



## Detection of *Clostridium perfringens* toxin genes in the gut microbiota of autistic children



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### ARTICLE INFO

#### Article history:

Received 7 November 2016

Received in revised form

5 February 2017

Accepted 11 February 2017

Available online 17 February 2017

Handling Editor: Laura M Cox

#### Keywords:

Autism

*Clostridium perfringens*

Toxin gene

Beta2 toxin

Microbiome

### ABSTRACT

We studied stool specimens from 33 autistic children aged 2–9 years with gastrointestinal (GI) abnormalities and 13 control children without autism and without GI symptoms. We performed quantitative comparison of all *Clostridium* species and *Clostridium perfringens* strains from the fecal microbiota by conventional, selective anaerobic culture methods. We isolated *C. perfringens* strains and performed PCR analysis for the main *C. perfringens* toxin genes, alpha, beta, beta2, epsilon, iota and *C. perfringens* enterotoxin gene. Our results indicate that autistic subjects with gastrointestinal disease harbor statistically significantly ( $p = 0.031$ ) higher counts of *C. perfringens* in their gut compared to control children. Autistic subjects also harbor statistically significantly ( $p = 0.015$ ) higher counts of beta2-toxin gene-producing *C. perfringens* in their gut compared to control children, and the incidence of beta2-toxin gene-producing *C. perfringens* is significantly higher in autistic subjects compared to control children ( $p = 0.014$ ). Alpha toxin gene was detected in all *C. perfringens* strains studied. *C. perfringens* enterotoxin gene was detected from three autistic and one control subject. Beta, epsilon, and iota toxin genes were not detected from autistic or control subjects.

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## 1. Introduction

Intestinal clostridia contribute to the clinical picture of autism [1]. Our microbiological studies have revealed a higher incidence and higher counts of clostridia in autism subjects' fecal samples compared to control samples and there is a significantly reduced overall bacterial diversity in the feces of the autistic group children [2–4]. Real-time PCR studies showed that *Clostridium* cluster I was statistically significantly higher in counts in autistic children in comparison to control children [4]. Similarly, Parracho *et al.* compared the fecal microbiota of autistic children with healthy

siblings and unrelated healthy children and noted a higher incidence of *Clostridium* clusters I and II in autistic children than in healthy controls, but an intermediate level of these clostridia in siblings of the autistic children [5].

It is generally recognized that cluster I forms the basis of the genus *Clostridium* [6]. *Clostridium perfringens* is a clinically important *Clostridium* species within *Clostridium* cluster I. *C. perfringens* are anaerobic, Gram-positive, and spore-forming bacteria which are common in many different microbiota, and are found in soil, marine sediment, decaying vegetation, and in the intestinal tract of humans and other organisms. They are thought to be among the most common pathogens in existence, and have a short generation time, enabling them to proliferate very rapidly, given optimal conditions. *C. perfringens* is classified by strain types, separated into 5 groups, denoted A–E. These groups are established based on which particular toxins each one can produce. The major toxins used for strain classification are alpha toxin, beta toxin, epsilon toxin, and iota toxin [7]. While these toxins are used for grouping purposes and are responsible for most of the symptoms in the

**Abbreviations:** AAD, antibiotic associated diarrhea; ASD, autism spectrum disorder; ATCC, American Type Culture Collection; CFU, colony forming units; CPE, *Clostridium perfringens* enterotoxin; DNA, Deoxyribonucleic acid; GI, gastrointestinal; PCR, Polymerase chain reaction; SD, sporadic diarrhea; UV, ultra violet.

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variety of diseases caused by *C. perfringens*, strains are able to produce an assortment of other toxins as well as enterotoxins. *C. perfringens* can cause a variety of symptoms depending on the strain and toxin present, resulting in a range of conditions from mild enteric disease to severe soft tissue necrosis and gas gangrene. More recently, a new *C. perfringens* toxin, beta2-toxin, was described from animal and human disease, especially antibiotic-associated gastrointestinal disease [8,9]. *C. perfringens* can be present in the gut microbiota of humans and animals alike without the host individual displaying any negative signs or symptoms.

This study examined the presence of *C. perfringens* and *C. perfringens* toxin genes in the gut of autistic children in comparison with control children, utilizing conventional culture and PCR methods. We describe the importance of the *C. perfringens* beta2-toxin in autism.

