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Pathogenesis and Toxins

Detection of toxigenic *Clostridium perfringens* and *Clostridium botulinum* from food sold in Lagos, Nigeria

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ABSTRACT

Food-borne diseases contribute to the huge burden of sickness and death globally and in the last decade, have become more frequently reported in Africa. In line with this, food safety is becoming a significant and growing public health problem in Nigeria. Diarrhoea is a common problem in Nigeria and has been reported but there has been little data on the possibility of clostridia as aetiological agents. *Clostridium* species are ubiquitous in the environment and in the gastrointestinal tract of man and animals and can serve as a marker for faecal contamination.

We set out to determine the potential of these foods to transmit *Clostridium* species. A total of 220 food commodities from six local governments in Lagos State were sampled. Isolates obtained were identified based on cultural, morphological and biochemical characteristics. Toxinotyping was done using multiplex-PCR with primers specific for alpha, beta, epsilon and iota-toxin genes, enterotoxigenic *cpe* gene and neurotoxigenic *BoNt* gene.

Fifty (22.7%) clostridial species were isolated of which 29 (58%) were identified as *C. perfringens*. Toxinotyping of the 29 strains showed that 28 (96.6%) were toxin producing *C. perfringens* type A while one (3.4%) was *C. perfringens* type D. Two (4%) *C. botulinum* species were isolated and identified by 16S rRNA sequencing, both harbouring *BoNt/A* gene. The contamination rates of food with *Clostridium* species show that food hygiene is a problem and *Clostridium* species may be a source of food borne disease in Lagos State, Nigeria.

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1. Introduction

Clostridium species form a heterogeneous group of environmental bacteria containing about 15 pathogenic species, which produce some of the most potent toxins known to man [1]. These toxins have molecular weights ranging from 22 to 600 kDa (kDa) [2]. The central feature of many clostridial toxins is their lethality for animals [3]. The most important pathogenic species are *Clostridium tetani, Clostridium perfringens, Clostridium botulinum* and *C. difficile.* Although these species share many similarities, the diseases caused by these bacteria, as well as the toxins responsible for various symptoms are unique for each specie and essentially so for *C. perfringens* [4]. The enterotoxin produced by type A *C. perfringens* is responsible for acute diarrhoea and cramping [4].

According to the United Nations Children Education Fund (UNICEF), the diarrhoea prevalence rate in Nigeria is 18.8% and is one of the worst in sub-Sahara Africa and above the average of 16% [5]. The World Health Organization (WHO) estimates 200,000 deaths from diarrhoea each year in Nigeria [6], as many as 70% of which may be attributable to contaminated food and water. WHO in a press statement ahead of the World Health Day 2015 stated that unsafe food is linked to the deaths of an estimated two million people annually [7]. The Federal Ministry of Health reported 90,000 cases of food poisoning in 2007 [8]. As food supply becomes increasingly globalized, the need to strengthen food safety systems in and between all countries is becoming more and more evident.

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That is why the WHO is promoting efforts to improve food safety, from farm to plate.

Food-borne illness due to *Clostridium* species consists of the rare but highly fatal food-borne botulism, an intoxication that follows the consumption of food containing preformed neurotoxin [9]; *C. perfringens* food poisoning as well as Infant botulism and clostridial necrotizing enteritis. Infant botulism (also referred to as floppy baby syndrome) is an uncommon disease that occurs when ingested spores of soil-dwelling *Clostridium botulinum* germinate and produce botulinum neurotoxin in the colon [10]. Human enteritis necroticans (pigbel) is a very rare but fatal disease caused by *Clostridium perfringens* type C strains which produces an ulcerative β-toxin that results in a hemorrhagic, inflammatory, or ischemic necrosis of the small intestine [11].

Toxins produced by *Clostridium* species are responsible for diverse diseases ranging from mild self-limiting diarrhoea, antibiotic associated diarrhoea to severe life threatening gas gangrene, enterotoxaemia, pseudomembraneous colitis, botulism and tetanus [12]. *Clostridium* species including *C. perfringens, C. sordellii* and *C. novyi* as well as *C. septicum* are responsible for myonecrosis, [13]. Botulinum neurotoxins (BONTs) produced by some species of the genus *Clostridium*, in particular *C. botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinense* [14,15] causes botulism. These toxins are divided into serotypes A through G based on their serological properties. *C. botulinum* strains produce toxins of types A through F, and *C. argentinense* strains produce type G toxin. Some strains of *C. butyricum* and *C. baratii* produce type E and F toxins respectively [14,15].

