



Molecular typing of *Clostridium perfringens* strains isolated from cooked beef, by toxin genes amplification

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ABSTRACT

The aim of the study was to determine the presence of gene coding for alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) from *Clostridium perfringens* strains isolated from cooked beef sold in the streets. A total of 395 samples of cooked beef were collected from vendors at Abidjan Côte d'Ivoire and subjected to *Clostridium perfringens* isolation and identification by using biochemical tests and API 20A system. Then, the multiplex polymerase chain reaction (PCR) method was used for toxin typing for confirmed isolates. The results obtained show that the prevalence of *C. perfringens* in cooked beef sold in the streets varied from 0 to 14% according to the area of study. The mean value was 5.06% and spore count was 1.11 ufc/g. The 20 confirmed isolates belonged to two toxinotypes: type A (95%) and type B (5%). Two groups of *C. perfringens* type A were present in cooked beef sold in the streets. One group was *cpe* negative and represented 55% of the isolates. The second group harbored *cpe* gene in addition to *cpa* gene. This group was found at 40%.

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INTRODUCTION

Clostridium perfringens is a spore-forming, Gram-positive, anaerobic, non-motile rod which form large, regular, round and slightly opaque and shiny colonies on the surface of agar plates. It is an ubiquitous bacterium found in virtually all environments tested including soil, water, milk, dust, sewage and the intestinal canal of humans and animals (Hatheway, 1990). This bacterium is one of the most widespread pathogenic bacteria, and has been associated with a wide range of histotoxic and gastrointestinal diseases in both humans and animals

(Labbé, 1989; McClane, 2007). *C. perfringens* is also widely distributed in a variety of foods, especially meat and poultry products, and is recognized as an important cause of food poisoning throughout the world (Stringer et al., 1980). Cooked meat products, such as ham, roast beef and corned beef, are frequently associated with foodborne outbreaks of *C. perfringens* gastroenteritis (Hall and Angelotti, 1965; Gross et al., 1989; CDC, 1994).

Food poisoning associated with *C. perfringens* is caused by the ingestion of a large number ($\geq 10^6$) of viable vegetative cells of the organism in temperature-abused foods. Once in the small intestine, the cells sporulate, releasing a toxin that is responsible for the pathological effects in humans as well as the typical

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symptoms of diarrhoea and abdominal pain (Juneja et al., 2006).

This species is assigned to 5 toxinotypes (A, B, C, D and E) on the basis of the production of 4 major toxins, namely alpha (α), beta (β), epsilon (ϵ) and iota (i) (Yamagashi et al., 1997; Petit et al., 1999). Type A produces only alpha toxin, type B produces alpha, beta and epsilon toxin, type C produces alpha and beta toxin, type D produces alpha and epsilon toxin and type E produces alpha and iota toxin. Each toxin type is associated with specific enteric infections of various animal species (El Idrissi and Ward, 1992; Hunter et al., 1993; Gkiourtzidis et al., 2001; Greco et al., 2005). Enterotoxin is also one of the important toxins of *C. perfringens* (Songer, 1996; Gkiourtzidis et al., 2001; Engström et al., 2003). All types of *C. perfringens* can produce enterotoxin that is responsible for provoking disease in both humans and animals (Van Immerseel et al., 2004). Enterotoxin relates to food poisoning and is produced during sporulation in the infected host intestine, where it binds to the intestinal epithelium, forms pores and causes diarrhoea (Nakamura et al., 2004). Thus, detection of *C. perfringens* toxin types and subtypes is critical for a better understanding of the epidemiology of *C. perfringens* infections and may be helpful in the development of effective preventive measures.

The classification of *C. perfringens* isolates into toxigenic types could be performed by various methods. Polymerase chain reaction has been applied in several studies and highlighted as a rapid and accurate method for the detection of low copy numbers of genes. This method is more accurate and faster than conventional methods such as serum neutralization test and enzyme-linked immunosorbent assay (ELISA) (Yoo et al., 1997). In spite of the importance of *C. perfringens* as pathogenic bacterium widely distributed in meat and meat products, there are no published data on molecular typing of *C. perfringens* in meat in Côte d'Ivoire. In the present study, the multiplex PCR was used in order to determine the presence of alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) toxin genes from *C. perfringens* strains isolated from cooked beef sold in the streets.

