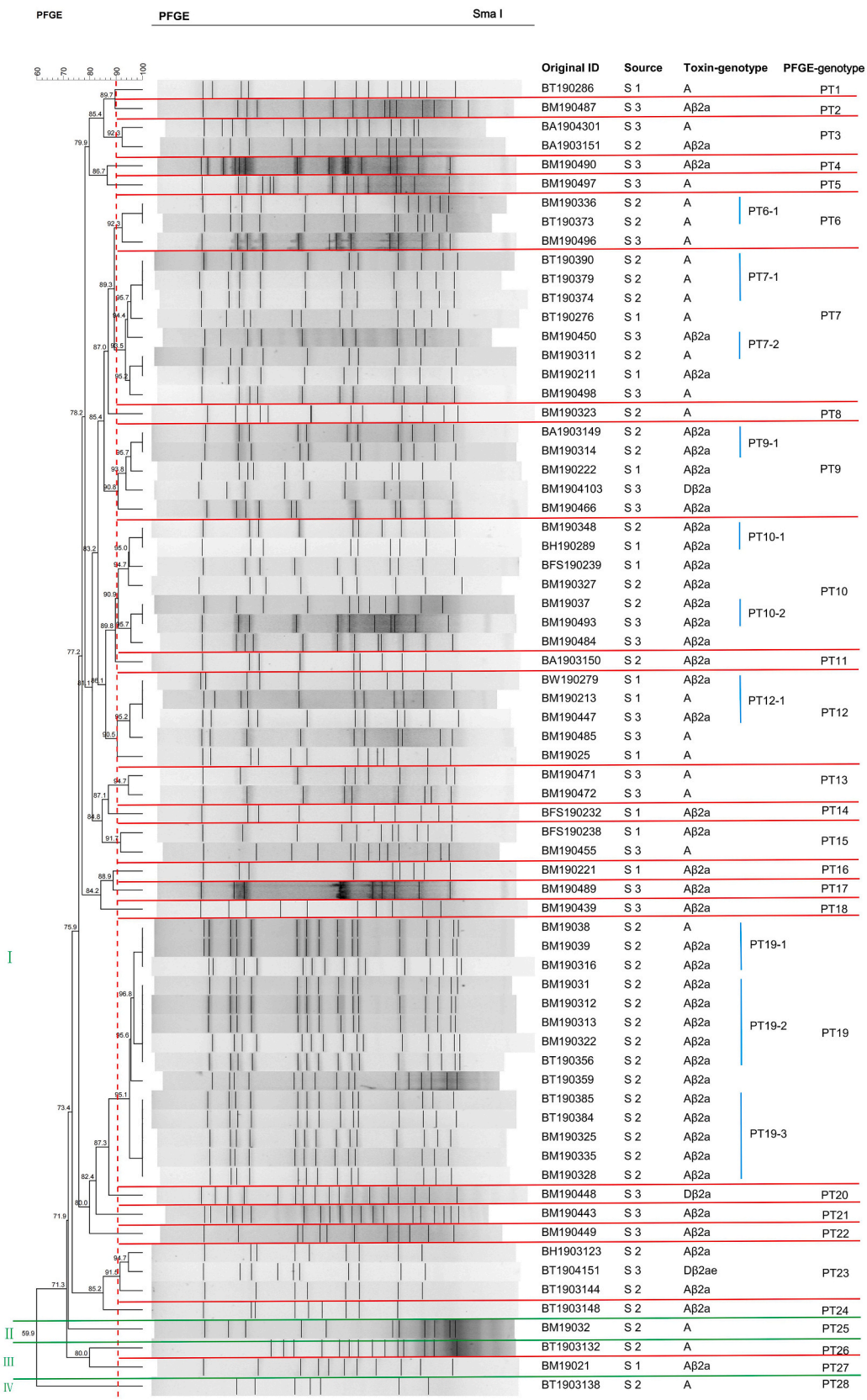

Fig. 5. Dendrogram of 69 *C.perfringens* isolates by PFGE analysis. Note: B (Bovine)+A/T/FS/H/M (air, tools, feces, hide, meat) +Year (18/19) +Sampling times (01, 02, 03, 04)+Number; Source: S1–S3 represents three slaughterhouses, respectively; All isolates were divided into four clades (I-IV) when the similarity>75.9%; PT1-PT28 represents different PFGE genotype with the similarity≥90%; PT6-1, …, PT19-3 represents different clusters with 100% similarity which were the same clone, respectivly. Toxin-genotype: same as Table 3.

