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Toxin profile of fecal *Clostridium perfringens* strains isolated from children with autism spectrum disorders

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ABSTRACT

Infectious factors are taken into consideration in pathophysiology of autism spectrum disorders (ASD). ASD patients often suffer from gastrointestinal disorders. The intestinal microbiota of autistic patients significantly differs from that in healthy individuals. The aim of the study was to compare the profile of toxins produced by *C. perfringens* strains isolated from feces of children with ASD, with healthy individuals and obese subjects.

This study included 111 strains of *C. perfringens*: 49 isolates from 29 children with ASD, 30 - from 17 healthy individuals and 32 - from 24 young obese subjects. Alpha, beta, beta2, epsilon, iota and enterotoxin genes were detected using appropriate PCRs.

The alpha toxin gene (*cpa*) was present in all 111 examined strains (100%). The beta2 gene (*cpb2*) was detected in 45/49 strains (91.8%) isolated from children with ASD, 17/30 (56.7%) isolates from healthy subjects, and 12 of 32 (37.5%) isolates from obese subjects. *C. perfringens* strains with *cpb2* gene were detected in 27/29 ASD patients (93.1%), 10/17 healthy subjects (58.8%) and 11/24 (45.8%) obese subjects.

Beta2 toxin encoding *cpb2* gene was significantly more common in strains isolated from ASD patients, with no significant difference between control subjects regardless of diet. Further research to explain observed phenomena and pathomechanism of beta2 toxin is required.

1. Introduction

Autism spectrum disorders (ASD) are a range of conditions characterized by persistent deficit in social interactions. The disability commonly manifests the impairment of verbal and nonverbal communication. Autistic patients present a restricted, repetitive pattern of behavior. Behavioral changes usually begin in early childhood [1]. Depending on the symptoms severity, some patients are functionally independent, but some require long-lasting support or lifelong care. Autism affects about 1% of the population [2].

The pathogenesis of the disorder has not been admittedly explained, yet. The development of central nervous system alterations may be due to coexistence of genetic, infectious and pregnancy complications [1]. Frequent occurrence of gastrointestinal complaints in these patients may indicate certain influence of intestinal microbiota on the course of the disease in concert with different other factors [3].

Patients with ASD have shown a number of abnormalities in the intestinal microbiota, which correlates with the severity of symptoms [4]. In some studies, significantly higher amount of the *Desulfovibrio* spp. and *Bacteroides* spp. was detected, with the lower amount of *Bifidobacterium* spp. [5,6] - while other study showed lower prevalence of *Bacteroides* spp., as well as an increase in amount of *Lactobacillus* spp. [7]. Nevertheless, the increase in number of *Clostridium* spp. colonies in comparison to the healthy population was consistent between studies [8]. In the blood of ASD patients, higher concentrations of propionic acid and short chain

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fatty acids have been reported [9]. These components are generally produced by *Clostridium* spp. These substances may presumably affect behavioral changes [10].

C. perfringens is a bacterium producing numerous toxins, responsible for gastrointestinal disorders as well as systemic symptoms. In a study performed by Sandler et al. [11], a short-term improvement in ASD children has been reported after treatment with oral vancomycin – a bactericidal antibiotic with spectrum of activities covering *Clostridium spp*. All major *C. perfringens* toxins produce cytotoxic effects. The alpha toxin gene presents in majority of *C. perfringens* strains; while genes encoding other toxins are detected in a certain percentage of strains. Beta toxin is a major factor in the course of necrotizing enterocolitis. Enterotoxin is a main cause of the symptoms of food poisoning. In 1997 Gibert et al. [12] described the beta2 *C. perfringens* toxin for the first time. In subsequent years beta2 toxin has been associated with gastrointestinal diseases such as necrotizing enterocolitis, diarrhea and food poisoning in both humans and animals [13]. The beta2 toxin is encoded by the *cpb2* gene. Unlike the other toxins, the *cpb2* gene is found in a significant percentage of strains isolated from feces of healthy patients [14]. However, Finegold et al. [15] found a higher occurrence of *cbp2* gene in strains isolated from feces of ASD children (79% in autism group compared to 38% in controls). Possible effects of chronic exposure are unknown. We were inspired by Dr Finegold's [15] publication about differences between *C. perfringens* strains isolated from feces of autistic children and control group, so we decided to compare a toxin profile of *C. perfringens* strains isolated from feces of ASD children, with those of apparently healthy subjects with normal body weight and with obesity, to additionally figure out if *C. perfringens* toxin profile may be affected by eating habits alone.

