Diversity of toxin genotypes and antimicrobial susceptibility of *Clostridium perfringens* isolates from feces of infants

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Abstract

Introduction **This study investigated the most important epidemiological characteristics of *C. perfringens* strains colonizing the intestine of Jordanian infants.**

Methods **A total of 302 fecal samples were collected from Jordanian infants aged ≤ 1 year from patients hospitalized in the neonatal intensive care unit and from the outpatient department. Samples were cultured for detection of *C. perfringens* and evaluation of their antimicrobial resistance; identification of their potential toxins genes was performed using PCR.**

Results **Overall the *C. perfringens* colonization rate was 27.2% (82/302). Infants aged ≤ 6 months showed significantly higher ($p<0.004$) colonization than older infants. The occurrence rates of *C. perfringens* isolates carrying potential specific toxin genes were as follows: alpha toxin 95.1% (78/82), beta-2 toxin 69.5% (57/82), beta toxin 14.6% (12/82), and only 2.4% (2/82) epsilon toxin. No isolate carried the iota toxin. Genotype A was the most prevalent among *C. perfringens* isolates (78.1%). The antimicrobial resistance rates of *C. perfringens* isolates were 20% to metronidazole and erythromycin, 16.7% to levofloxacin, and 6.7% to vancomycin.**

Conclusions **This study demonstrates that the majority of *C. perfringens* isolates from feces of Jordanian infants were classified as type A, few isolates were classified as type C and type D, and all were negative for potential enterotoxin genes causing diarrhea.**

Keywords ***Clostridium perfringens*, infants feces, toxins genotypes, antimicrobial resistance.**

Introduction

Clostridium perfringens is a Gram-positive, non-motile, anaerobic bacillus widely present in the gastrointestinal tract of healthy humans and animals.[1-2] C. *perfringens* has been associated with a wide variety of pathological conditions that include gas gangrene, necrotic enteritis, food poisoning and sporadic cases of antibiotic-associated diarrhea.[3-4]

The importance of this organism arises from its ability to produce many toxins. Various combinations of C. *perfringens* toxin genes are localized on either the chromosome or within transferable plasmids that may possess lethal hemolytic and necrotizing properties.[5]

The association of many toxin genes with insertion sequences and conjugative plasmids enhances the virulence of the organism in causing intestinal infections.[6]

C. *perfringens* strains carrying enterotoxin (CPE) gene on the chromosome are more heat-resistant than those carrying this gene on a plasmid; these are associated with human foodborne disease.[5] Currently, there are five different confirmed toxigenic types of C. *perfringens*, named A-E.[5,7] However, a recent study has reported that two additional candidates of toxinotypes may be included in a future list.[8]

Received: 05 November 2018; revised: 13 January 2019 and 29 Jan 2019; accepted: 05 February 2019.

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Published March 2019

ISSN 2248 – 2997
ISSN – L = 2248 – 2997

This study investigated the occurrence rate of C. *perfringens* isolates and their potential toxins in fecal samples of Jordanian infants aged ≤ 1 year using culture and polymerase chain reaction (PCR) procedure.

Methods

Study design and population

This cross-sectional study included 302 infants ≤ 1 year of age in the Pediatrics Department, The Jordan University Hospital (JUH) in Amman, Jordan, over a 10-month period from May 2016 through February 2017. A total of 302 fresh fecal samples from soiled diapers were collected randomly using cotton swabs from infants hospitalized in the neonatal intensive care unit (NICU) or examined in the outpatient department (OPD). All fecal specimens were sent within 2-3 h to research microbiology labs. For each sample, the following data were recorded: date of collection, patient number, hospital ward, mode of delivery, infant's name, sex, age, birth weight, type of feeding, presence of diarrhea, and the recent use of one or more antibiotics. Permission for the study was obtained from the Ethical Review Board (ERB) at the JUH, no. 118/May 2016. Verbal consent was obtained from all mothers of infants after explaining the aim of the study.

