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Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children

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Children with autistic spectrum disorders (ASDs) tend to suffer from severe gastrointestinal problems. Such symptoms may be due to a disruption of the indigenous gut flora promoting the overgrowth of potentially pathogenic micro-organisms. The faecal flora of patients with ASDs was studied and compared with those of two control groups (healthy siblings and unrelated healthy children). Faecal bacterial populations were assessed through the use of a culture-independent technique, fluorescence *in situ* hybridization, using oligonucleotide probes targeting predominant components of the gut flora. The faecal flora of ASD patients contained a higher incidence of the *Clostridium histolyticum* group (*Clostridium* clusters I and II) of bacteria than that of healthy children. However, the non-autistic sibling group had an intermediate level of the *C. histolyticum* group, which was not significantly different from either of the other subject groups. Members of the *C. histolyticum* group are recognized toxin-producers and may contribute towards gut dysfunction, with their metabolic products also exerting systemic effects. Strategies to reduce clostridial population levels harboured by ASD patients or to improve their gut microflora profile through dietary modulation may help to alleviate gut disorders common in such patients.

INTRODUCTION

Autism is a spectrum of developmental disorders, with onset in early childhood, affecting social, communicative and imaginative development (Wing, 1997). The condition is prevalent (5/1000) (Baird *et al.*, 2003), and boys are four times more likely to have autistic spectrum disorders (ASDs) than girls. Numerous theories have been proposed regarding the aetiology of ASDs (including pathogenesis), yet the condition remains poorly understood. Recent studies have correlated gut dysfunction with ASD group and suggested a possible role of the gastrointestinal (GI) microflora in symptomatology and/or severity of symptoms in autistic children (Shaw *et al.*, 1995; Bolte, 1998). However, the evidence is rather speculative, as little is known on the gut flora of ASD sufferers compared with that of healthy controls. Many autistic children experience severe dietary and/or GI problems (including abdominal pain, constipation, diarrhoea and bloating). Such symptoms may be due to a disruption of the indigenous gut flora promoting the overgrowth of potentially pathogenic (toxin-producing) micro-organisms (Bolte, 1998). Typically, parents claim that GI problems and behaviour symptoms are manifest in parallel. Interestingly, restricted diets [such as gluten-free and/or casein-free (GF/CF) diets] have been associated with reduced GI disorders, and improved behaviour, in ASD individuals (Knivsberg *et al.*, 2002). Indeed, food intolerance is suspected to play a role in ASDs, but the underlying cause of GI symptoms remains unclear.

The human gut flora is a complex microbial ecosystem, which appears to be of key importance in health and disease (Gibson & Roberfroid, 1995). A history of multiple courses of antibiotic therapy is common in individuals with ASDs, raising questions regarding the composition and stability of the gut flora. Repeated antimicrobial use may disrupt the protective commensal microflora and create an environment more favourable to colonization by one or more toxin-producing species (Bolte, 1998). One group of bacteria known to produce powerful neurotoxins is clostridia, and several species have been implicated in clinical infections (Brook, 1995). To date, however, a very limited amount of work has investigated the clostridial populations in ASD individuals. Cultivation studies by Finegold *et al.* (2002) demonstrated that certain clostridial species were specific to autistic samples and not seen in faecal samples from healthy subjects. Subsequent work by this group employed real-time

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Abbreviations: ASD, autistic spectrum disorder; FISH, fluorescence *in situ* hybridization; GF/CF, gluten-free and/or casein-free; GI, gastrointestinal.

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PCR assays to quantify selected *Clostridium* clusters (namely, clusters I, XI and XIVab) and the species *Clostridium bolteae* (Song *et al.*, 2004). However, numerical differences in the gut flora of ASD patients and healthy subjects remain poorly studied.

Modern molecular techniques, such as fluorescence *in situ* hybridization (FISH) using specific 16S rRNA-based oligonucleotide probes, have proved superior to cultivation methodology for accurate evaluation of the predominant gut microflora. Such molecular methods may be used to detect the bacterial content of samples at different taxonomic levels, for example genus or species (Amann *et al.*, 1995). In the present study, FISH using group-specific oligonucleotide probes was employed to compare the bacterial composition of faecal samples obtained from children diagnosed with ASDs with those of two healthy control groups.