MATERIALS AND METHODS

Sampling

A total of 395 samples of cooked beef (172 samples of kidney and 223 samples of flesh) were purchased from street food vendors in Abidjan, Côte d'Ivoire. Only one sample of kidney or flesh was purchased from each vendor. The sampling places were randomly selected throughout eight municipalities (Abobo, Adjame, Cocody, Koumassi, Marcory, Port-Bouet, Treichville, Yopougon) within Abidjan. Samples were collected aseptically in

sterilized plastic bags by using sterilized utensil and immediately transported to the laboratory in ice-cooled containers within 2 h. The analysis for bacteria isolation started as soon as samples arrived to the laboratory (Kouassi et al., 2014).

Isolation and identification of *C. perfringens*

Ten grams (10 g) of each sample was placed into a sterile plastic bag containing 90 mL of sterilized Buffered Peptone Water and homogenized for 1 min using a stomacher (Seward, West Sussex, UK). In order to quantify spores, 40 mL of the homogenate solutions were heated in screw cap tubes in a water bath at 80°C for 10 min. Anaerobic total plate and spore counts were performed in duplicates on tryptose sulfite cycloserine (TSC) medium (Bio-Rad, France). After incubation at 37°C for 24 h, black colonies were purified in TSC agar and identified by Gram staining and the following standards biochemical tests: gas production, lecithinase and lipase productions, motility, starch hydrolysis, indole production, coagulation and retraction of cysteinated milk. Suspected isolates were preliminarily identified as *C. perfringens* by using a commercially available identification system, API 20A (BioMerieux, France). Isolates were stored in 25% glycerol at -80°C for further analyses.

C. perfringens toxin genes detection by multiplex PCR

Multiplex PCR was used to determine the presence of alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*iap/ibp*) and enterotoxin (*cpe*) toxin genes. Five sets of primers were synthesized (Eurobio, France) for the amplification purpose based on previous reports (Baums et al., 2004). The primer sequences and their product sizes are shown in Table 1. For total genomic DNA extraction, *C. perfringens* isolates were grown on TSC agar (BioMerieux, Paris, France) supplemented with egg yolk potassium tellurite prepared and poured into Petri dishes. The plates were incubated at 37°C for 24–48 h under anaerobic conditions according to the procedure described by Imhof and Hentzer (1996). One loop of biomass was scraped off the agar, suspended in 200 mL of sterile distilled water, placed in an ice bath for 5 min and then boiled for 10 min. After centrifugation at 1500 rpm for 10 min at 4°C, the supernatants were used as templates for PCR. The PCR reactions were performed a Techne TC-32 thermal cycler (Barloworld Scientific Ltd, Milano, Italy). Each multiplex PCR reaction mix contained 5 μ L *C. perfringens* template DNA, 1 μ L (10 pmol/ μ L) each of *cpa*, *cpb*, *cpe*, *etx* and *iap/ibp* primer sequences, 2.5 μ L of 2 mM deoxyribonucleotide triphosphates

Table 1. Primer for multiplex PCR detection of *C. perfringens* toxin genes (Baums et al., 2004).

Gene (Toxin)	Primers	Primer sequence (5'3')	Types	Fragment length (bp)
<i>cpa</i>	CPA5L	AGTCTACGCTTGGGATGGAA	ABCDE	900
	CPA5R	TTTCCTGGGTTGTCCATTTTC		
<i>cpb</i>	CPBL	TCCTTTCTTGAGGGAGGATAAAA	BC	611
	CPBR	TGAACCTCCTATTTTGTATCCCA		
<i>cpe</i>	CPEL	GGGGAACCCTCAGTAGTTTCA	ABCDE	506
	CPER	ACCAGCTGGATTTGAGTTTAATG		
<i>etx</i>	CPETXL	TGGGAACTTCGATACAAGCA	BD	396
	CPETXR	TTAACTCATCTCCCATAACTGCAC		
<i>iap</i>	CPIL	AAACGCATTAAGCTCACACC	E	293
	CPIR	CTGCATAACCTGGAATGGCT		

Table 2. Prevalence of *C. perfringens* in cooked beef sold in the streets.