2. Methodology

2.1. Sample collection

The samples were collected twice a month for a period of one year from May 2014 to April 2015. Study sites were mapped to reflect the three senatorial districts in Lagos state and two local governments were randomly selected from each of the zones. Local food commodities, grouped into categories namely; meat and meat products, milk products, vegetables, canned foods and honey were collected from open markets, cafeterias and supermarkets all around Lagos (Table 2). Samples were collected into sterile plastic bags and transported to the laboratory for processing within 2 h of collection. Samples were inoculated into Robertson cooked meat broth for enrichment and incubated anaerobically for 24 h at 37 °C.

2.2. Bacterial identification by culture

Aliquots of 100 μL from bacterial growth were transferred to plates with the following media Fastidious anaerobic agar (Lab M, Lancashire-UK), Columbia Blood Agar (Anaerobe systems, California-USA), Tryptose Sulphite Cycloserine agar –TSC (Oxoid, Basingstoke- UK) and *Clostridium perfringens* Agar - CPA (Oxoid, Basingstoke- UK). They were prepared according to manufacturer's instructions. Plates were incubated at 37 °C in an anaerobic atmosphere using anaerobic jar (Merck, Darmstadt-Germany) and Gas pak (AnaeroGen 3.51 Oxoid, Basingstoke UK); and examined after 48 h. The colonies showing typical characteristics such as double zone of beta-haemolysis on blood agar, black colonies on TSC or CPA were selected for morphological and biochemical identification.

2.3. DNA extraction

DNA was extracted by phenol-chloroform method [16]. The bacteria pellets were added to 500 μL of sterile water and incubated with 70 μL of lysozyme (10 mg/mL) at 37 °C overnight. Subsequently, 20 mg/mL proteinase K and 20% sodium dodecyle sulfate (SDS) were added and the mixture vortexed and put in the water bath at 55 °C for 1 h. DNA was extracted using equal volumes of phenol-chloroform, vortexed and centrifuged at 10 min × 14,000 revolutions per minutes (rpm). The supernatant was precipitated using 10% sodium acetate and cold isopropanol. The tube was left for 1hr in refrigerator to allow for maximum DNA precipitation and later centrifuged for 10 min × 14,000 rpm. Finally, the DNA was washed with 70% Ethanol and eluted using 200 μL of Tris-EDTA (TE). The concentration of the DNA was quantified by spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, USA) and then, stored at - 80 °C until use.

2.4. Toxin typing of Clostridium perfingens using multiplex PCR assay

The presence of genes encoding the toxins alpha (α), beta (β), epsilon (ε), iota (ι) and enterotoxin production was detected by a multiplex PCR assay [17]. The DNA amplifications were performed by using final volumes of 25 μL containing 10 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 U Platinum *Taq DNA* polymerase (Invitrogen, SP-Brazil), 0.4 mM of each primer and 1 ng of DNA. The thermocycler (PE Applied Biosystems Gene Amp PCR System 9700, USA) was programmed to: 1 cycle of 95 °C (3 min), followed by 35 cycles of 95 °C (1 min), 56 °C (1 min) and 72 °C (2 min) and a final cycle of 72 °C (5 min) to allow the final DNA extension. All primers used are shown in Table 1. PCR products were analyzed in 1% agarose gel stained with ethidium bromide (0.5 mg/mL) and photographed by using a Kodak Digital System DC-120, USA. The reference strain *C. perfringens* ATCC 13124 (α-toxin positive) was used as control.

2.5. Identification of Clostridium botulinium toxin genes

A 25 μL reaction mixture contained 2× multiplex PCR master mixture, 0.3 μM of each primer (Table 1) and 3 μL of purified DNA template as described by De Medici et al. [18]. The reaction mixture was heated at 95 °C for 5min and then subjected to 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 90 s, followed by a final extension at 72 °C for 7 min. PCR products were analyzed by 2% agarose gel electrophoresis at 90 V for 70 min, and the fragments sizes were determined by relating their positions on the gel to those of standard DNA fragments (100-bp molecular ruler; Invitogen, SP-Brazil).