METHODS

Subjects. Fifty-eight children diagnosed with ASDs participated in the study (48 males and 10 females, between 3 and 16 years of age). Many of the children had undertaken numerous courses of antibiotic treatment from an early age and suffered from common GI disorders (the most frequent complaints being diarrhoea and/or constipation). Most of the children were on GF/CF diets and many were taking probiotics/prebiotics and other supplements prior to entering the study. Two control groups were included: a non-autistic sibling group (seven males and five females, from 2 to 10 years of age) and an unrelated healthy group (six males and four females, from 3 to 12 years of age). Inclusion in the unrelated healthy control group required that volunteers had not used functional foods (such as probiotics and/or prebiotics) for at least 6 months prior to the study. The healthy sibling group was included to examine any impact of host genetics, environment and/or lifestyle. Written informed consent was obtained from each individual, their parent or guardian prior to inclusion in the study. In addition, a written questionnaire was used during recruitment of volunteers to assess GI habit, dietary characteristics and antibiotic usage, and to look for any correlation between the characteristics of individuals and bacterial profiles. This study was carried out under the guidance of the Ethics and Research Committee of the University of Reading.

Sample preparation and FISH analysis. Fresh faecal samples were collected in sterile stool tubes (Sarstedt) and immediately frozen at −20 °C for future analysis. The faecal samples were diluted 1 : 10 (w/v) in pre-reduced PBS (0·1 M, pH 7·0; Oxoid). Tubes were centrifuged for 2 min at 2000 ***g*** to remove debris. Aliquots of the faecal slurries were then fixed in 4 % (w/v) paraformaldehyde solution (Sigma) overnight at 4 °C (Amann *et al.*, 1995). Following paraformaldehyde fixation, cells were washed twice in PBS (centrifuged for 5 min at 13 000 ***g***), resuspended in a PBS/ethanol mixture (1 : 1) and stored at −20 °C for at least 4 h prior to hybridization.

Faecal bacterial populations were assessed by FISH analysis using a collection of 5′ Cy3-labelled 16S rRNA oligonucleotide probes (commercially synthesized; MWG Biotech) to cover the predominant bacterial groups of human faeces: Bif164 (Langendijk *et al.*, 1995), Bac303 (Manz *et al.*, 1996), Chis150 (Franks *et al.*, 1998), Erec482 (Franks *et al.*, 1998) and Lab158 (Harmsen *et al.*, 1999), specific for bifidobacteria, bacteroides, the *Clostridium histolyticum* group (*Clostridium* clusters I and II), the *Clostridium coccoides/Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb) and lactobacilli/enterococci, respectively. The nucleic acid stain 4′,6-diamidino-2-phenylindole was used to enumerate the total bacterial load of samples.

The hybridized cells were washed and vacuum-mounted on filters (0·2 μm isopore type GTBP, black membrane filter; Millipore) for enumeration using an epifluorescence microscope (Eclipse E400 UV microscope; Nikon). Fifteen random microscopic fields were counted per assay and used to calculate the number of cells on the filter. The number of cells (g faecal sample)$^{-1}$ was then calculated using the equation: bacteria (g sample)$^{-1}$ = dilution factor $\times$ b $\times$ number of fields on filter$\times$correction factor = $155{\cdot}56 \times b \times 14873{\cdot}74 \times (1000/c)$, where b is the mean number of cells observed per microscope field of view and c is the volume of hybridization mix applied to the filter.

Statistical analyses. Data were analysed by using Minitab statistical software (version 13). A chi-square test was performed on different characteristics (age, gender, GI symptoms) to estimate variance ratio among the subject groups (ASD and healthy groups). Differences in bacterial populations among the subject groups were analysed by using ANOVA (generalized linear model).