Municipalities	Number of samples	Means of spore counts (ufc/g)	Number of samples positive for <i>C. perfringens</i>	Prevalence (%)
Abobo	50	0.99	06	12.00
Adjame	57	0.53	03	05.26
Cocody	43	1.90	06	13.95
Yopougon	54	0.10	01	01.85
Port-Bouet	47	0.20	01	04.08
Koumassi	49	0.29	02	10.20
Marcory	45	0.14	01	02.22
Treichville	50	< 1	00	00.00
Total	395	1.11	20	05.06

(dNTPs), 2.5 µL of 10×PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl₂, 0.1% TritonTMX-100), 0.25 µL of 5 U/µL of Taq DNA polymerase (Roche) and water to 25 µL. The DNA was initially denatured for 5 min at 95°C then amplified for 30 cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C for denaturation, annealing and extension phases, respectively), and followed by an additional period of extension for 3 min at 72°C (Baums et al., 2004). The reference strain *C. perfringens* ATCC 3624 type A was used as the positive control. Samples (10 µL) of PCR products were separated by electrophoresis for 45 to 60 min at 80 V in a 2% (w/v) agarose gel with ethidium bromide 0.5 µg/mL. Amplified bands were visualized and photographed under ultra-violet (UV) illumination.

RESULTS

Prevalence of *C. perfringens* in cooked beef samples

As shown in Table 2, twenty cooked beef sold in the streets out of 395 samples collected throughout eight

municipalities in Abidjan were contaminated by *C. perfringens* (05.06%). *C. perfringens* was isolated from all the municipalities except Treichville and the rates of contamination ranged from 1.85 to 13.95%. The highest prevalence was obtained at Cocody (13.95%) followed by Abobo (12%) and Koumassi (10.20%) while the lowest at Yopougon.

C. perfringens spore counts were found between 1.90 and 0.10 ufc/g. Among the municipalities examined, spore count was the highest at Cocody, Abobo, Adjame and Koumassi, respectively.

Toxinotypes of *C. perfringens* isolated from cooked beef sold in the streets

The twenty *C. perfringens* strains isolated from cooked beef sold in the streets were analysed by multiplex PCR in order to determine the toxin genes for the molecular typing. In all twenty *C. perfringens* isolates, the *cpa* gene encoding the α-toxin production was detected. The amplified band size was 900 pb as expected (Figure 1). Thus, all the isolates were confirmed as *C. perfringens*.