## 2. Materials and methods

### 2.1. Sample collection and subject characteristics

This study used previously processed and homogenized stool specimens stored frozen at  $-80^{\circ}\text{C}$ . We studied stool specimens from 33 children diagnosed with autism spectrum disorder (ASD) aged 2–9 years with GI abnormalities and 13 age- and sex-matched control children without autism and without GI symptoms. The study was carried out in accordance with the guidelines of the Institutional Review Board of the VA Greater LA Health Center.

### 2.2. Clostridium culture

We performed quantitative comparison of *Clostridium* species and specifically *C. perfringens* strains from the fecal microbiota of autistic and control children by conventional, selective anaerobic culture methods. Aliquots of the homogenized specimens were diluted 1:10 in pre-reduced thioglycollate broth (Anaerobe Systems, Morgan Hill, CA). For the selective isolation of *Clostridium*, these diluted aliquots of the stool specimens were processed two ways, utilizing ethanol and heat treatments, to select spores. For ethanol treatment, a 1 ml aliquot of thioglycollate broth was incubated with 100% ethanol for 10 min. For heat treatment, the rest of the thioglycollate tube was incubated at  $80^{\circ}\text{C}$  for 10 min. Subsequently, 10-fold serial dilutions were plated (100 $\mu\text{l}$ /plate) onto Brucella and CDC agar plates (Anaerobe Systems) from both preparations. The plates were incubated under anaerobic conditions at  $37^{\circ}\text{C}$  for 72 h before initial inspection and re-incubated up to 7 days [10]. Anaerobic conditions consisted of a gas mixture of 5%  $\text{CO}_2$ , 5%  $\text{H}_2$ , and 90%  $\text{N}_2$ ; residual oxygen was removed by palladium catalysts. *Clostridium* colonies were counted and the counts were adjusted to dry weight of stool. Similarly, characteristic *C. perfringens* colony types were counted. These consisted of large, usually irregular or spreading, colonies surrounded by a double zone of  $\beta$ -hemolysis. The counts were adjusted to dry weight of stool. Single *C. perfringens* colonies were selected, described and subcultured on Brucella agar and incubated for 48 h, as described above. Additionally, as a back-up enrichment culture, the heat-treated thioglycollate tubes were incubated at  $37^{\circ}\text{C}$  for 72 h and then let stand at room temperature for one week before subculturing.

### 2.3. Reference strains

*C. perfringens* reference strains ATCC 3626 and ATCC 14809 were obtained from the American Type Culture Collection (ATCC). The strains were grown 48 h on Brucella agar and processed as described below.

### 2.4. DNA extraction

For PCR analysis of *C. perfringens*, several single colonies obtained after 48 h cultivation on Brucella agar were inoculated into 250  $\mu\text{l}$  nuclease-free water and boiled for 20 min to lyse the cells. After centrifugation at 13000g for 5 min at room temperature, the supernatant fluid was removed and used in the PCR reaction.

### 2.5. Toxin gene PCR

We performed PCR analysis for the main *C. perfringens* toxins to detect the genes for *C. perfringens* alpha-toxin (*cpa*), beta-toxin (*cpb*), beta2-toxin (*cpb2*), iota-toxin (*iA*), and enterotoxin (*cpe*) using primer pairs as shown in Table 1. The *C. perfringens* strains were typed utilizing methods previously described [11,12] with the exception that instead of multiplex PCR, single PCRs were performed with each primer pair. The PCR conditions were as follows: DNA was denatured for 2 min at  $95^{\circ}\text{C}$  and amplified for 35 cycles (1 min at  $94^{\circ}\text{C}$ , 1 min at  $55^{\circ}\text{C}$ , 1 min at  $72^{\circ}\text{C}$  for denaturation, annealing and extension phases, respectively), followed by an additional period of extension for 10 min at  $72^{\circ}\text{C}$ . PCR products were separated by electrophoresis in a 2% (w/v) agarose gel stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

### 2.6. Verification of PCR products

Verification of the PCR products was done by DNA sequencing of purified PCR product. The PCR products were excised from an agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were sequenced directly with a Biotech Diagnostic Big Dye sequencing kit (Biotech Diagnostics, CA) on an ABI 3130 Avant sequencer (Applied Biosystems, Foster City, CA). The sequencing data were analyzed by comparison of the consensus sequences with GenBank sequences by using Ribosomal Database Project (RDP-II) (Michigan State University, East Lansing) [13], and Basic Local Alignment Search Tool (BLAST) [14]. Analyses of the sequences were performed by comparing with the sequences of the type strains retrieved from GenBank by using the program Clustal W [15].