RESULTS AND DISCUSSION

Subjects

Data relating to GI problems, dietary characteristics and antibiotic use were collected for each study group from the individual questionnaires (Table 1). The total number of antibiotic courses was recorded retrospectively for each individual and information on the exact treatment regimes taken (type of antibiotic, daily dose and duration of treatment) was incomplete. Erythromycin and amoxycillin were the most commonly named antibiotics (when data were available), and two autistic children were known to have had a course of flucloxacillin (data not included).

A high proportion of ASD patients suffered from GI disorders (91·4 %). In contrast, 25 % of the sibling group and none of the healthy unrelated children acknowledged gut problems. The chi-square test was used to estimate population variance due to GI disorders between the ASD group and all healthy controls (both siblings and unrelated children). GI problems were significantly more frequent in ASD patients than in controls ($P < 0{\cdot}05$), demonstrating a significant association between GI symptoms and autism. Diarrhoea was the most common GI symptom (75·6 % of ASD patients), followed by excess wind (55·2 %), abdominal pain (46·6 %), constipation (44·8 %) and abnormal faeces (43·0 %). Some autistic individuals were recorded to suffer from multiple GI problems, including having had episodes of both diarrhoea and constipation.

A history of extensive and repeated broad-spectrum antibiotic treatment (more than six courses, usually for respiratory tract disorders or ear infections) was also common in ASD children (34·5 %) and the sibling group (33·3 %), compared with the healthy unrelated group (0 %). Furthermore, it was noted that a higher proportion (54·5 %) of the ASD* group (i.e. those ASD individuals for whom healthy siblings were included in the study) had received more than six courses of antibiotics than that seen for the total ASD group (34·5 %).

Implementation of a GF/CF diet is very common in ASD subjects, due to evidence that foods containing such proteins

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Table 1. Characteristics of the ASD and healthy control groups

Characteristics	Total ASD ($n = 58$)	Healthy ($n = 10$)	ASD* ($n = 11$)	Siblings ($n = 12$)
Age (mean ± SD)	7 ± 3·76	6 ± 2·88	5 ± 1·33	6 ± 2·15
Gender (*n*/%)				
Females	10/17·2	4/40	0	5/41·7
Males	48/82·8	6/60	11/100	7/58·3
GI disorders (*n*/%)				
No	5/8·6	10/100	1/9	9/75
Yes	53/91·4	0	10/91	3/25
Antibiotics (*n*/%)†				
None	6/10·3	5/50	1/9·1	1/8·4
1–5 Courses	32/55·2	5/50	4/36·4	7/58·3
⩾6 Courses	20/34·5	0	6/54·5	4/33·3
Diet (*n*/%)‡				
Varied	20/34·5	10/100	3/27	11/91·7
Restricted	38/65·5	0	8/73	1/8·3
Probiotics (*n*/%)				
No	27/46·6	10/100	4/36·4	7/58·3
Yes	31/53·4	0	7/63·6	5/41·7
Other supplements (*n*/%)§				
No	20/34·5	10/100	3/27	12/100
Yes	38/65·5	0	8/73	0

*ASD patients whose siblings were included in the study.

†Total number of courses taken during lifetime.

‡Varied diet corresponds to a normal diet; restricted diet refers to a GF/CF diet.

§Other supplements in diet, such as vitamins and minerals, fatty acids, etc.

contribute significantly to autistic behaviour. Sixty-six per cent of ASD subjects and 8 % of the sibling group were following a restricted diet.

Over half of the autistic patients (53·4 %) were taking probiotic treatments. Five of the six families in which ASD-affected children were receiving probiotics also gave them to healthy siblings. This may reflect parental awareness/belief in the potential health-promoting impact of such products. Nutritional supplements (such as vitamins, minerals and essential fatty acids) have also been recommended for ASD patients. Approximately two-thirds of the ASD patients were receiving such supplements, in contrast to none of the healthy controls (including siblings).