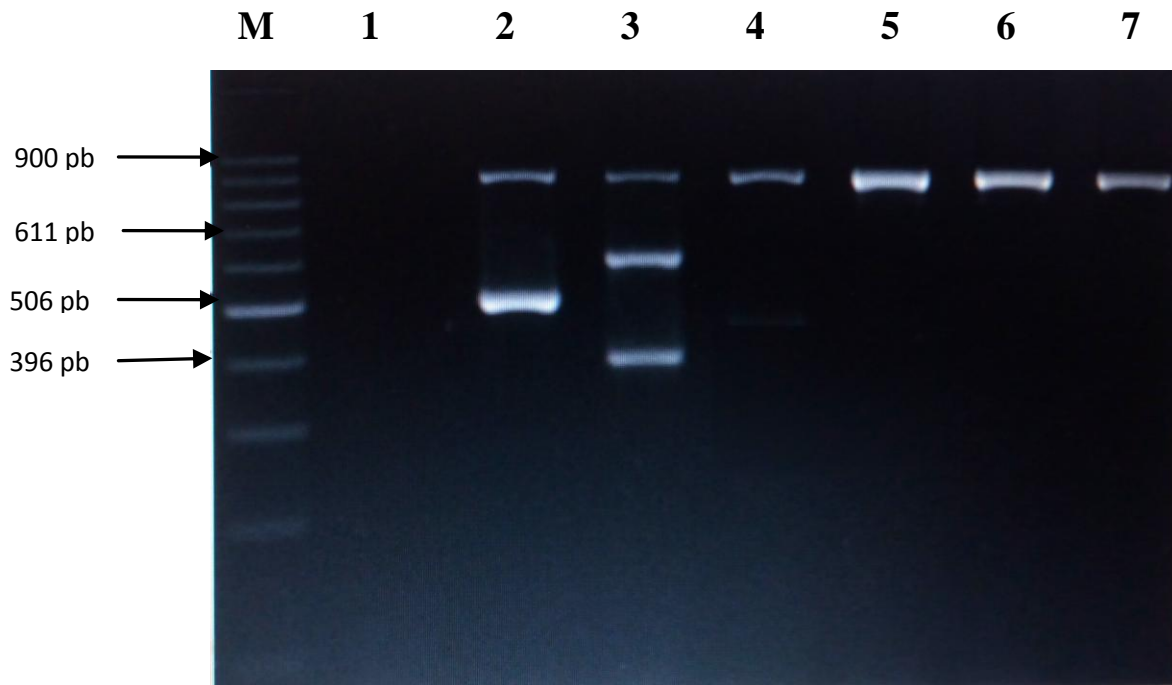


Figure 1. Multiplex PCR detection of toxin genes in *C. perfringens* isolated from cooked meat sold in the streets. **Lane M:** 100 bp molecular marker (TriDye™, BioLabs); **lane 1:** negative control; **lanes 2-6:** *C. perfringens* strains isolated from cooked meat; **lane 7:** positive control (*Clostridium perfringens* ATCC 3624 type A).

Table 3. Toxinotypes of *C. perfringens* isolated from cooked beef sold in the streets.

Profiles	Toxin genes					Toxin type	Isolates	% of <i>C. perfringens</i> isolates (n=20)
	<i>Cpa</i>	<i>cpb</i>	<i>iap/ibp</i>	<i>etx</i>	<i>Cpe</i>			
I	+	-	-	-	+	A	AbBr4, AbBr5, KsBr2, CoBr1, CoBr4, YopBr1, MaBr1, CoBr6,	40
II	+	-	-	-	-	A	CoBr5, AdBr1, KsBr1, CoBr3, AbBr2, AbBr3, CoBr2, PBr1, AbBr6, AdBr3, AbBr1	55
III	+	+	-	+	-	B	AdBr2	5

Moreover, eight isolates harbored the *cpe* encoding the enterotoxin production. Only one isolate was found to harbor *cpb* and *etx* genes while none of the tested strains was found to carry *iap/ibp* gene.

According to the multiplex PCR analyses, results showed that two groups of *C. perfringens* type A were present in cooked beef sold in the streets (Table 3). One group was *cpe* negative and represented 55% of the isolates. The second group harbored *cpe* gene in addition to *cpa* gene. This group was found at 40%. One strain (5%) was classified as type B (possessing *cpa*, *cpb* and *etx* genes). Types C, D and E were not identified among

the isolates.

DISCUSSION

In the present study, the multiplex PCR method was chosen in order to determine the presence of major toxin genes (*cpa*, *cpb*, *etx* and *iA*) and *cpe* gene from *C. perfringens* isolates obtained from cooked beef sold in the streets. According to the literature, *C. perfringens* was isolated from 66% of fresh chicken samples and from 67% of frozen samples (Nowell et al., 2010). Here, we

found the prevalence of 5.06%. Many surveys have shown that *C. perfringens* is found in raw and processed foods, most notably, raw meat products and spices (Labbé, 2000). This species lacks the ability to produce 13 of the 20 essential amino acids and is therefore associated with protein-rich foods, and 75% of the foodborne outbreaks can be traced to meat and meat products (Johnson and Gerding, 1997). The differences in the prevalence of the bacteria from meat and meat products reported by different authors will be mainly caused by the use of different detection methods.

The source of *C. perfringens* in cooked beef sold in the streets is not clear. The raw beef may become contaminated with faecal material or from the environment during the slaughtering process as *Clostridia* are members of the gut microbiota in animals. They can proliferate in large numbers and produce several toxins when the intestines are altered by sudden changes in various conditions such as diet or other factors (Uzal, 1996). Contamination may also occur through transmission by food handlers during processing and sale. The hands are the most important vehicle for the transfer of organisms from faeces, nose, skin or other sites to food (WHO, 1989). The spore-forming ability and rapid growth rates at a range of temperatures are features which allow the bacteria to multiply and survive in food.