2.6. 16S rRNA gene sequencing

The two *C. botulinum* strains were subjected to 16S rRNA gene sequencing in other to confirm their identity. The bacterial DNA extracts and control were amplified as described by Wilson et al. [19]. The PCR products were purified using the PCR DNA and gel band purification kit (Illustra, GE healthcare, SP-Brazil) according to manufacturer's instructions. Purified PCR products were sequenced with an automated sequencer ABI 3730 DNA Analyser (Life Technologies- Applied Biosystem) using BigDye ®Terminator V3.1 cycle sequencing kit. The sequences of the PCR products were compared with known 16S rRNA gene sequences in the GenBank (http://www.ncbi.nlm.nih.gov) by multiple sequence alignment using the Clustal W program.6.

3. Results

The food product sampled includes meat and meat products, milk products, vegetables, canned foods and local honey. Majority

Table 1
Base sequence of *Clostridium* primers used in this study.

Genes	Oligonucleotide sequence (5′- 3′)	Annealing temperature	Size of PCR product	Reference
Cpa	AGTCTACGCTTGGGATGGAA; TTTCCTGGGTTGTCCATTTC	56	900	[17]
Cpe	GGGGAACCCTCAGTAGTTTCA; ACCAGCTGGATTTGAGTTTAATG	56	506	[17]
cpb	TCCTTTCTTGAGGGAGGATAAA; TGAACCTCCTATTTTGTATCCCA	56	611	[17]
Etx	TGGGAACTTCGATACAAGCA; TTAACTCATCTCCCATAACTGCAC	56	396	[17]
iap	AAACGCATTAAAGCTCACACC; CTGCATAACCTGGAATGGCT	56	293	[17]
10AF (Bot. Toxin A)	GGGCCTAGAGGTAGCGTARTG; TCTTYATTTCCAGAAGCATATTTT	55	101	[18]
CBML Br (Bot. Toxin B)	CAGGAGAAGTGGAGCGAAAA; CTTGCGCCTTTGTTTTCTTG	55	205	[18]
16S rRNA 27F/1492R	AGAGTTTGATCCTGGCTCAG; ACGGCTACCTTGTTACGACTT	56	1.4 kb	[19]

Table 2
Distribution of food products sampled and isolates.

Food Products	No sampled (%) N = 220	No of positive isolates (%)	No positive for *C.perf.* (%)	No positive for *C.bot.* (%)
Meat/Meat product				
Meat pie	15	4 (26.7)	2 (50)	0 (0)
Sausage roll	10	5 (50)	4 (80)	0 (0)
Spiced meat (*Suya*)	20	2 (10)	1 (50)	1
Raw meat	20	6 (30)	4 (66.7)	0 (0)
Total	**65(29.6)**	**17(34)**	**11(64.7)**	**1(5.9)**
Milk product				
Local yoghurt	20	2 (10)	1 (50)	1 (50)
Raw milk (*Fura dununu*)	20	0 (0)	0 (0)	0 (0)
Total	**40(18.2)**	**2(4%)**	**1(50)**	**1(50)**
Vegetables				
Water leaf	10	4 (40)	2 (50)	0 (0)
Green peas/Green pepper	10	10 (100)	5 (50)	0 (0)
Pumpkin leaf (*Ugu)*	10	4 (40)	1 (25)	0 (0)
Cabbage	10	8 (80)	6 (75)	0 (0)
Carrot	10	2 (20)		0 (0)
Total	**50(22.7)**	**28(56%)**	**14(50)**	**0(0)**
Local canned foods				
Mackerel	10	0 (0)	0 (0)	0 (0)
Sardines	10	2 (20)	2 (100)	0 (0)
Corned beef	10	0 (0)	0 (0)	0 (0)
Tuna	10	1 (10)	1 (100)	0 (0)
Tin tomato	10	0 (0)	0 (0)	0 (0)
Total	**50(22.7)**	**3(6%)**	**3(100)**	0 (0)
Local honey	15**(6.8)**	**0(0)**	0 (0)	**0(0)**
TOTAL	**220(100)**	**50(100)**	**29(58)**	**2(4)**

Bold represents the food categories sampled, the total number and percentage sampled and total isolate from each group.

