

More Evidence of Altered Microflora in Patients with Autism Spectrum Disorder (ASD): A Controlled Analysis of qPCR Stool Results in 147 Cases of ASD

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Abstract

Aim: Autism Spectrum Disorder (ASD) is a prevalent neuropsychiatric condition affecting a person's ability to interact and socialize. It varies in severity, but the majority are unable to work or develop partnerships. There are currently no treatments. Microflora abnormalities have been investigated in relation to the neuropsychiatric manifestations in ASD. Various abnormalities have been identified, and to date there are no refutation studies discoverable.

Methods: A total of 147 patients with ASD had undergone quantitative Polymerase Chain Reaction (qPCR) stool analysis at initial presentation and had been free from antibiotics for the previous three months. The proportion of firmicutes to bacteroidetes phyla were calculated in a percentage ratio. Controls were taken from patients who had attended the outpatient clinic around the same time, had not had antibiotics for three months and did not have a diagnosis of ASD.

Results: There was a significant increase in the proportion of firmicutes in the patients with ASD versus controls (63:37 in ASD patients (n=147) versus 55:45 in controls (n=12), mean difference of 8.6% $P=0.005$, CI=3.1 – 12.9). Significance remained after correction for age and sex.

Conclusion: In this retrospective analysis, patients with ASD had a significantly different composition of microflora than unhealthy controls. This adds to the growing body of evidence that ASD patients have abnormal microflora. Longitudinal population studies examining a potential causal link between microflora and ASD onset/symptomology are crucial for examining preventative, harm-reduction and treatment interventions in this often-debilitating condition.

Keywords: Autism spectrum disorder; Polymerase chain reaction; Constipation

Introduction

Autism Spectrum Disorder (ASD) is a heterogeneous condition affecting an individual's ability to communicate and socialize. It often presents with repetitive movements or behaviours [1,2]. It tends to be severe with less than 10% achieving independent living with a marked variation in the progression of the condition [3-5]. To date the literature supports a multifactorial model with the largest, most detailed twin study demonstrating strong environmental contribution to the development of the condition [2,6]. Currently there are no recommended treatments for the condition [1,2].

Microflora

Microflora refers to the collective sum of all the microorganisms inhabiting the large intestine. Microflora can be considered at the species level. It is reported that 'roughly' 150 individual species dominate the grown adult's large intestine (the number varies with age and methodology of the study used) [7]. Microflora can also be considered at the broader phyla level. In this regard, the evidence suggests there are two main phyla that make up the majority of the microflora - bacteroidetes and firmicutes. Indeed, it is generally considered that over 90% of all microflora in the human gut consists of either firmicutes or bacteroidetes phyla [7,8]. There is some evidence that shifts in firmicutes to bacteroidetes ratio lead to systemic disease [9].

Beyond this, the biology of these micro-organisms and how they affect host immune and neurological function is only beginning to be

understood. We are confident they are crucial for life [10]. We are clear they do influence the immune system [9]. And it is believed they are likely important in brain function [11]. Evidence though is lacking. Both basic physiological mechanisms involved in the interplay between the microflora and host, as well as the involvement of the various microflora compositions and species in the evolution of disease remain woefully under-researched.

Microflora and Autism

Abnormal compositions of microflora have been found in ASD (Table 1). Higher proportions of certain species (such as Clostridium) and lower proportions of other species (such as bifidobacterium) have also been discovered. Methodology differs significantly between trials.