Faecal microflora

The *C. histolyticum* group varied markedly between the total ASD group and the unrelated healthy children group (Table 2). Significantly higher levels were observed in the total ASD group, compared with both healthy unrelated children and healthy siblings ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively). However, when comparisons were made between the two sibling groups (ASD* and healthy siblings), no significant difference was seen in this bacterial population (*Clostridium* clusters I and II). This reflected the intermediary levels of the ASD sibling subset compared with the levels observed for the total ASD group and the healthy siblings group. In addition, the healthy sibling group displayed intermediate levels (compared with the healthy unrelated and ASD groups), indicating that environmental factors (such as diet and living conditions) and host genetics may impact on this bacterial population of the human gut microflora.

The only significant difference observed for the remaining bacterial populations was between the *Bacteroides* population of the two healthy groups (i.e. unrelated healthy group and healthy siblings) ($P < 0{\cdot}05$). In fact, the healthy sibling group harboured the lowest levels of bacteroides of all the subject groups. The reason for this observation was unclear, but may reflect the higher incidence of probiotic and/or antibiotic administration in this group compared with the control group. In addition, host and/or environmental factors may play a role. All other bacterial groups examined showed similar levels across all subject groups, including *Clostridium* clusters XIVa and XIVb (determined using the Erec482 probe). This finding is in agreement with that of Song *et al.* (2004), who used real-time PCR to monitor these bacteria.

ANOVA was performed to examine associations between subject characteristics (age, gender, antibiotic usage, GI disorders, diet, probiotics and/or prebiotics, and other supplements) and bacterial profiles of the different groups.

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Table 2. Faecal bacterial numbers, determined by FISH analysis, for patients diagnosed with ASDs and control groups

Data are presented as the mean (SD) of $\log_{10}$[no. of cells (g faeces)$^{-1}$].

Bacterial group	Total ASD ($n = 58$)	Healthy children ($n = 10$)	ASD* ($n = 11$)	Siblings ($n = 12$)
Bifidobacteria	9·58 (0·27)	9·54 (0·18)	9·56 (0·23)	9·62 (0·24)
Bacteroides	9·59 (0·29)	9·63 (0·25)	9·50 (0·35)	9·18 (0·60)‡
C. histolyticum group	9·75 (0·36)†	9·38 (0·34)	9·69 (0·34)‡	9·51 (0·35)§
Lactobacilli/enterococci	8·93 (0·34)	8·88 (0·34)	8·83 (0·35)	8·68 (0·29)
C. coccoides/*E. rectale* group	9·76 (0·35)	9·81 (0·18)	9·95 (0·11)	9·62 (0·43)
Total bacteria	10·55 (0·21)	10·41 (0·24)	10·51 (0·18)	10·45 (0·28)

*ASD patients whose siblings were included in the study.

†Significantly different to healthy children ($P < 0·01$).

‡Significantly different to healthy children ($P < 0·05$).

§Significantly different to total ASD ($P < 0·05$).

By employing the general linear model option of ANOVA, any biasing due to different sample sizes (between groups) was eliminated.

No relationship was evident between the levels of any of the bacterial populations examined and age, gender, antibiotic history or diet type (i.e. varied or restricted). Furthermore, bifidobacterial, *C. coccoides*/*E. rectale*, lactobacilli/enterococci and total bacterial loads did not show significant variation among the different groups of volunteers or the remaining characteristics tested (GI disorders, probiotics and other supplements).

GI problems were associated with high levels of clostridia ($P < 0·001$) in patients with ASD compared with the unrelated healthy children group, i.e. a significant link was observed between the levels of the *C. histolyticum* group and GI problems in ASD patients. Statistical analysis also showed an association between high clostridial counts and individuals consuming probiotics ($P < 0·01$). However, this may reflect the fact that most of the individuals consuming probiotics were ASD patients (31/36; 86 %). This suggested that clostridial populations did not appear to be affected by probiotic ingestion. Similarly, an association between clostridial levels and the use of other supplements was observed ($P < 0·05$). No statistical correlation was seen between the subject characteristics and bacteroides levels.