Culture and identification

Fresh fecal samples were treated by absolute ethanol (v/v) for 1 hour before inoculation onto Perfringens Tryptose-Sulphite-Cycloserine (TSC) agar plates with egg-yolk (EY) (Oxoid, Altrincham, England). Samples were carefully mixed, and 0.1 mL inoculum was directly inoculated on EY-TSC agar plates and incubated in anaerobic jar for 48 hours at 37°C by using Anaerobe bag (AnaeroGen AN25; Oxoid). All suspected colonies resembling C. *perfringens* which appeared black and surrounded by egg yolk precipitate were examined by the catalase-negative reaction. Five to ten colonies suspected to be C. *perfringens* were picked from each plate and streaked on blood agar plates (Oxoid) supplemented with human blood (5%), and incubated for 48 h at 37°C under anaerobic conditions. All isolates that formed white colonies surrounded by hemolysis and displayed Gram-positive rod shape with subterminal spores were identified primarily as C. *perfringens* isolates.[9] C. *perfringens* control strain (CMUL Clo. Per. SL IX-004) was obtained from Prof. Monzer Hamze, Laboratoire de Microbiologie Santé et Environnement (LMSE), Faculty of Public Health, Lebanese University, Tripoli, Lebanon. The control strain contains the toxins alpha, beta and epsilon and was included for quality control in biochemical identification of C. *perfringens* isolates, antibiotic susceptibility and as a positive control in genetic identification of C. *perfringens* toxins. All 82 C. *perfringens* isolates were inoculated in 1.8 mL of brain-heart infusion broth (Oxoid) with 20% glycerol and were stored at -70°C for further characterization of the toxin genes.

Antimicrobial susceptibility testing

All C. *perfringens* isolates were tested using E-test. The minimum inhibitory concentration (MIC) breakpoints were used to indicate the resistance to each tested drug according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI). [10]

DNA and plasmid extraction

All C. *perfringens* isolates stored in cryogenic tubes at -70°C were thawed at room temperature and cultured onto TSC agar. After incubation at 37°C for 48 h, a few colonies (6-7 colonies) were picked from the agar and inoculated into 5 mL Mueller Hinton broth and incubated at 37°C overnight. According to the manufacturer's protocol, the bacterial DNA was isolated using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA); bacterial plasmid was extracted using EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic, Markham, ON, Canada).

16S rDNA confirmation for *C. perfringens* identification

PCR confirmation for C. *perfringens* isolates was performed by amplification of the 16S rRNA gene using C. *perfringens* specific primers

(Table 1). The PCR assay was performed at the final volume of 20 µL using the following mixture: 4 µL of KAPPA Fast ready mix (5X) master mix (Solis BioDyne, Tartu, Estonia), one pair of primers were used to detect 16S rDNA. The primer concentration used was 10 µL of each primer, 12 µL of nuclease free water, and a volume of 2 µL of the extracted DNA. A PCR mix with DNA from the C. *perfringens* control strain was used as positive control, whereas a PCR mix containing nuclease free water was used as negative control. All samples were amplified in a PCR thermal cycler (Bioer XP Cycler, Hangzhou, China), with cycling condition of the initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min then extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products, controls and 100 bp ladder marker (Biolabs, Ipswich, MA, USA) were detected in 2.0% agarose gel (Promega) stained with red safe (CONDA Pronadisa, Madrid, Spain).

Detection of toxin genes using PCR

All primers and PCR assays for the targeted toxin genes were carried out according to Asten et al.,[11] as shown in Table 1. A total volume of 20 µL was used for each reaction, with the following mixture: 4 µL of the KAPPA Fast ready mix (5X), 12 µL of nuclease free water, and a volume of 2 µL of the extracted genetic materials (DNA or plasmid). One pair of primers was used to detect the toxin gene. The primer concentration used was 10 µL of each primer. The primers, methods and their references used for detection of the different toxin genes with the anticipated size of the amplified product are presented in Table 1. The PCR products were detected using 2.0% agarose gel.

Statistical analysis

Data generated from the study were tabulated with Microsoft Excel and uploaded to Statistical Package for Social Sciences (SPSS, version 20, IBM Corp, Armonk, NY, USA).

Frequency and percentage were calculated for the categorical data, and Pearson's Chi-squared test or Fisher's exact test were applied to determine potential factors associated with C. *perfingens* and to determine whether there are any statistical differences between groups. The level of significance was set at a $p<0.05$ to test the hypothesis of no association. Fisher's exact test replaced the Chi-squared test when the minimum expected count was less than five.