### 2.7. Statistics

The CFU/g comparisons were determined by comparing means using the *t*-test and Wilcoxon *t*-test model. The proportions of the beta2-toxin gene were compared by Fisher exact test. Significance reported for any analysis was defined as  $p \leq 0.05$ . Spearman's rank correlation coefficient was calculated between *C. perfringens* CFU/g and beta2-toxin gene-producing *C. perfringens* CFU/g.

## 3. Results

We performed quantitative, selective anaerobic culture from stool specimens of 33 autistic children and 13 control children (Table 2). Overall, the mean colony forming unit (CFU) *Clostridium* cell count obtained from stool samples of the autistic children was  $9.2 \times 10^7$  CFU/g dry weight, and  $4.73 \times 10^7$  CFU/g dry weight from normal control children. 30/33 and 10/13 autism and control samples, respectively, yielded *C. perfringens*. The mean CFU *C. perfringens* cell count obtained from stool samples of the autistic children was  $2.12 \times 10^5$  CFU/g dry weight, and  $1.7 \times 10^4$  CFU/g dry weight from normal control children.

Altogether 134 *C. perfringens* colonies from the autism and control samples were subjected to toxin PCR. A single band on electrophoresis of expected size corresponding to the type strain

**Table 1**

Primers for PCR detection of *Clostridium perfringens* toxin genes cpa (alpha toxin), cpb (beta toxin), cpb2 (beta2 toxin), cpe (enterotoxin), etx (epsilon toxin), and cpia (iota toxin).

	Forward primer	Reverse primer	Size	Ref.
cpa	GCTAATGTTACTGCCGTGA	CCTCTGATACATCGTGAAG	324	11
cpb	GCGAATATGCTGAATCATCTA	GCAGGAACATTAGTATATCTTC	196	11
cpb2	AGATTTTAAATATGATCCTAACC	CAATACCCCTCACCAAACTC	548	12
cpe	GGAGATGGTTGGATATTAGG	GGACCAGCAGTTGTAGATA	233	11
cpetx	TGGGAACTTCGATACAAGCA	AACTGCACTATAAATTCCTTTCC	655	11
cpia	ACTACTCTCAGACAAGACAG	CTTTCCTTCTATTACTATACG	446	11

**Table 2**

*Clostridium perfringens* mean counts (CFU/g) and toxin typing of isolates from fecal specimens of autistic and control children.

Specimens	CFU/g		Number of positive specimens								
	Total	<i>C. perfringens</i> isolated	<i>Clostridium</i>	<i>C. perfringens</i>	Beta2-Toxin gene-positive <i>C. perfringens</i>	Beta2-Toxin	CPE	Alpha	Beta	Epsilon	Iota
Autism	33	30	$9.20 \times 10^7$	$2.12 \times 10^5$	$6.55 \times 10^4$	26/33 (79%)	3/33 (9%)	30	0	0	0
Control	13	10	$4.73 \times 10^7$	$1.70 \times 10^4$	$1.48 \times 10^3$	5/13 (38%)	1/13 (8%)	10	0	0	0
p-value			0.17	0.031	0.015	0.014					

band was considered positive (Fig. 1). A sequence analysis of the purified PCR product was done on 14 isolates, and the identity of the PCR product was verified as the target sequence with 99–100% sequence similarity to GenBank type strain entries and to each other. All tested *C. perfringens* harbored *C. perfringens* alpha toxin gene, 26 autism samples and 5 control samples harbored genes for beta2-toxin, and 3 autism and 1 control sample harbored genes for *C. perfringens* enterotoxin; sequences corresponding to genes for beta toxin, epsilon toxin, or iota toxin were not detected (Table 2). The PCR analysis for clostridial toxins of *C. perfringens* isolates revealed that autism samples had statistically significantly ( $p = 0.014$ ) higher incidence of *C. perfringens* beta2-toxin gene (Table 2). The mean CFU of beta2-toxin gene-producing *C. perfringens* cell count obtained from stool samples of the autistic children was  $6.55 \times 10^4$  CFU/g dry weight, and  $1.48 \times 10^3$  CFU/g dry weight from normal control children ( $p = 0.015$ ). *C. perfringens* CFU/g and the CFU/g of beta2-toxin gene-producing *C. perfringens* were moderately, positively correlated (Spearman corr = 0.7125) (Fig. 2).