Research Methodology

The study utilized a database of 289 patients with confirmed ASD

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Study Group	Methodology	Significantly Higher in ASD	Significantly Lower in ASD	Study Reference
13 ASD, 8 CON	Bacterial culture, faeces	<i>Clostridium</i> and <i>Ruminococcus</i> spp.	--	[12]
15 ASD, 8 CON	Quantitative PCR, faeces	<i>Clostridium</i> clusters I and XI, <i>Clostridium bolteae</i>	--	[13]
58 ASD, 12 SIB, 10 CON	Fluorescent in situ hybridization, faeces	<i>Clostridium histolyticum</i> group (<i>Clostridium</i> clusters I and II)	--	[14]
33 ASD, 7 SIB, 8 CON	Pyrosequencing, faeces	Severe ASD (11 subjects) versus CON: Phylum level: bacteroidetes and proteobacteria Genus level: <i>Alkaliflexus</i> , <i>Desulfovibrio</i> , <i>Acetanaerobacterium</i> , <i>Parabacteroides</i> , <i>Bacteroides</i>	Severe ASD (11 subjects) versus CON: Phylum level: firmicutes and actinobacteria Genus level: 14 genera, most significant and abundant: <i>Weissella</i> , <i>Turcibacter</i> , <i>Clostridium</i> , <i>Anaerofilum</i> , <i>Pseudoramibacter</i> , <i>Ruminococcus</i> , <i>Streptococcus</i>	[15]
23 ASD, 22 SIB, 9 CON	Quantitative PCR, faeces	<i>Bacteroides fragilis</i> in ASD subjects with GI symptoms only (9 of 23)	<i>A. muciniphila</i> (ASD and SIB); <i>Bifidobacterium</i> spp. (ASD only)	[16]
58 ASD, 39 CON	Bacterial culture, faeces	<i>Lactobacillus</i> spp.; <i>Bacillus</i> spp.	<i>Bifidobacterium</i> spp., <i>Enterococcus</i> spp., <i>Klebsiella oxytoca</i>	[17]
15 ASD, 7 CON	Pyrosequencing and quantitative PCR, ileal and cecal biopsies	Cumulative level of firmicutes + proteobacteria; <i>Sutterella</i> spp.	bacteroidetes	[18,19]
20 ASD vs. 20 Controls	Quantitative PCR, faeces		Diversity <i>Prevotella</i> , <i>Coprococcus</i> , and unclassified <i>Veillonellaceae</i>	[20]
10 ASD vs. 10 PDD-NOS vs. 10 siblings	Quantitative PCR, faeces	Clostridiaceae, Sutterellaceae, Enterobacteriaceae (e.g., <i>Proteus</i> , <i>Shigella</i>)	<i>Bifidobacterium</i> spp.,	[21]
23 ASD, 22 SIB, 9 CON	Quantitative PCR, faeces	(<i>Ruminococcus gnavus</i> and <i>Ruminococcus torques</i>)		[22]
40 ASD, 40 CON	Quantitative PCR, faeces	Increased proportion of Firmicutes phyla		[25]
59 ASD, 44 CON	Quantitative PCR, faeces	Increased proportion of Firmicutes phyla		[26]
18 ASD, 20 CON	Quantitative PCR, faeces		Reduced diversity	[27]
14 ASD, 15 CON	Quantitative PCR, faeces	Clostridiales	<i>Dorea</i> , <i>Blautia</i> and <i>Sutterella</i>	[28]
21 ASD, 23 CON	Quantitative PCR, faeces		<i>Prevotella copri</i> , <i>Faecalibacterium prausnitzii</i> , <i>Haemophilus parainfluenzae</i>	[29]

Table 1: A summary of microflora studies in ASD patients.

who had attended an outpatient clinic in London between 2012 to 2014. Most cases had been referred to the clinic due to concerns regarding allergies, malnourishment or suspicion about an undiagnosed cause of pain. As part of the work-up most patients (62%) would undergo a stool analysis at initial visit. The stool analysis was conducted by Metamatrix laboratories via Biolab Medical Unit, London and included a number of parameters such as faecal elastase, calprotectin, *H. pylori* stool antigen and a quantitative PCR measure of firmicutes and bacteroidetes.

During routine review of these stool results it was noted that many of the ASD patients maintained a higher than expected firmicutes level. Given the clinical observation of exaggerated positive response to treatment that would affect microflora levels and knowledge of the research in the area, a study was proposed to examine whether the clinical observation of compositional dysbiosis had merit i.e., whether the observed increase in the proportion of firmicutes was statistically significant.

Patient selection

The first 60 cases of confirmed ASD who had qPCR stool analysis conducted at their first clinic appointment were included. The case files were scrutinized further for recent (within 3 months) antibiotic use, and such cases were excluded from further analysis. The remaining cases had the results of their qPCR stool analysis extracted and recorded as a percentage ratio of firmicutes: bacteroidetes. The initial results

were compared with 7 non-ASD controls who had also attended the clinic between 2012 to 2014, had undergone qPCR analysis at their first consult and had not had antibiotic use for at least 3 months preceding the test.

The ASD case files were then each examined for evidence of gut symptoms, supplement use, medication use, dietary modification and severity. The results were collated and analysed.

Both parametric and non-parametric analysis was undertaken as normal distribution could not be inferred from these numbers or the general knowledge pertaining to qPCR stool analysis in ASD. QPCR stool analysis was undertaken through an in-house 16s rDNA targeting quantitative PCR technique.

After the initial 60 cases were examined and significant correlation detected, the entire database of 289 patients were analysed further. Specifically, qPCR results were extracted from patients with confirmed ASD (n=178). Each case file underwent a limited review to insure inclusion criteria were met. The results were again collated and analysed through SPSS, as detailed above.

Results

Out of the 60 patients selected, 54 had undergone PCR stool analysis at initial assessment and were considered relatively naïve to treatment. 29 of the 54 had reliable record of age, sex, diet, supplements and

medications. Seven controls were identified with reliable demographics. Age and sex ratios were reasonably well matched between controls and ASD-patients. In the larger cohort of 54 ASD-patients the sex ratio was 2.8:1 male predominance (Table 2).