Results

A rate of 27.2% (82/302) positivity for C. *perfringens* was recovered from fecal samples of examined infants aged less than one year. The rate of intestinal colonization was significantly associated with certain neonatal conditions related to low age (≤ 6 months), birth weight, absence of diarrhea and caesarean delivery ($p<0.05$), as shown in Tables 2 and 3. The distribution of toxin genes among the 82 C. *perfringens* isolates is shown in Table 4. A total of 78 C. *perfringens* isolates (95.1%) were positive for the alpha toxin gene, followed by 69.5% for the β2-gene, but only 12 (14.6%) and 2 (2.4%) were positive for the beta and epsilon toxin genes (Table 4). All C. *perfringens* isolates were negative for both iota and enterotoxin genes (Table 4). Toxigenic C. *perfringens* isolates were classified into 5 toxin genotypes based on the presence of the four main toxins detected using PCR (Table 5). Genotype A was the most prevalent type (78.1%), followed by genotype C (14.6%) and genotype D (2.4%). The other 2 genotypes (B and E) were not found. Genotype D was found in infants aged 9 and 10 months old who were fed with formula and mix.

Most C. *perfringens* isolates were susceptible to vancomycin, levofloxacin, metronidazole, and erythromycin. The resistance rate was 6.7% to vancomycin and levofloxacin, 16.7% to metronidazole, and 20% to erythromycin, respectively (Table 6).

Discussion

Detection of C. *perfringens* and its various toxins became straightforward after introducing the PCR typing method, which was first developed by Yoo et al.[12] This study shows that intestinal colonization with *Clostridium perfringens*

Table 1. Oligonucleotide primer sequences used in this study

Toxin gene	Primers	Sequence (5'-3')	Product size (bp)	Reference
16S rRNA	16S rRNA F 16S rRNA R	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279	7
cpa (α-toxin)	CPAlphaF CPAlphaR	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	11
cpb (β-toxin)	CPBetaF 3CPBetaR3	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	195	11
etx (ε-toxin)	CPEpsilonF CPEpsilonR2	TGGGAACTTCGATACAAGCA AACTGCACTATAATTTCCTTTTCC	376	11
iap (ι-toxin)	CPIotaF2 CpIotaR	AATGGTCCTTTAAATAATCC TTAGCAAATGCACTCATATT	272	11
cpe (enterotoxin)	CPEnteroF CPEnteroR	TTCAGTTGGATTTACTTCTG TGTCCAGTAGCTGTAATTGT	485	11
cpb2 (β2-toxin)	CPBeta2totalF2 CPBeta2totalR	AAATATGATCCTAACCAAM[a]AA CCAAATACTY[d]TAATYGATGC	548	11

Table 2. Demographic characteristics of 302 infants with positive *C. perfringens* cultures

Variables		No. (%) positive for *C. perfringens*	P-value	OR (95%CI)
Age	1-29 days 1-12 months	47/213 (22.1) 35/89 (39.3)	0.002	0.437 (0.256, 0.746)
Gender	Male Female	47/170 (27.6) 35/97 (26.5)	0.826	1.059 (0.635, 1.767)
Diarrhea	Yes* No	0 82/265 (30.9)	<0.001	
Antibiotic treatment	Yes No	6/95 (6.3) 76/207 (36)	<0.001	0.116 (0.049, 0.278)
Birth weight	≤ 2500 g > 2500 g	51/134 (38.1) 31/168 (18.5)	<0.001	2.716 (1.61, 4.58)
Mode of delivery	Vaginal delivery Caesarean section	27/144 (18.8) 55/158 (34.8)	0.001	0.432 (0.254, 0.735)
Type of feeding	Breastfeeding Formula and mix	23/97 (23.7) 59/205 (28.8)	0.355	0.769 (0.441, 1.343)

OR – odds ratio; **95%CI** – 95% confidence interval.
*37/302 (12.3%) had diarrhea but all were negative for *C. perfringens*.