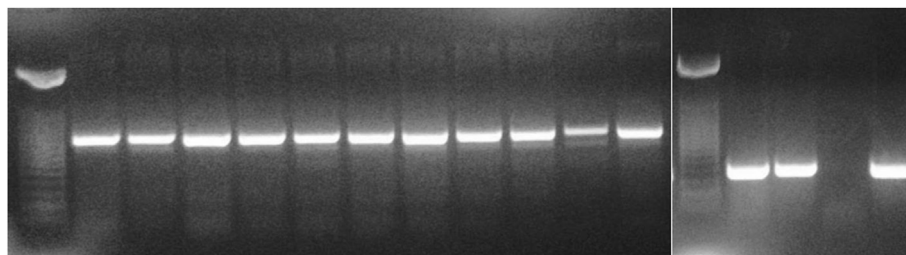
#### 4. Discussion

Gastrointestinal symptoms are increasingly reported in autistic children [16–18]. Published data suggests that an alteration in colonic microbiota contributes to autistic symptoms [2,3,5]. Similarly, reports have linked gastrointestinal dysfunction with affective symptoms [19]; and dietary induced shifts in bacterial diversity have been linked with changes in animal behavior indicating a role for gut bacterial diversity in memory and learning [20]. Recent

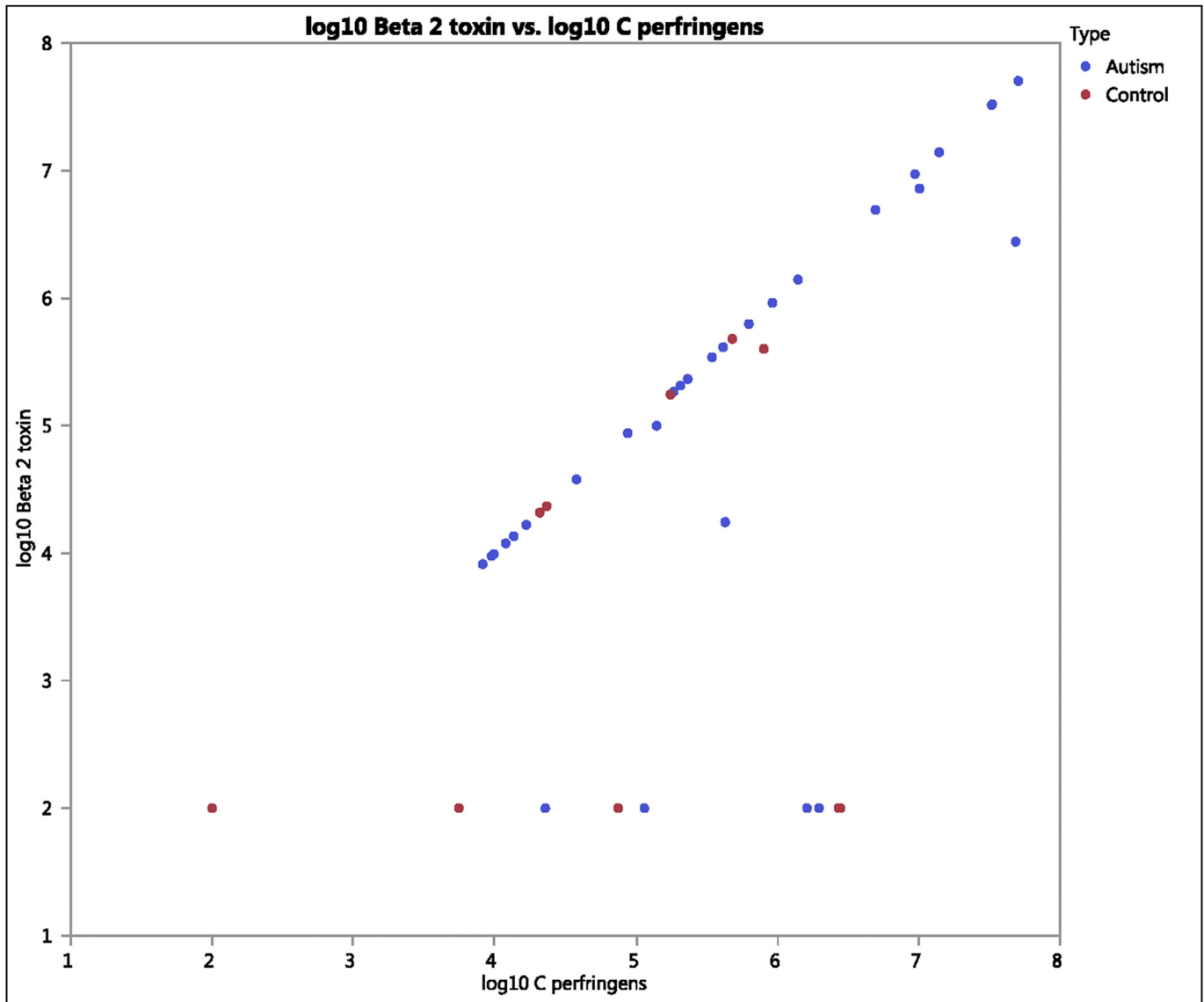
reports have also documented increased oxidative stress in autism [21,22]. Our previous studies indicated that *Clostridium* Cluster I was statistically significantly higher in autism samples as compared to control samples [4]. *C. perfringens* is a common Cluster I gut *Clostridium* and is capable of production of a great diversity of biologically active proteins including toxins, many of which are important in disease and may play a role in gastrointestinal dysfunction and oxidative stress. It has been reported that healthy people carry less than  $10^5$  *C. perfringens* CFU/g feces [23]. In the present study, we found that samples from autistic subjects have statistically significantly higher counts of the species *C. perfringens* ( $p = 0.031$ ) compared to samples from control subjects with mean counts of over  $10^5$  CFU/g.