2. Materials and methods

The study included 49 strains of *C. perfringens*, isolated from fecal specimens from 29 patients diagnosed with ASD at the age from 3.5 to 18 years, treated in Upper Silesian Pediatric Health Center, Katowice, Poland (23 boys, 6 girls). The control group consisted of 30 strains of *C. perfringens* isolated from fecal specimens of 17 healthy individuals and 32 strains of *C. perfringens* isolated from fecal specimens from 24 young obese subjects.

In the ASD group, 17 out of 29 children (59%) experienced gastrointestinal symptoms: 17 (59%) reported abdominal pain, 15 (52%) diarrhea, 14 (48%) loss of appetite, 16 (55%) presented with selective eating, 4 (14%) children were overweight and 8 (28%) suffered from too low body weight compared to age.

2.1. C. perfringens and DNA isolation

All feces samples were diluted 1:100, subjected to heat shock and cultured onto Columbia blood and Reinforced Clostridial agars for 5 days in anaerobic conditions (GENbox anaer, bioMerieux, Marcy L'Etoile, France). All Gram-positive anaerobic bacilli were evaluated for hemolysis (double), lecithinase and lipase production (on Egg-Yolk-Agar) and identified with use of ANC cards in VITEK 2 compact (bioMerieux, Marcy L'Etoile, France). Strains were stored in Microbanks (Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics, UK). After thawing, all strains were cultured on Columbia blood agar and incubated under anaerobic conditions (Whitley A35 Anaerobic Workstation, UK) at 37 °C for 24 h. The grown singular colonies were subcultured in BHI broth, which was used for DNA isolation. The GeneMATRIX DNA Purification Kit by DNA Gdansk, PL was used for isolation of DNA.

2.2. Genetic analysis

The presence of toxin *alpha* - *cpa,* toxin *beta* - *cbp,* enterotoxin –*cpe, iota*toxin - *cpiA, epsilon* toxin -*etx* genes was detected by multiplex PCR according to Meer et al. [16] and Heikinheimo et al. [17] using the primers presented in Table 1. Multiplex-PCR reaction condition was: 120 s at 94 °C as initial denaturation, followed by 35 cycles of 60 s at 94 °C for denaturation, 60 s at 53 °C as annealing, 60 s at 72 °C for extension, and final extension at 72 °C for 10 min (see Table 2).

The *cpb2* gene was detected using subsequent primers: GCGAATATGCTGAATCATCTA(F),GCAGGAACATTAGTATATCTTC (R) according to Garmony et al. [18]. Reaction conditions: 120 s at 92 °C as initial denaturation, followed by 35 cycles of 60 s at 92 °C for denaturation, 60 s at 53 °C as annealing, 60 s at 72 °C for extension and final extension at 72 °C for 10 min. The amplification was

Table 1
Primers used in multiplex PCR.

Gene	F sequence	R sequence	Product size (bp)	Ref
cpa	TGCATGAGCTTCAATTAGGT	TTAGTTTTGCAACCTGCTGT	400	[17]
cpb	GCGAATATGCTGAATCATCTA	GCAGGAACATTAGTATATCTTC	196	[16]
cpe	GGAGATGGTTGGATATTAGG	GGACCAGCAGTTGTAGATA	233	[16]
cpiA	ACTACTCTCAGACAAGACAG	CTTTCCTTCTATTACTATACG	446	[16]
etx	TGGGAACTTCGATACAAGCA	AACTGCACTATAATTTCCTTTTCC	655	[16]

cpa – *alpha* toxin; *cpb* – *beta* toxin; *cpe* – *epsilon* toxin; cp*iA* – *iota* toxin; *etx* - enterotoxin.

Table 2
Toxin profiles.

Gene	ASD		Healthy		Obese	
	n	%	n	%	n	%
By strains						
alpha (*cpa*)	49	100	30	100	32	100
beta2 (*cpb2*)	45	91,8	17	56,7	12	37,5
Other	0	0	0	0	0	0
By patients						
alpha (*cpa*)	29	100,0	17	100	24	100
beta2 (*cpb2*)	27	93,1	10	58,8	11	45,8
Other	0	0	0	0	0	0