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

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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.

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 °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 °C for 10 min. Subsequently, 10-fold serial dilutions were plated (100μl/plate) onto Brucella and CDC agar plates (Anaerobe Systems) from both preparations. The plates were incubated under anaerobic conditions at 37 °C for 72 h before initial inspection and re-incubated up to 7 days [10]. Anaerobic conditions consisted of a gas mixture of 5% CO_2, 5% H_2, and 90% 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 β-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 °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 μl nuclease-free water and boiled for 20 min to lyse the cells. After centrifugation at 13000*g* 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 °C and amplified for 35 cycles (1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C for denaturation, annealing and extension phases, respectively), followed by an additional period of extension for 10 min at 72 °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 9.2×10^7 CFU/g dry weight, and 4.73×10^7 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 2.12×10^5 CFU/g dry weight, and 1.7×10^4 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