food poisoning for humans but there is great argument as to the source of the *cpe* isolates. It is generally considered that the strains originate from human contamination and that the relation with beef relates to the increased resistance of the spores in these isolates (Sarker et al., 2000; Raju and Sarker, 2005; Miki et al., 2008; Abbona and Stagnitta, 2016). Our studies showed the absence of type F (*cpe*, *cpa* positive strains) in cattle in this survey and tend to support the argument. Therefore, 208 *C. perfringens* isolates were toxin-genotyped to gain further insights into the presence of its different types on beef cattle slaughterhouse.

The prevalence of *C. perfringens* from the 3 slaughterhouses were showed in Fig. 4. In two previous studies, the *C. perfringens* isolation rate from diarrheal and slaughtered cattle and sheep from Xinjiang was 42.4% (67/158) including 82.1% type A and 17.9% type D (Wang et al., 2019), and 13 isolates from sudden death cattle including 92.3% type A and 7.7% type D in Heilongjiang of China (Liu et al., 2019). Similarly, the isolates from feces were assigned to type A (n = 442) and 0.3% type D (n = 2) from dairy farms in Germany (Fohler et al., 2016). The toxin-genotype of the isolates in this study was consistent with these previous studies, and the difference of portion of type D may relate the health status of the sampled animals since type D is known to cause enterotoxemia in small ruminants and calves (Uzal et al., 2014). It seems the predominant toxin-genotype of cattle is type A with a minority of type D in cattle and dairy farms. However, a recent study showed that 59.7% isolates were type A and the least prevalent was type C from neonatal calves cattle and buffalo in India (Athira et al., 2018). In our study, none of the investigated isolates was found in types B, C, E, and/or F.

The beta-2 toxin is assumed to have a causative role in enteric disease of calves since *cons. cpb2* gene is widely found in cattle with enterotoxemia (mainly in calves) but the role of *atyp. cpb2* is still not clear (Manteca et al., 2002; Kukier and Kwiatek, 2010; Fohler et al., 2016). In present study, 68.8% isolates harbored *atyp. cpb2* gene and similar rates were found by Gurjar et al. (2008) and Jost et al. (2005), while others studies *cpb2* positive rate from 21.4% to 47.3% from cattle and calves suffering from enteric disease (Bueschel et al., 2003), and Fohler et al. (2016) found *atyp.cpb2* was detected about twice as often as the consensus variant and 0.3% of the isolates carried both allelic variant, but a previous study showed 50% (4/8) type A isolates carried both allelic variant in China (Liu et al., 2019). Further, 82.5% type D isolates harbored *atyp. cpb2* gene (type Dβ2a) in present study, Fohler et al. (2016) found 50% (1/2) type D from fecal samples harbored *cons.cpb2*. Interestingly, no *cons. cpb2* was found in any isolates in our study, in similar to that of Schlegel et al. (2012). Though the varying detection frequencies and those of previous studies demonstrate that both allelic variants occur frequently in cattle, whereby our results indicated the *atyp. cpb2* seems to dominate, but whether the *atyp.cpb2* plays a role in the pathogenesis of type D needs to be further explored.

CPE-positive strains of *C. perfringens* type A, which have been named *C. perfringens* type F recently (Rood et al., 2018), have been shown to be responsible for human food-poisoning and non-foodborne human gastrointestinal (GI) diseases, including some instances of AAD. In present study, no type F were found, although 72.5% (29/40) type D isolates carried both *cpe* and *aty.cpb2* (type Dβ2ae), including 13 isolates with 4.2% (7/165) prevalence rate isolated from beef meat samples. A similar rate (4%) of *cpe*-positive isolates from with *cpe* locating on plasmid was reported from Japan (Miki et al., 2008). Jang et al. (2020) found all *C. perfringens* isolates were type A and negative for the *cpe* gene in retail beef in Seoul. A recent study showed 33.3% (6/18) *C. perfringens* isolates carried chromosomal *cpe* and 27.8% (5/18) carried plasmid *cpe* gene from bovine (Park and Rafii, 2019). Moreover, the *cpe* loci of type C, D, and E strains differ from the *cpe* loci of type F(A) strains, and the *cpe* loci of *C. perfringens* have remarkable diversity, the chromosomal *cpe* strains which are responsible for most food poisoning cases have distinct genetic characteristics, enabling these strains unique biological properties, such as the formation of highly heat-resistant spores, and plasmid *cpe* isolates also play a role in *C. perfringens* type A/F food poisoning cases (Miyamoto et al., 2012). Notably, 43.26%(61/141) *C. perfringens* strains involved in foodborne outbreaks in France were *cpe*-negative type A strains according to a recent study (Mahamat Abdelrahim et al., 2019), which suggests, the presence of *cpe*-negative *C.perfringens* type A in slaughter house and food-processing plants may be a possible threat to public health, and its possibility to cause food poisoning should not be ignored.