was detected in 27.2% of feces from infants aged ≤ 1 year at JUH. Infants aged ≤ 6 months had a higher (p<0.004) colonization rate than older infants. A similar study from Japan found *C. perfringens* in the feces of 18.2% healthy infants approximately 30 days old.[13] A three-year follow up study of 89 healthy infants in Japan showed that the incidence of early life intestinal colonization with α-toxigenic and enterotoxigenic *C. perfringens* was found only in 3% of infants at one week of age, and was later increased to 33-39% after 1-3 months.[14] This increased incidence probably occurred because the infant's gut is first colonized primarily by facultative anaerobes that create a reduced environment and contribute to the establishment of strict anaerobic bacteria in the intestine, including *Clostridium* species.[14]

The present study shows that the *C. perfringens* colonization rate was lower but not significantly in breast-fed compared to formula-fed infants (23.7% versus 28.8%). We found also that infants born by caesarean delivery were significantly (p=0.001) more frequently colonized by toxigenic *C. perfringens* than those born by vaginal delivery (Table 2). These results are in agreement with a recent Japanese study, which

Table 3. Distribution of 82 *C. perfringens* isolates in fecal samples of infants according to their age

Age group*	Colonized infants no. (%)	P-value	OR (95%CI)
1-29 days	47/213 (22.1)	0.001	0.437 (0.256, 0.746)
1-3 months	17/40 (42.5)	0.019	2.24 (1.127, 4.451)
4-6 months	14/32 (43.7)	0.025	2.31(1.091, 4.894)
7-9 months	3/11 (27.3)	>0.999	1.006 (0.260, 3.889)
10-12 months	1/6 (16.7)	0.964	0.531 (0.061, 4.613)
Total	82/302 (27.2)		

OR – odds ratio; **95%CI** – 95% confidence interval.
*1-6 months 78/285 (p-value=0.004)

Table 4. Distribution of potential toxin gene types among 82 *C. perfringens* isolates

Type of gene	No. (%) of positive isolates
α-gene	78 (95.1)
β-gene	12 (14.6)
β2-gene	57 (69.5)
ε-gene	2 (2.4)
ι-gene	0
enterotoxin	0

has also reported that caesarean-born infants had a higher carriage of C. *perfringens* compared to vaginally-born infants.[14] However, the Japanese study demonstrated that the rate of colonization by C. *perfringens* was much lower in breast-fed (17%) than in formula-fed infants (43%). It appears that the composition of *Clostridium* species in the fecal microbiota of infants can be affected by the type of feeding during the first month of birth. Higher carriage of toxigenic C. *perfringens* in caesarean-born infants needs further investigation of its sources and potential clinical significance.

This study shows that there was a significant association ($p<0.05$) between C. *perfringens* colonization and certain neonatal condition related to age, birth weight, mode of delivery, and hospital ward, while the presence of diarrhea, antibiotic treatment, type of feeding and gender were not significantly associated with C. *perfringens* colonization ($p>0.05$).

The pathogenicity of C. *perfringens* is related to the potential presence of its lethal toxin genes. The pattern of potential toxin genes is different depending on the C. *perfringens* toxigenic type prevalent in each community, and it is still unknown why such difference occurs and which conditions activate these genes.[5-6,15]

The present study indicates that almost all C. *perfringens* isolates were positive for potential presence of the a-toxin gene (95.1%), followed by β2-toxin gene (69.5%), whereas the other toxin genes were detected in few samples, and no single isolate was positive for iota or enterotoxin genes (Table 3). All our C. *perfringens* isolates were obtained from infants without diarrhea, and this might explain the absence of enterotoxin-producing isolates in their intestine. It has been reported that α-toxin, encoded by the *cpa* gene, is common to all 5 C. *perfringens* types (A–E),[5] and especially C. *perfringens* type A can cause fatal diseases in human and several animal species.[5-6]

This study shows also that 78.1% of C. *perfringens* isolates can be classified for epidemiological purposes as genotype A and *cpe*-negative (PCR positive for the alpha toxin gene but negative for genes encoding beta, epsilon, iota, and enterotoxins) as reported by Badagliacca et al.,[15] whereas the other B to E genotypes were found less commonly (0-14.6%) in our isolates (Table 4).

