



Genetic variation among *Clostridium perfringens* isolated from food and faecal specimens in Lagos



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ABSTRACT

Background: *Clostridium perfringens* is an anaerobic Gram-positive bacterium which is commonly present in the gastrointestinal tract of man and animals and causes enteric diseases in animals and food poisoning in humans. Previous studies have looked at the epidemiological relationship between *C. perfringens* isolates from outbreak source. In this study, the genetic diversity of *C. perfringens* strains from non-outbreak food and faecal specimens was investigated for epidemiological purposes.

Methods: We analyzed thirty-eight (38) *Clostridium perfringens* strains isolated from food and faecal specimens in Lagos State. Bacterial identification was done using colonial morphology, Gram stain reaction, conventional biochemical tests and PCR. Genetic analysis was performed using arbitrary primed polymerase chain reaction (AP-PCR) technique with oligonucleotide primer of random sequences (OPA-3) to determine the genetic diversity of *C. perfringens*. The distance between the different bands produced were analyzed using numerical taxonomy and multivariate system software (NTSYS).

Results: Seventeen (44.7%) *C. perfringens* strains showed at least one polymorphic DNA patterns when genotyped. However, this method identified polymorphisms among the *C. perfringens* species from which four genetic groups (1, 2, 3 and 4) were established.

Conclusions: Our findings suggest that there may be faecal contamination of food products and similar clones of *Clostridium perfringens* may be incriminated.

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1. Introduction

Clostridium species are ubiquitous in nature found in soil, decaying vegetation, marine sediment, and the intestinal tract of humans. *Clostridium perfringens*, the most frequent clinical isolate of *Clostridium*, is classified into five types, A through E, according to four major lethal toxin produced (*alpha*, *beta*, *epsilon*, *iota*). They are responsible for a variety of infections including gas gangrene [1], skin and soft tissue infection [2], liver abscess [3], bacteremia and septic shock [1,4,5]. It is also an important pathogen of human gastrointestinal (GI) tract diseases such as food poisoning, antibiotic-associated, and sporadic diarrhoeas as

well as nosocomial diarrhoeal disease outbreaks [6,7].

Toxinogenic *C. perfringens* has been isolated in foods, human faeces, and the environment [8–12]. High numbers may be recovered from food samples during an outbreak but the numbers present in non outbreak related food samples are usually few [11]. The strains involved sometimes vary from one region to another and this is put into consideration when managing related cases especially under epidemic and emergency conditions. Understanding the diversity of toxigenic strains in food products and faecal specimens will lead to a greater appreciation of the epidemiology and pathogenesis of *C. perfringens* which may help in developing effective intervention methods for preventing transmission and outbreaks. Genotyping allocates distinctive labels to bacterial isolates and facilitate identification of transmission routes and sources [13]. Common typing methods that have been used to type *C. perfringens* include Multilocus sequence typing (MLST) [14],

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Multiple-locus variable-number tandem repeat analysis (MLVA) [15], Pulsed-field gel electrophoresis (PFGE) [16,17], Randomly amplified polymorphic DNA (RAPD) typing [18,19], Amplified Fragment Length Polymorphism [20] and Toxinotyping [12].

In Nigeria, food poisoning is not often investigated because many patients tend not to go to hospital. We therefore assessed the occurrence and diversity of pathogenic *C. perfringens* from food and faecal specimens of patients with suspected food poisoning for epidemiological purposes.

2. Materials and methods

2.1. Study design and sample collection

Four hundred and twenty (420) samples comprising 220 food products and 200 faecal specimens were randomly selected from food commodities and faecal specimens of patients with suspected food poisoning indicated by diarrhoea. The study sites include six randomly selected local government areas (Epe, Shomolu, Mushin, Ikeja, Surulere and Lagos Island), two from each of the three senatorial districts in Lagos state (Lagos east, Lagos west and Lagos central). Faecal specimens were collected from the General hospitals located in the selected local governments while food products were collected from different markets and grocery stores located in the six local governments. All samples were collected into a pre-reduced transport medium (Thioglycollate broth) or sterile plastic bag (for food products) and transported to the laboratory for processing within 2 h of collection.