C.perfringens type D strains produce enterotoxemia in sheep, goats and cattle, but are not known to cause spontaneous disease including food poisoning in humans(Uzal et al., 2018). The virulence of type D isolates is heavily plasmid-dependent and can carry multiple virulence plasmids with up to three different toxin genes. In some *cpe*- and/or *cpb*2-positive type D isolates, the *etx*, *cpe*, and *cpb2* located on either the same plasmid or different plasmids (Sayeed et al., 2007). Though, the location of *cpe* have not been distinguished and characterized in our study, we speculated that *cpe*, *atyp.cpb2* and *etx* might locate on the same plasmid since all *cpe* + type D isolates (29/40) were identified in combination with *atyp.cpb2*, suggesting that these isolates is no risk of food poisoning in humans.

The contamination of *C. perfringens* of beef gradually increased during the slaughtering processes. The prevalence of *C. perfringens* changed dramatically which decreased before carcass exposure and increased after carcass exposure at the slaughtering stage, then significantly increased of beef samples at the cutting step. This observation consists with the PFGE analyzed results (Fig. 5) as well as other studies carried out from beef slaughterhouses for microbiological testing and other foodborne pathogens inspecting (Sofos et al., 1999; Wang et al., 2010; Dong et al., 2019; Camargo et al., 2019). Our data supports the idea that the bacteria shaken off from the hide and flied in the air during the hide removal process of slaughtering then dropped on the carcass and induced the contamination of carcass, and the cross contamination mainly includes meat to meat, meat to tools/personnel as well as tools/personnel to meat during the cutting stage afterwards. Low temperature in the chilling step did not lead to the reduction of *C. perfringens,* as the positive rate kept at a level in similar with the rate after ending washing in the slaughtering step, possibly due to the small number of samples. However, other foodborne bacteria in the chilling step showed a significant decrease of the contamination of chicken carcass (Rosenquist et al., 2006).

Among the 28 PTs, 18 different PTs only contains one isolate in each PT, the rest 8 PTs were all isolated from different slaughterhouses besides PT13 from S3 and PT19 from S2. The single isolate from PT28 had the greatest difference from the other PTs with a similarity of 59.9%. These data indicate that some of *C. perfringens* isolates had the same PFGE genotype at different regions while the PFGE type in each slaughterhouse was diverse, in similar with the finding by Melero et al. (2012) in tracing the entire production chain for *Campylobacter jejuni* from farm to retail. Our study also showed PFGE to be superior to toxinotyping for providing links to epidemiologic data, this is consistent with that PFGE to be superior to serotyping reported by Maslanka et al. (1999) since when the same PFGE type with 97.1% probability of the same toxinotype.

In our study, by tracing *C. perfringens* in the whole beef slaughtering process using PFGE, we found that the *C. perfringens* in same slaughterhouse have genotype diversity, but meanwhile, in different regions of Shaanxi the bacteria also have homology. From the obtained data, we also concluded that rectum contents and hide are the main carcass contamination source, and cross-contamination is common in slaughtering and cutting workshops. These are the three key points to control the contamination of the bacteria in cattle slaughtering. Therefore, animals should be fasted and showered before slaughtering, the anus ligation should be operated strictly to prevent contents contaminating the carcass during slaughtering, which can reduce the chance of fecal contamination of beef (Pointon et al., 2012). Timely disinfection of cutting tools, sharpening sticks and operating tables in the cutting workshop can also avoid cross-contamination. Other studies have shown

that spraying organic acids, in combination with high-pressured water washing and ozone water spray and antimicrobial agent on the carcass after ending washing in slaughterhouse can reduce the proliferation rate of microorganisms (Van Ba et al., 2018; Valenzuela-Martinez et al., 2010; Fu et al., 2019). Thus, the standard operation and hygienic management of slaughterhouses is crucial important for the production of beef with guaranteed quality.