It is well documented that enterotoxigenic C. *perfringens* is one of the most common causes of

Table 5. Classification of toxin genotypes among 82 *C. perfringens* isolates

C. perfringens genotype	Toxin type	No. (%) of positive *C. perfringens*
A	alpha	64 (78.1)
B	alpha, beta and epsilon	0 (0)
C	alpha and beta	12 (14.6)
D	alpha and epsilon	2 (2.4)*
E	alpha and iota	0 (0)
***Clostridium* genotype not confirmed**	-	4 (4.9)

*The isolates were found in infants aged 9 and 10 months, respectively, who were fed with formula and had not been treated with antibiotics.

Table 6. Antimicrobial MIC for 30 *C. perfringens* isolates

Antimicrobial agents	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Resistance breakpoint (µg/mL)	MIC (µg/mL) range	No. (%) resistant
Vancomycin	0.49	0.89	32	0.5-4	2 (6.7)
Metronidazole	0.47	0.84	32	0.19-8	5 (16.7)
Erythromycin	0.83	1.49	8	0.5-16	6 (20)
Levofloxacin	0.61	1.1	8	0.19-3	2 (6.7)

food-poisoning in humans worldwide.[4-6] A study in Jordan reported that no enterotoxin-positive C. *perfringens* found in clinical cases was associated with necrotic enteritis in commercial poultry using multiplex PCR, since all their isolates were classified as enterotoxin negative type A.[16] It would be highly important epidemiologically to initiate a large follow up study to search for the incidence of toxin types among C. *perfringens* isolates from food sources and food-poisoning cases in Jordan.

Most published antimicrobial susceptibility studies tested isolates from food items or animals. This study demonstrates that most C. *perfringens* isolates from fecal samples of infants were susceptible to vancomycin, levofloxacin, metronidazole, and erythromycin (Table 5). The minimum inhibitory concentrations for 50% of isolates (MIC_{50}) for vancomycin, levofloxacin, metronidazole, and erythromycin were 0.49, 0.61, 0.47, and 0.83 µg/mL, respectively. Only 22% of C. *perfringens* isolates were resistant to erythromycin. A case study of clindamycin-resistant C. *perfringens* human cellulitis was reported by Khanna in England, while it is well known that C. *perfringens* is highly susceptible to all penicillin drugs.[17]

A previous study done in Jordan found that all C. *perfringens* isolates from animal sources showed a very high minimal inhibitory concentration (MIC_{50}> 256 mg/mL) for lincomycin, erythromycin, and tilmicosin.[16] A recent study has reported that all C. *perfringens* isolates from cooked beef sold in Côte d'Ivoire were vancomycin susceptible, and 80% and 45% of the isolates were resistant to norfloxacin and erythromycin, respectively.[18] A recent study done also in Brazil found that all C. *perfringens* isolates from insects (*Tinamidae*, *Cracidae* and *Ramphastidae* species) were susceptible to vancomycin and metronidazole, while 22.2% were resistant to erythromycin.[19] Additionally, a recent study from Thailand reported that 54.9% of C. *perfringens* isolates from diarrheal newborn piglets were resistant to erythromycin and exhibited a high MIC_{50} value of 128 µg/mL.[20] A study done in Egypt found that 34%, 46%, 58%, 67%, 94% and 98% of C. *perfringens* isolated from broiler chickens were resistant to rifampicin, chloramphenicol, colistin, ciprofloxacin, norfloxacin, and doxycycline, respectively.[21] Overall, studies from various countries showed generally an elevated rate of antibiotic resistance among C. *perfringens* and among other bacterial pathogens isolated from poultry.[21-22]

Conclusions

This study demonstrated a high diversity of potential toxin genes found among *C. perfringens* isolates from fecal samples of Jordanian infants, but no single infant carried an isolate with potential enterotoxin genes causing diarrhea.

Authors' contributions statement: AAR performed all laboratory tests, EFB supervised all clinical issues of patients, AAS supervised all laboratory tests and writing of the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest: All authors – none to disclose.

Funding: This work was supported by grant from The University of Jordan, Dean of Research (No. 15/2016).

Acknowledgment: We are thankful for the Jordan University Hospital for allowing us to collect clinical specimens and data from the admitted patients.

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Please cite this article as:
AL Radaideh AJ, Badran EF, Shehabi AA. Diversity of toxin genotypes and antimicrobial susceptibility of *Clostridium perfringens* isolates from feces of infants. GERMS 2019;9(1):28-34. doi: 10.18683/germs.2019.1154.