of the isolates were obtained from vegetables (56%) and meat products (34%). Lower contamination rate was observed in canned foods (6%) and milk product (4%). The local honey sampled had no significant growth of *Clostridium* species. *Clostridium perfringens* were the most common isolates constituting 29 (58%) of the 50 *Clostridium* species isolated and all of them harboured the gene for alpha (*cpa*) toxin confirming them to be *C. perfringens*. One strain (3.4%) harboured the enterotoxin gene (*cpe*) in addition to alpha toxin. Furthermore, one strain also had epsilon toxin gene in addition to alpha toxin. None of the strains tested were positive for beta (*cpb*) and iota (*iap*) toxin genes. As a result, 28 (96.6%) strains were typed as toxin producing *C. perfringens* type A and one (3.4%) *C. perfringens* type D. Out of the 28 strains of *C. perfringens* identified as Type A, only one (3.6%) strain from vegetable harboured the enterotoxigenic *cpe* gene (Table 3). Fig. 1 shows the agarose gel photo with representative bands for *C. perfringens* toxin genes.

Duplex PCR for the detection of botulinum neurotoxin A (*BoNt/A)* and botulinum neurotoxin B (*BoNt/B)* indicated that the two *C. botulinum* were positive for *BoNt/A,* thereby confirming both as *C. botulinum* type A.

PCR analysis of these isolates using 16S ribosomal RNA oligonucleotide primer showed bands at about 1400 bp. 16S rRNA gene sequencing of the *C. botulinum* strains revealed that the two have nucleotide identity to known *C. botulinum* type A. The accession number and percentage nucleotide identity to the closest match in GenBank is shown in Table 4.

4. Discussion

We isolated toxigenic *C. perfringens* most frequently in food products (29/50) in Lagos State. The most prevalent toxinotype was type A detected at the rate of 28/29 (96.6%). *Clostridium perfringens* type A is a spore forming, toxin producing bacterium found in soil, water, dust, sediments and food commodities and is a common inhabitant of the human and animal gastrointestinal tract [20]. Our finding is similar to previous reports by other researchers [21–23]. The food commodity groups "vegetables (56%) and meat products (34%)." pose the highest risk with respect to contamination with *C. perfringens*. This data suggest that these food products maybe an important source of clostridial food borne infection.

Out of the 28 strains of *C. perfringens* identified as type A, only one (3.6%) strain from vegetable harboured the enterotoxin (*cpe+*) gene (Table 3). *Clostridium perfringens* type A isolates producing enterotoxin (CPE) are an important cause of food poisoning and non-food-borne human gastrointestinal (GI) diseases, including antibiotic-associated diarrhoea (AAD) [20]. The predominant disease symptom is watery diarrhoea and abdominal cramp. However, only about 2–5% of *C. perfringens* strains produce enterotoxin gene [20]. In this study, approximately 3.4% of the investigated *C. perfringens* strains possessed the gene encoding the enterotoxin

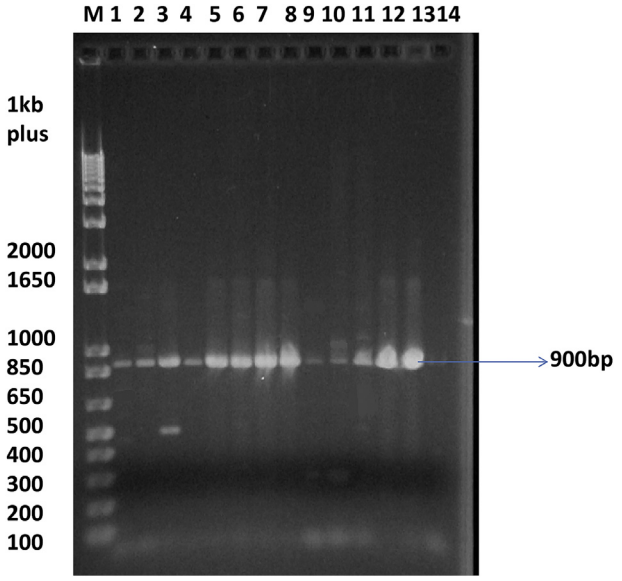

Fig. 1. Agarose gel containing representative amplicon for the detection of *C. perfringens* toxins genes using Multiplex PCR. Lane M: Molecular weight marker (1 kb plus). Lane 13: Positive control *C. perfringens* ATCC 13124. Lane 1–12 were PCR amplicon positive for *cpa* toxin gene with 900 bp. Lane 3 was positive for both *cpa* and *cpe* toxin genes with 900bp and 506 bp respectively.