5. Conclusion

C. perfringens exists in the whole beef slaughtering and butchering process with high prevalence, and the contamination rate gradually increases as the handling process going on. While type A is the predominant toxinotype, the PFGE genotype of the isolates showed great diversity. To prevent and control the contamination of *C. perfringens*, the key points (rectal contents, hide, tools and personnel, etc) should be strictly monitored. This is for the first time the scientific data along the whole beef slaughtering process were provided to prevent and control the contamination of *C. perfringens*, and eventually to reduce the potential risk of posing *C. perfringens* foodborne disease in China.

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Auhors' contributions

Yanfen Jiang, Yanming Zhang and Kangkang Guo contributed to the conception and design of this study. Yanfen Jiang, Yinghui Ma, Qianqian Liu, Tianmei Li, Yiming Li carried out the experiments, collected and analyzed the data. Yanfen Jiang and Yanming Zhang wrote and revised the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Appendix A

Description

The documents including a PCR detection gel picture and PFGE gel picture and 2 tables of the isolation rate and toxin-genotypes of *C.perfringens* from each slaughterhouse just for reference.

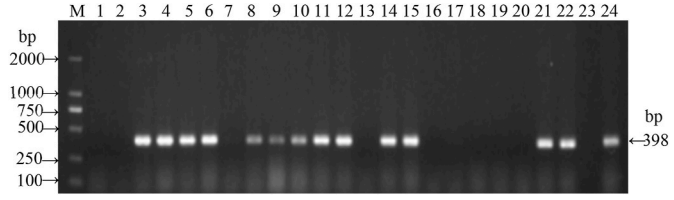

Fig. 6. PCR detection of *cpa* from partial suspected positive samples.

M.DL2000 DNA Marker; 1–22.Samples; 23.Negative control; 24.Positive control (*C. perfringens* type A)

Sample 3–6, 8-12, 14–15, 21-22 showed *cpa* positive, means the samples carried *C.perfringens*; the rest samples showed *cpa* negative, means samples didn't carry *C.perfringens.*

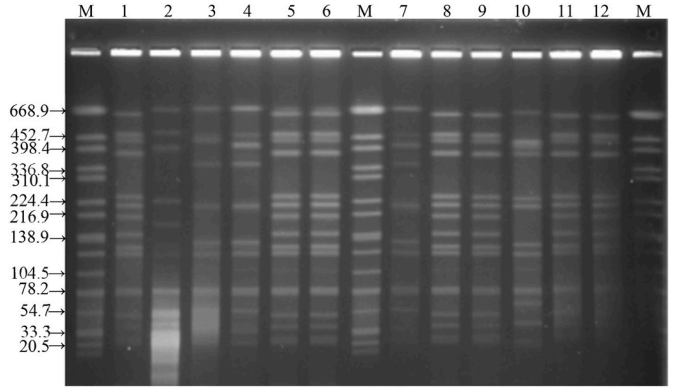

Fig. 7. PFGE profile of genomic DNA of partial *C.perfringens* isolates.

M.*Salmonella Braenderup* H9812 (restricted by *Xba* I); 1–12.Isolates.

Note: *C.perfringens* isolates were digested by *Sma* I restriction enzymes and analyzed by PFGE. The DNA fragments of those isolates are separated to

11–21 well distributed bands of 30–700 kb which provided good discrimination between isolates.

Table 4
Isolation rate of *C. perfringens* from the whole slaughtering and butchering process of each slaughterhouse.