2.2. Bacterial strains

Bacterial colonies showing a double zone of beta-haemolysis on blood agar (Fastidious Anaerobic agar- Lab M), black colonies on Tryptose Sulphite Cycloserine agar-TSC and/or *Clostridium perfringens* agar-CPA (Oxoid, Basingstoke- UK), were Gram stained and identified using conventional biochemical tests. The isolates were further confirmed to be *C. perfringens* by the presence of alpha toxin using PCR as described in our previous study [12].

2.3. Toxin typing of *C. perfringens* using multiplex PCR assay

Bacterial DNA was extracted using GeneJET Genomic DNA Purification kit (Thermo Scientific #K0722 Lot 00232256). The presence of genes encoding the toxins α , β , ϵ , ι and enterotoxin were detected by a multiplex PCR assay as described in our previous study [12]. Alpha-toxin positive *C. perfringens* ATCC 13124 was used as control.

2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility of thirty-eight *C. perfringens* isolates to 5 commonly used antibiotics; Amoxicillin, Clindamycin, Erythromycin, Tetracycline (Luper Ind Farm Ltd, Sao Paulo, SP-Brazil) and Metronidazole (Aventis Farm Ltd, Sao Paulo, SP-Brazil) was determined by agar dilution method as recommended by Clinical Laboratory Standard Institute [21]. The antibiotics were reconstituted according to the manufacturer's instructions and serial two-fold dilutions (ranging from 0.125 to 64 $\mu\text{g/ml}$) were prepared on the day of the test and added to Wilkins-Chalgren agar supplemented with hemin (5 $\mu\text{g/ml}$), vitamin K (1 $\mu\text{g/ml}$). A final inoculum concentration of 1.5×10^5 cfu/spot was delivered using a Steers replicator (Cefar Ltd, Sao Paulo, Brazil). Control plates without drugs were inoculated before and after each drug containing plates. All the plates were incubated in anaerobic atmosphere (90% N_2 , 10% CO_2) at 37 °C for 48 h. A Reference strain

C. perfringens ATCC 13124 was included in each batch as control.

2.5. Determination of genetic diversity of *C. perfringens*

Genetic analysis was performed using arbitrary primed polymerase chain reaction (AP-PCR) technique with a 10-base oligonucleotide of random sequences (OPA-3: AGTCAGCCAC) as described by Llanco et al. [19]. Amplifications were carried out in final volumes of 25 μL in a thermocycler programmed to one cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 42 °C for 2 min, 72 °C for 2 min, and a final extension cycle of 72 °C for 10 min. A negative control without a DNA template was included in each AP-PCR run. Amplified products (20 μL) were separated using 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetic, 2 mM EDTA [pH 8.3]) at 70V for 2.5 h. Gels were stained with 0.5 $\mu\text{g/ml}$ of ethidium bromide for 45 min and washed with water for 20 min. Stained gels were examined under ultra-violet (UV) trans-illuminator and photographed using a digital Kodak Science 120 system. A DNA ladder digest of 1 kb plus and 100 bp (Invitrogen, SP-Brazil) was used as a molecular weight marker.

2.6. AP-PCR data collection and statistical analysis

A rectangular binary data matrix was obtained by scoring each DNA fragment as a discrete variable and statistical analysis was performed using the Numerical Taxonomy and Multivariate Analysis System NTSYS-pc statistical package [22]. Pair wise similarities were computed by the NTSYS program (Applied Biostatistic, inc. version 2.21) using UN1 coefficient of similarity and unweighted pair group method with arithmetic averages (UPGMA) clustering. A dendrogram was developed and a batch mode of NYSYS-pc was used to show the inter-relationship and genetic similarity matrix among the strains. Cross contamination was assessed based on the similarities of the fingerprints of *C. perfringens* isolated from the different sources. Data obtained was used to assess the health risk of these contaminated food products.

2.7. Discriminating index

Simpson's index of diversity was used to determine the discriminating power of AP-PCR in typing local isolates of *C. perfringens* according to the following formula described by Hunter and Gaston [23].

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^S n_j(n_j - 1) \quad (1)$$

Where N is the total number of strains in the sample population for which data were obtained. S is the total number of types/subtypes described. And n is the number of strains on the jth type.