Table 3
Toxinotyping of *Clostridium perfringens* strains isolated from food commodities.

S/N	Strain No	Food source	Toxin genes	Genotype/Toxin type
1	82C	Yoghurt	*cpa*+	*C. perfringens* type A
2	88C	Meat pie	*cpa*+	*C. perfringens* type A
3	94C	*Suya*	*cpa*+	*C. perfringens* type A
4	95C	Sausage roll	*cpa*+	*C. perfringens* type A
5	96	Sausage roll	*cpa*+	*C. perfringens* type A
6	97A	Sausage roll	*cpa*+	*C. perfringens* type A
7	20C	*Ugu* leaf	*cpa*+ *cpe*+	*C. perfringens* type Ae
8	21A	Water leaf	*cpa*+	*C. perfringens* type A
9	23A	Cabbage	*cpa*+	*C. perfringens* type A
10	27	Cabbage	*cpa*+	*C. perfringens* type A
11	25B	Cabbage	*cpa*+	*C. perfringens* type A
12	32C	Cabbage	*cpa*+	*C. perfringens* type A
13	26A	Green peas	*cpa*+	*C. perfringens* type A
14	35A	Cabbage	*cpa*+	*C. perfringens* type A
15	215	Cabbage	*cpa*+	*C. perfringens* type A
16	37B	Green peas	*cpa*+	*C. perfringens* type A
17	89T	Meat pie	*cpa*+	*C. perfringens* type A
18	39	Water leaf	*cpa*+	*C. perfringens* type A
19	43A	Green peas	*cpa*+	*C. perfringens* type A
20	44A	Green pepper	*cpa*+	*C. perfringens* type A
21	50A	Raw meat	*cpa*+	*C. perfringens* type A
22	91A	Sausage roll	*cpa*+	*C. perfringens* type A
23	99A	Raw meat	*cpa*+	*C. perfringens* type A
24	70B	Canned tuna	*cpa*+	*C. perfringens* type A
25	63A	Sardine	*cpa*+	*C. perfringens* type A
26	62B	Sardine	*cpa*+	*C. perfringens* type A
27	52B	Raw meat	*cpa*+ *etx*+	*C. perfringens* type D
28	22B	Green peas	*cpa*+	*C. perfringens* type A
29	51C	Raw meat	*cpa*+	*C. perfringens* type A

and therefore potentially pathogenic. With this finding, the estimated risk for diarrhoeal disease by *C. perfringens* is less than estimated on the basis of the prevalence of the microorganism. This may be attributed to the fact that the food samples used in this study were non-outbreak random samples. Nevertheless, the obtained result was near to that of previous studies [22,23]. In a study by Gurmu et al. [24], of the 33 *C. perfringens* isolated from foods of animal origin in Guwahati, India, 5 (15.15%) were found to be positive for enterotoxin gene (*cpe*) by PCR. However, higher rate of enterotoxin gene production has been reported in outbreak isolates of *C. perfringens* [21,25]. Genotyping of *C. perfringens* isolates solved the problems of enterotoxin detection which is only produced during sporulation [17]. PCR is more accurate and faster than seroneutralization with mice or guinea pigs. Multiplex PCR assays have been established to genotype *C. perfringens* with respect to the genes *cpa, cbp, etx, iap* and *cpe* encoding the α, β, ε, ¡ and enterotoxins, respectively [17,26].

One strain from raw meat also had epsilon toxin gene in addition to alpha toxin gene. This was identified as *C. perfringens* type D (3.4%). *Clostridium perfringens* type D infection occurs via food, water, animal litter or soil and has been reported to cause severe, often fatal enterotoxaemia mainly in sheep, goat and cattle [22,27]. Its presence in raw meat may be indicative of environmental contamination of the meat with animal litter. Epsilon toxin (ε-toxin) produced by *C. perfringens* types B and D is one of the most potent clostridia toxins. Unfortunately, there is paucity of literature on the role of *C. perfringens* type D in human infections. Nevertheless, ε-toxin producing *C. perfringens* (type D) is regarded as a public health risk since this toxin is considered a potential biological weapon [28]. It damages cell walls causing potassium and fluid leakage from cells and also has the ability to cross the blood-brain barrier attacking the nervous system and leading to neural effects [27,29].