Typing of *C. perfringens* isolates by multiplex PCR revealed that type A was the most important type of *C. perfringens* in cooked beef sold in the streets in Côte d'Ivoire (95% of isolates). Wen and McClane (2004) found that 80% of 278 *C. perfringens* isolates collected from contaminated foods were determined as type A. Similar results were obtained by Greco et al. (2005), who reported that type A was the dominant type of *C. perfringens* in lambs and sheep worldwide. In various studies, type A has been also reported to be the dominant type of *C. perfringens* worldwide (Meer and Songer, 1997; Yoo et al., 1997; Engström et al., 2003; Nauerby et al., 2003). Our results also revealed the presence of type B in cooked beef samples (one isolate). *C. perfringens* type B strains was reported to cause diseases in small ruminants but not associated with human diseases (Lindström et al., 2011).

About 40% of all *C. perfringens* isolates, belonging to type A, harbored the *cpe* gene encoding the enterotoxin (CPE), a 35-kDa single polypeptide (Songer and Meer, 1996). This finding is contrary to that of Heikinheimo and Korkeala (2005) who found that none of the broiler chicken isolates tested were *cpe*-positive. Wen and McClane (2004) found that 4.3% of the isolates carried *cpe* gene by multiplex PCR, which was different from our study. CPE has been shown to be the major virulence factor in the common form of food poisoning. Type A strains producing CPE are the second most common cause of bacterial food poisoning in the United States, with ~1,000,000 cases/yr at an estimated economic cost

of >\$300 million USD/yr (Scallan et al., 2011; Batz et al., 2012). Additionally, CPE-producing type A strains are associated with 5-15% of non-foodborne human intestinal diseases, including antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD) (Carman, 1997). The enterotoxin gene (*cpe*) can be located chromosomally or on plasmids, with ~70% of food poisoning strains harboring a chromosomal copy of *cpe*, whereas the remaining ~30% of food poisoning strains, and virtually all AAD/SD strains, carry a plasmid-borne *cpe* gene (Grant et al., 2008; McClane et al., 2013). All of these strains cause disease when *C. perfringens* sporulates in the intestine and produces CPE. During this *in vivo* sporulation, CPE accumulates in the cytoplasm and is finally released into the intestinal lumen when the mother cell lyses (McClane et al., 2013). In previous studies, the *cpe* gene has been estimated to be present in <5% of global *C. perfringens* isolates (Labbé, 2000; Engström et al., 2003). Here, we found up to 40%.

Optimal conditions for food poisonings arise when food contaminated with *cpe*-positive *C. perfringens* spores is slowly chilled or held or served at a temperature range of 10-54°C, allowing germination and rapid growth of *C. perfringens* (Li and McClane, 2006). As the temperature at which the cooked beef samples are served fall in this temperature range, the spores could grow rapidly. Upon ingestion of large numbers of vegetative *C. perfringens* cells, they sporulate in the intestinal lumen and produce CPE (McClane, 2001). Abdominal cramps and diarrhea appear within 8-12 h after ingestion, followed by recovery within 24 h. Fatalities are rare but possible in elderly or debilitated humans (Smith, 1998; Bos et al., 2005).

The occurrence of *cpe*-negative *C. perfringens* type A strains was found at 55%. This low level (compare to 95% reported by literature) could be led to the conjugative transfer of *cpe* located on plasmid. Indeed, it was reported that the transfer of *cpe* from positive strains to negative strains in situations where CPE production is an advantage, presumably in kitchen environments and in the gut, could account for the appearance of new *cpe*-positive strains (Brynstad and Granum, 2002). Brynstad et al. (2001) showed transfer rates up to 10^3 transconjugates/donor. Non-CPE-producing strains of type A *C. perfringens* which were thought only to be associated with gas gangrene in humans were recently reported to be implicated in two food-borne gastroenteritis outbreaks in Japan (Yonogi et al., 2014).

In conclusion, our study showed that the prevalence of *C. perfringens* in cooked beef sold in the streets at Abidjan, Côte d'Ivoire, varies up to 14% according to area of study with mean of 5.06%. *C. perfringens* type A is the most common type in cooked beef and it is highly composed of *cpe*-positive strains which are responsible of food poisoning and non-foodborne human intestinal diseases. Thus, cooked beef sold in the streets at Côte d'Ivoire poses a public health hazard. In order to remove

these problem bacteria, we recommend good disinfection routines and attention to proper food handling practices.

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