3. Results

Thirty-eight *C. perfringens* strains harbouring alpha toxin were obtained and included in this study, 29 (76.3%) from food and 9 (23.7%) from faecal specimens. Thirty six (94.8%) of these were typed as *C. perfringens* toxin type A, one (2.6%) *C. perfringens* type C and one (2.6%) *C. perfringens* type D. One (2.8%) of the 36 strains of *C. perfringens* identified as Type A, harboured the enterotoxin gene. All *C. perfringens* strains were susceptible to Metronidazole but showed varying degrees of resistance to Amoxicillin (3.3%), Tetracycline (16.7%), Erythromycin (33.3%) and Clindamycin (36.6%). [Table 1.](#)

AP-PCR typing of *C. perfringens* using the primer OPA 3 was able

Table 1
Susceptibility pattern of *Clostridium perfringens* isolates to five commonly used antibiotics.

Antibiotics	^a CLSI ^b MIC (μg/ml)	Range (μg/ml)	^c MIC ₅₀	^d MIC ₉₀	Resistance (%)	Susceptible (%)
Amoxicillin	≥2	0.125–8	≤0.25	0.5	3.3	96.7
Metronidazole	≥32	0.125–8	1.0	4	0	100
Clindamycin	≥8	0.25 – ≥ 64	1.0	≥64	36.6	63.4
Tetracyclin	≥16	0.125–32	2.0	8.0	16.7	83.3
Erythromycin	≥8	4–64	2.0	64	33.3	66.7

^a Clinical Laboratory Standard Institute.

^b Minimum Inhibitory Concentration.

^c MIC at which growth of 50% of the isolates was inhibited.

^d MIC at which growth of 90% of the isolates was inhibited.

to type 17/38 (44.7%) strains. The typeable strains were differentiated into four distinct groups (1, 2, 3 and 4) showing genetic differences that exists among the different strains of *C. perfringens* (Fig. 1). The discriminating capability of this method was high (0.95) as calculated using the Simpson's index of diversity but the degree of amplification by OPA-3 was low (44.7%). S1 Fig. shows fingerprints of *Clostridium perfringens* obtained using primer OPA-3. In general, all amplified and typeable isolates had approximately 20% similarity coefficient. The genetic relatedness of the strains ranged from 30 to 100% indicating a wide genetic variation. Any band differences between two strains were considered sufficient to distinguish between two AP-PCR types and sub-types. Based on the food source, presence of toxin genes and common antimicrobial resistance; the typeable strains of *C. perfringens* were categorized into groups and sub-groups (Table 2).

Group 1 comprises two subgroups A and B. Subgroup A (1A) showed two strains from food and stool with 60% similarities while subgroup B (1B) consists of two strains also from stool and food with about 65% similarity. Group 2 was divided into two sub-groups 2A and 2B. Sub-group 2A harboured four strains which were further sub-divided into two; 2Ai consisting of two strains with 75% similarity and 2Aii with two strains from food belonging

to the same clone (100% similarities). Sub-group 2B contains only one strain. Group 3 harboured two strains from yoghurt and stool with 30% similarity (Table 2). Group 4 consists of six strains which were divided into two sub-groups 4A and 4B. Group 4A harboured four strains with about 90% similarity. Three of the strain which were obtained from food and stool had AP-PCR profiles with indistinguishable pattern (100% similarity), while the fourth strain (4Aii) obtained from raw meat (identified as *C. perfringens* type D) was multi-resistant and belonged to a separate clone. The sub-group 4B contained two strains possessing indistinguishable genetic fingerprints suggesting that they were from the same clone. Interestingly, the strains were obtained from stool samples of two men with diarrhoea though from two different local government areas (Epe and Ikeja respectively).

4. Discussion

The high prevalence of *C. perfringens* type A recorded in this study is consistent with previous reports [24–26]. Approximately 2.6% of the investigated *C. perfringens* strains possessed the gene encoding the enterotoxin and therefore potentially pathogenic. Studies have shown that the occurrence of *cpe* in non-outbreak