Toxinotyping of the 29 strains of *C. perfringens* showed that all (100%) possessed *C. perfringens* α-toxin gene (*cpa*). Majority of the food isolates were obtained from vegetables (56%) and meat products (34%). This toxin is a 43-kDa protein encoded by the chromosomal *plc* gene and has phospholipase C, sphingomyelinase, and lethal properties [30]. The presence of high level of α-toxin producing *C. perfringens* in food commodities sold in Lagos State (100%) is worrisome since they are involved in initiation and progression of food-borne enterotoxaemia and are also capable of contaminating wounds causing gas gangrene [31]. However, the presence of α-toxin gene alone may not represent a virulence factor of *C. perfringens* type A, other factors, such as adhesion and invasion, could be involved and needs to be investigated [32].

The present study also reveals the presence of toxigenic *Clostridium botulinum*. Duplex PCR for the detection of botulinum neurotoxin A (*BoNt/A)* and botulinum neurotoxin B (*BoNt/B)* indicated that the two *C. botulinum* isolated possessed toxin genes positive for *BoNt/A* thereby confirming both as *C. botulinum* type A. The two toxigenic *C. botulinum* were isolated from local spiced beef (*Suya*) commonly consumed in Nigeria and home bottled street vended yoghurt. Of particular concern is the isolate recovered from the bottled yoghurt. Home canned and bottled food products create

Table 4
Identification of *Clostridium botulinum* isolates by 16S ribosomal RNA (rRNA) gene sequencing.

Isolate	GenBank AC. version number	GI number	Species identification by 16S rRNA gene sequencing	Strain number	% Nucleotide identity[a]
83B	NC_009698.1	GI:153934468	*C. botulinum* type A	ATCC 3502	97.0
92A	NC_009698.1	GI:153934468	*C. botulinum* type A	ATCC 3502	98.0

AC = Accession number.
GI = GenInfo Identifier number.
[a] % Nucleotide identity of 16S rRNA gene sequence to the closest match in GB.

a favourable environment for germination of *C. botulinum* spores and production of neurotoxins. Food-borne botulism develops after consumption of contaminated foods containing botulinum neurotoxin (BoNT) [33]. Nonetheless, there are other forms of botulism in which *C. botulinum* produces toxin *in vivo*, including infant botulism, adult intestinal colonization botulism, and wound botulism [33]. Mazuet et al. [34] suggested that home-canned foods and homemade or small-scale commercial ham are the common source of human botulism [34].

In this study, by using 16S rRNA gene sequencing, we were able to confirm the first report of *C. botulinum* in our local environment. Our findings showed that both strains have nucleotide identity to known *C. botulinum* type A (Table 4). Previously, all clostridia producing neurotoxins able to cause paralysis symptomatic of botulism were believed to be *C. botulinum* [35]. This greatly complicated the identification and classification of this organism. Defining *C. botulinum* only on the basis of BoNT production resulted in the species encompassing a range of metabolically very diverse microorganisms and six phenotypically distinct groups of clostridia are known to be capable of producing botulinum neurotoxin [36]. However, with the availability of molecular techniques, most *C. botulinum* species have been subjected to 16S rRNA gene sequence analysis, and phylogenetic clusters have been defined [37,38]. As PCR and sequencing techniques are becoming more readily accessible, 16S rRNA gene analysis is probably the most practical approach to confirm the identity of *C. botulinum* isolates.

In the last two decades there has been an increase in small and medium industries producing ready to eat foods and drinks. Many of these are hawked on the streets or sold at small shops and stalls. The safety of the production process cannot always be assured. The presence of toxigenic *C. botulinum* in these ready to eat street vended food commodities (*Suya* and Yoghurt) brings to question the hygiene level of these food commodities. The source of contamination in the streets is not clear. Food products may become contaminated with faecal material. Contamination may also occur from the environment or 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.

5. Conclusion

Toxinogenic clostridia contaminated food products are sold in Lagos markets. There is a need to investigate possible clinical cases of *Clostridium* food borne infections in the study area. It is also necessary to educate the public on proper food processing and for ready to eat food commodities to be monitored in order to facilitate proper hygiene and ensure food safety.

Conflict of interest

The Authors declare that no competing interest exists.

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