Generally, a low percentage (1–5%) of *C. perfringens* isolates from healthy human fecal microbiota produce enterotoxin [24]. Similarly, in our study the proportion of enterotoxin gene-positive *C. perfringens* was low in both autistic (3 positive samples) and control (1 positive sample) subjects. The significance of enterotoxin-positive *C. perfringens* in healthy subjects is not clearly understood; however, it cannot be ruled out that some healthy subjects may serve as a reservoir for toxin-positive *C. perfringens* and therefore may be more prone to developing *C. perfringens*-associated diseases, particularly in case of damaged intestinal mucosa, impaired immunity or dysbiosis.

*C. perfringens* beta2-toxin was first studied and described in 1997 by Popoff's group [7]. Initially it was found in enteric disease in animals (piglets, horses) and in food. Subsequently, it was found in other animals and in soil, and finally in humans. *C. perfringens* isolates carrying beta2-toxin have been described in human



**Fig. 1.** *Clostridium perfringens* toxin PCR on autistic subjects. 1, MW marker; 2–12, *C. perfringens* beta2-toxin (12 = ATCC 3626); 13, MW marker; 14–16, *C. perfringens* entero-toxin (CPE, 16 = ATCC 14809).



**Fig. 2.** Spearman's rank correlation of *C. perfringens* CFU/g and the CFU/g of beta2-toxin-producing *C. perfringens* presented in log 10 scale blue = autism, red = control, log (value + 100). Spearman's corr = 0.7125 showing moderate, positive correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gastrointestinal diseases, including food poisoning, antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD). Altogether 24% (7/29) of isolates from humans with food poisoning were shown to harbor beta2-toxin whereas 76% (34/46) isolates from humans suffering with either AAD or SD were beta2-toxin positive [25]. Thirty-four of these AAD- and SD-isolates produced beta2-toxin *in vitro*, whereas only one food poisoning isolate produced the toxin. From the one healthy person tested, a beta2-toxin-harboring *C. perfringens* was also isolated and was capable of producing the beta2-toxin *in vitro* [25]. The descriptions of AAD did not indicate the severity of the AAD or whether *Clostridium difficile* was also present. Another study characterizing *C. perfringens* isolates of SD patients demonstrated the presence of beta2-toxin in 87% (12/14) isolates; ten of these isolates produced the beta2-toxin *in vitro* [26]. In a study of fecal samples of 43 healthy North Americans, 13 (30%) of the people had beta2-toxin-harboring *C. perfringens* [27].

Our findings suggest that the involvement of beta2-toxin in autistic patients with gastrointestinal disease is significant, with 79% of the 33 autism patients showing the presence of beta2-toxin gene in feces, compared to 38% of the 13 control subjects ( $p = 0.014$ ). Our results are remarkably similar to reports where subjects with AAD and SD were shown to harbor 76–87% of beta2-toxin producing *C. perfringens* [25,26], and normal healthy subjects 30% [27]. The apparent occurrence of high levels of beta2-toxin positive *C. perfringens* in autistic children's gut is particularly relevant given the importance of such opportunistic pathogens in hosts with immature or compromised immune system. Generally, *C. perfringens* colonizes the intestinal tract in the early postnatal period; however, the source of toxin gene-producing *C. perfringens* strains in our study population remains to be determined. Further studies are also required to determine whether the strains identified as positive for the beta2-toxin and enterotoxin genes are capable of producing biologically active toxin.

## Acknowledgments

This work was supported in part by the Autism Research Institute, San Diego, CA. We thank Jeff Gornbein, Ph.D., UCLA, for statistical analysis.

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