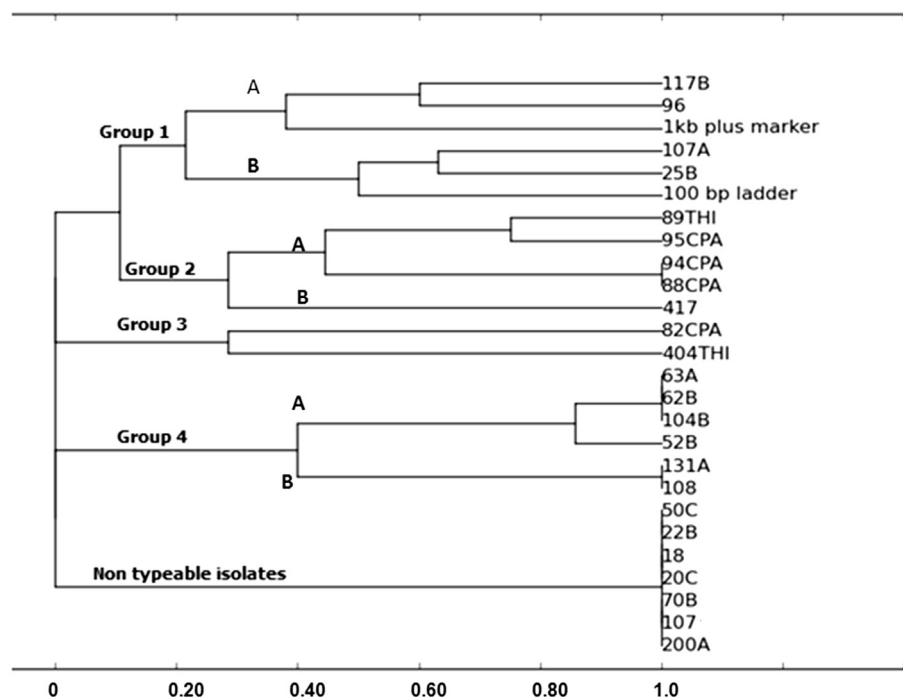


Fig. 1. Dendrogram showing the genetic distance of *C. perfringens* obtained with the use of OPA-3. The tree was constructed using NTSYS statistical program (Applied Biostatistic, inc. version 2.21) to compute the pair wise similarities. The isolates were placed into groups 1, 2, 3, and 4.

Table 2
AP-PCR types detected among *Clostridium perfringens* from food products and faecal specimens in Lagos State.

Strain no	Source	Local government areas	Toxin genes	Resistance to antimicrobials	AP-PCR groups/sub-groups
117B	Stool	Surulere	<i>cpa</i> +	Ery, Tet	1A
96	Sausage roll	Surulere	<i>cpa</i> +	Tet	1A
107A	Stool	Surulere	<i>cpa</i> +	—	1B
25B	Cabbage	Lagos Island	<i>cpa</i> +	—	1B
89THI	Meat pie	Mushin	<i>cpa</i> +	Clin, Ery	2Ai
95CPA	Sausage roll	Epe	<i>cpa</i> +	Clin, Ery	2Ai
94CPA	Suya	Epe	<i>cpa</i> +	—	2Aii
88CPA	Meat pie	Shomolu	<i>cpa</i> +	—	2Aii
417	Stool	Surulere	<i>cpa</i> +	Clin	2B
82CPA	Yoghurt	Ikeja	<i>cpa</i> +	Tet	3
404THI	Stool	Mushin	<i>cpa</i> +	—	3
63A	Sardine	Shomolu	<i>cpa</i> +	Clin, Ery	4Ai
62B	Sardine	Mushin	<i>cpa</i> +	Clin, Ery	4Ai
104B	Stool	Mushin	<i>cpa</i> +	Clin, Ery	4Ai
52B	Raw meat	Mushin	<i>cpa</i> + <i>etx</i> +	Amx, Clin, Ery, Tet	4Aii
131A	Stool	Epe	<i>cpa</i> +	Clin, Tet	4B
108	Stool	Ikeja	<i>cpa</i> +	Clin, Tet	4B

Key:

Amx	Amoxicillin
Ery	Erythromycin
Tet	Tetracycline
Clin	Clindamycin
Cpa	<i>Clostridium perfringens</i> alpha toxin
EtX	<i>Clostridium perfringens</i> epsilon toxin

strains is generally low compared with out-break strains of *C. perfringens* [10,27,28]. In an Iranian study, none of the *C. perfringens* type A isolated from non-outbreak food and human sources harbored the enterotoxin gene [29]. In our study, the estimated risk for *C. perfringens* food poisoning is less than estimated on the basis of the prevalence of the microorganism judging by the low prevalence (2.6%) of *cpe* positive *C. perfringens* recorded. Most of the strains were susceptible to Metronidazole (100%) and Amoxicillin (96.7%) which are the recommended drugs of choice for treating Clostridial infections (Table 1). These results are in keeping with the findings of Kouassi and colleagues [30] who reported Metronidazole and Penicillin G as the most potent agents against *Clostridium perfringens* and *Clostridium difficile* isolated from cooked beef sold in Cote d'Ivoire. Nonetheless, there is need for periodic antimicrobial susceptibility testing and surveillance to detect geographic or temporal changes in the resistance trends.