Process	Category of samples	Isolation rate/%				
		S1(Jan.)	S1(Sep.)	S2(Jan.)	S3(Mar.)	Total
Slaughtering	feces	22.22(4/18)^{ab}	66.67(4/6)^{ab}	16.67(1/6)^{ab}	11.11(2/18)^{bcd}	22.92(11/48)^{abc}
	hide	10.0(1/10)^{ab}	83.0(5/6)^{a}	9.1(2/22)^{b}	6.9(2/29)^{cd}	14.9(10/67)^{bcd}
	after head removal	0.0(0/6)^{b}	33.3(2/6)^{bc}	0.0(0/4)^{b}	0.0(0/3)^{d}	10.5(2/19)^{cd}
	after hide removal	4.6(1/22)^{b}	33.3(5/15)^{bc}	30.0(3/10)^{ab}	5.4(3/56)^{cd}	11.7(12/103)^{cd}
	after end washing	36.4(12/33)^{a}	0.00(0/6)^{c}	9.1(2/22)^{b}	–	23.0(14/61)^{abc}
	air	20.0(1/5)^{ab}	40.0(2/5)^{abc}	60.0(3/5)^{a}	22.2(2/9)^{abc}	33.3(8/24)^{ab}
	waste water	–	37.5(6/16)^{bc}	33.3(1/3)^{ab}	50.0(3/6)^{a}	40.0(10/25)^{a}
	rinse water	0.0(0/2)^{b}	–	0.0(0/3)^{b}	0.0(0/3)^{d}	0.0(0/9)^{cd}
	total	19.6(19/97)^{B}	33.3(24/60)^{A}	16.0(12/75)^{BC}	9.7(12/124)^{C}	18.8(67/356)^{B}
Chilling	halves carcass	–	–	20.0(1/5)^{ab}	–	20.0(1/5)^{abc}
	air	0.0(0/2)^{b}	–	0.0(0/4)^{b}	–	0.0(0/6)^{cd}
	total	0.0(0/2)	–	11.1(1/9)	–	9.1(1/11)
Cutting	meat samples	37.5(3/8)^{a}	60.0(9/15)^{ab}	48.7(18/37)^{a}	31.4(33/105)^{ab}	38.2(63/165)^{a}
	operating floor	0.0(0/5)^{b}	0.0(0/2)^{c}	8.3(1/12)^{b}	16.1(5/31)^{abc}	12.0(6/50)^{cd}
	conveyor	33.3(1/3)^{ab}	–	0.0(0/3)^{b}	50.0(2/4)^{ab}	30.0(3/10)^{abc}
	tools and personnel	12.5(1/8)^{ab}	15.0(3/20)^{c}	20.8(5/24)^{ab}	2.4(1/42)^{d}	10.6(10/94)^{cd}
	air	0.0(0/3)^{b}	0.0(0/5)^{c}	0.0(0/4)^{b}	0.0(0/10)^{d}	0.0(0/22)^{d}
	total	18.5(5/27)	28.6(12/42)	30.0(24/80)	21.4(41/192)	21.4(82/341)
Summary		19.1(24/126)^{B}	35.3(36/102)^{A}	22.7(37/164)^{B}	16.8(53/316)^{B}	21.2(150/708)^{B}

Note: Marked with different lowercase letters after the same column of data indicates significant differences ($P < 0.05$); With different capital letter following the same line of data indicates significant differences ($P < 0.05$).
-:Not sampled.

Table 5
Toxin-genotypes of *C.perfringens* isolates of the whole slaughtering process from 3 slaughterhouses.

Process	Category of sample	*C. perfringens types*														
		S1					S2					S3				
		A	Aβ2a	D	Dβ2a	Dβ2ae	A	Aβ2a	D	Dβ2a	Dβ2ae	A	Aβ2a	D	Dβ2a	Dβ2ae
Slaughtering	Feces	1	11		1		1					1	3			
	Hide	3	4			2	1	1				1	1			
	After head removal	3	4													
	After hide removal		10	2	1		2	1				3				
	After end washing	1	1	2		11		2				–				
	Air		1			2		3				2	1			
	Waste water	3	4			1	1					1	2			
	Rinse water															
	Total	11	35	4	2	16	5	7				8	7			
chilling	Halves carcass	–						1				–				
	Air											–				
	Total							1				–				
Cutting	Meat sample	4	9	3		12	5	14				15	23		2	1
	Operating floor						1	1				4	2			
	Conveyor	1										1	1			
	Tools and personnel		7				2	3				1				
	Air															
	Total	5	16	3		12	8	18				21	26		2	1
Summary		16	51	7	2	28	13	26	0	0	0	29	33		2	1

Note: "-": Not sampled. type A = *cpa*$^{+}$; type Aβ2a = *cpa*$^{+}$+*cpb2aty*$^{+}$; type D = *cpa*$^{+}$$_{+}$*etx*$^{+}$; type Dβ2a = *cpa*$^{+}$$_{+}$*etx*$^{+}$+*cpb2aty*$^{+}$; type Dβ2ae = *cpa*$^{+}$$_{+}$*etx*$^{+}$+*cpb2aty*$^{+}$+*cpe*$^{+}$.

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