Few studies have investigated the bacterial populations of the faecal flora of ASD patients, and relatively little information is available concerning the effects of the gut flora on the symptoms of ASD patients. Anecdotal reports by parents often describe evidence that autistic children suffer from disturbed GI tract function. Nevertheless, comprehensive assessments of GI abnormalities are still poorly understood. In the present study, comparisons between ASD and healthy children groups revealed that significant differences exist in the composition of the human gut flora of patients with ASD and healthy subjects (especially with regard to the *C. histolyticum* group). In addition, GI disorders were associated with high levels of clostridia. This suggests a possible link between clostridial levels and GI function in ASD patients. However, cause and/or effect remain to be determined.

Bolte (1998) suggested a possible role of clostridia in ASDs. Previous cultivation and PCR-based studies (Finegold *et al.*, 2002; Song *et al.*, 2004) have also shown distinctive clostridial populations in autistic children compared with healthy controls. Such alterations in the indigenous gut flora may impact/reflect colonization and/or activity of neurotoxin-producing bacteria (including several members of the genus *Clostridium*). Finegold *et al.* (2002) demonstrated that a similar number of clostridial species was harboured by the ASD patients and healthy controls. However, nine *Clostridium* species were exclusively isolated from faecal samples of autistic children (i.e. not found in the predominant faecal microflora of healthy controls). In addition, three species were only found in healthy samples. In a subsequent study, Song *et al.* (2004) identified significantly higher levels of *C. bolteae* and *Clostridium* clusters I and XI in autistic children ($n = 15$) than in healthy controls ($n = 8$). As such, there is now great interest in the clostridial population (composition, diversity and dynamics) associated with ASDs.

Clostridia are recognized toxin-producers, including neurotoxins (Hatheway, 1990). Theoretically, toxic products may be overexpressed in the autistic gut, which may lead to increased levels in the bloodstream and thus exert systemic effects. Interestingly, many anecdotal reports from parents of autistic children report worsening of behavioural symptoms coinciding with bouts of GI problems.

Overall, the major differences observed between the ASD patients and healthy control groups, in the current work, were the *C. histolyticum* group levels. Indeed, the numerically predominant bacterial population in samples from ASD

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patients was *C. histolyticum* (*Clostridium* clusters I and II) – an observation never previously seen in analyses of gut flora composition in human subjects within our laboratory (including investigations of clinical states such as ulcerative colitis, bowel cancer and irritable bowel syndrome). These results support the hypothesis of an association between clostridia and the development of certain autistic characteristics.

Importantly, the culture-independent technique used (FISH) circumvented recognized problems associated with cultivation and 'selective' plates, which are known to be unreliable in terms of characterization, recovery of species and selectivity (McCartney, 2002). An additional benefit of utilizing such molecular-based strategies is the reduced bias caused by storage and/or transport of samples (such as freezing samples prior to processing). Future studies should also encompass molecular-based profiling strategies (such as denaturing gradient gel electrophoresis; DGGE), to enable the diversity and dynamics of bacterial populations to be monitored. Unfortunately, PCR-DGGE primers are not currently available for the clostridial subgroups. Such investigations would, however, be of great interest – and the development of appropriate molecular strategies to monitor the diversity and dynamics of the clostridial populations is clearly of major importance.

Previous studies have reported increased resistance of clostridia to several antimicrobial agents. Sandler *et al.* (2000) demonstrated significant improvements in ASD children given vancomycin orally. However, the benefit was short term, with regression noted approximately 2 weeks after treatment ceased. These findings may be explained by vancomycin treatment reducing the *Clostridium* population, but due to the persistence of spores the clostridial levels return once treatment has stopped. Since orally administered vancomycin is only minimally absorbed, it is likely that the effect is mediated, in some way, through vancomycin activity on intestinal bacteria. Thus, it has been suggested that the short-term benefit from vancomycin treatment might be due to the temporary elimination of neurotoxin-producing micro-organisms.

There is now evidence that the gut microflora plays a role in autism. Modulation of the gut microflora by reducing the numbers of certain clostridia in ASD patients, while stimulating more beneficial gut bacteria, may help alleviate some of the related symptoms.

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