Several molecular methods have been used for typing *C. perfringens* in attempts to differentiate pathogenic strains from commensals and as an epidemiology tool to identify clusters or strains that are associated with disease outbreak [15,17,19,20]. Leflon-Guibout et al. [18] assessed the discriminatory capability of AP-PCR for typing *C. perfringens* strains and observed a discriminatory index of 0.97. The authors suggested that AP-PCR can be used as an epidemiological tool for *C. perfringens*. The aim of the present study was to demonstrate the general genetic diversity of *C. perfringens* isolated from food and faecal specimens in Lagos state, Nigeria. From our observations, 44.7% of the strains were typeable with OPA-3 with high (0.954) numerical index of discrimination. Contrary to our findings, Llanco and workers [19] recorded 100% typeability for *Clostridium perfringens* Type A using OPA-3. They recommended OPA 3 primer for Arbitrary primed PCR typing of *Clostridium perfringens*. The low typeability recorded in this study highlights the need for the local development of primers using our local strains.

Cluster analysis based on the genetic distance identified and established four distinct groups (1, 2, 3 and 4). The wide genetic diversity recorded among *C. perfringens* isolates in this study was not un-expected considering the wide variety of samples analyzed and the fact that these samples were non-outbreak random

samples. However, a number of strains showed high genetic similarity and clonal relationship. Comparison of the AP-PCR pattern of food and faecal isolates revealed clonal relationship among some isolates (group 1A&B, 4Ai) which cluster together in the dendrogram (Fig. 1) suggesting the possibility of faecal contamination of food. The result obtained could not establish any definite relationship between the source of isolates, the origin (local government areas) and the positions in the dendrogram.

Of particular concern is strain (4Aii) obtained from raw meat (*C. perfringens* type D) which was multi-resistant (Amoxicillin, Clindamycin, Erythromycin, Tetracycline) and belonged to a separate clone from the group. *Clostridium perfringens* type D has been reported to cause severe, often fatal enterotoxaemia mainly in sheep, goat and cattle [31]. Its presence in raw meat may be suggestive of contamination of the meat with animal litter. Unfortunately, there is paucity of literature on the role of *C. perfringens* type D in human infections. Based on the results from this study, some of the isolates from different sources may have originated from a single clone and transmitted through faecal contamination of food.

Previous studies have defined different clonal types among *C. perfringens* species [19,32]. Afshari et al. [29] used Randomly Amplified Polymorphic DNA (RAPD) technique to investigate the relatedness among 49 *C. perfringens* type A isolates from food and human source. They were able to divide the strains into 5 major groups based on genomic diversity. Different commercial OPA primers have been used to study bacterial cells but not every arbitrary primer produced useful banding pattern following amplification [33]. We decided to use OPA-03 based on high discriminating capacity and typeability observed in a previous study [19]. Based on the Simpson's index of diversity [23] this primer was quite rapid and discriminatory but showed low level of typeability because visible bands were observed in only 44.7% (17/38) of isolates studied. Further studies can be aimed at evaluating other typing techniques for their suitability for typing our local strains of *C. perfringens*.

5. Conclusion

Although high level of clonal differences was observed among

species, some of the isolates were genotypically identical and belonged to the same clone. Our findings suggest that there may be faecal contamination of food products and these similar clones may be associated with *Clostridium perfringens* food intoxication. However, there is a need for further verification.

Ethics approval and consent to participate

Ethical approval for this study was obtained from ethical Research Grants and Experimentation Ethics Committee of College of Medicine, University of Lagos (CM/COM/08/VOLXXV, 2014) All participants signed an informed consent form.

Consent for publication

Not Applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The Authors declare that no competing interest exists.

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Authors' contributions

The main contributor (EEC) designed the study, collected samples, carried out the conventional culturing and molecular analysis, wrote the draft of the manuscript. The second author (FON) participated in molecular assays and supervised the work. The authors AOC, FTO and MJA supervised the work, reviewed the drafts and provided suggestions. All authors contributed to the literature searches and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.micpath.2017.08.031>.

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