In the controlled analysis, diet and supplement use were consistent between the control group and ASD-cohort, with greater medication use in the ASD cohort versus controls (28% vs. 14% respectively). Controls were more likely to suffer GI symptoms than the ASD-cohort (86% vs. 67%).

Mean levels of firmicutes and bacteroidetes were calculated for both the ASD-cohort and the control group. Firmicutes was higher in the ASD group with a mean difference of 8.5% ($P=0.007$, CI 2.4 to 14.5) and bacteroidetes was lower in the same fashion.

A further 178 medical records were analysed for PCR stool analysis and, after checking for duplication were subject to the same exclusion criteria detailed above and then combined with the previous cohort (Table 3).

There remained a significant difference in firmicutes to bacteroidetes percentage ratio between the ASD-cohort and the non-ASD control group of 8.6% ($P=0.005$, CI 3.1 – 12.9).

Age and sex were then analysed within a multi-variant analysis to assess for confounding effects of either on the mean difference between levels of firmicutes in the ASD-cohort and the control cohort. The unadjusted correlation for the mean difference of firmicutes between groups was statistically significant ($P=0.005$). The sex and age adjusted correlation remained significant ($P=0.009$).

Discussion

The present study examined qPCR stool results in patients with ASD and non-ASD patient controls. There were significant compositional differences between the ASD cohort and the control (non-ASD) cohort. These compositional changes did not correlate with abdominal symptoms. Further, the ASD cohort did not show the expected fall in firmicutes with age.

Direct comparison to other studies in the area is difficult. Compositional analysis is often neither completed nor reported – many studies seeking species versus phylum abnormalities. The difficulty is compounded further by the differences in methodology and the population being studied.

Variables	ASD-Cohort n=29	Controls n=7
Age (yrs.)	6.14	7.2
Sex Ratio (M:F)	1.9	2.5
Autism Severity	5.8	N/A
Supplements	45%	43%
Diet	48%	43%
Medication	28%	14%
Firmicutes (%)	65.8 (SD 7.8)	54.1 (SD 10.9)
Bacteroidetes (%)	34.2 (SD 8)	45.9 (SD 10.9)
Abdominal Symptoms	67%	6/7 (86%)

Table 2: Characteristics of initial ASD and non-ASD cohorts.

Variables	ASD-Cohort n=147	Controls n=12
Age (yrs.)	6.03 (Range 2 to 21)	6.4 (Range 1 to 16)
Sex Ratio (M:F)	4.0	1.42
Firmicutes (%)	63.15 (SD 7.8)	54.75 (SD 10.4)
Bacteroidetes (%)	36.85 (SD 8)	45.25 (SD 10.4)

Table 3: Demographics and mean qPCR compositional scores.

Superficially, of the 15 studies listed in Table 1 ten reported elevation in at least one species belonging to the firmicutes phyla. Only two studies reported increases in species favouring the bacteroidetes phyla – the former studied 11 severe ASD patients, and the latter studied ASD patient with GI symptoms [12-16].

Certainly, the present study is consistent with the majority of studies in the area, and supports the notion that, firstly, patients with ASD may well have abnormal microflora compositions and secondly that such shifts in compositions may be consistently towards the firmicutes phyla.

Limitations

Whilst this retrospective analysis provides qPCR results on a significant number of ASD patients, there are several limitations conferring only a moderate power for this study. The control numbers were limited, and demographics also limited. Given the retrospective nature of the study, the recording of important potential confounders was unreliable and selection bias could not be accounted for.

Future Studies

Nonetheless, taken together with the other publications in the area, this study provides further argument for a large prospective controlled study of qPCR stool analysis in patients with ASD versus healthy (and unhealthy) controls. Additionally, there remains the paucity of literature pertaining to the ‘normal’ microflora compositions and hence a terminal limitation to the scientific understanding of what is normal and what is abnormal; what is compensatory and what may be causative or partially perpetuating. Unquestionably, it is suggested here, a population based longitudinal study of microflora from infancy through to childhood is warranted to progress the human microflora field further.

Clinical Implications

Whilst there are a number of studies identifying abnormal microflora species and/or compositions in patients with ASD, and indeed even a treatment trial indicating improvement in ASD symptomology following treatment targeted at the microflora, the presence of abnormal microflora in patients with ASD has not been adequately confirmed [17-23]. Further, even if one accepts the findings of the studies confirming abnormal microflora in ASD, it remains unknown as to the clinical relevance of such abnormalities. In saying that, the best available evidence is more suggestive of abnormal microflora in patients with ASD with no refutation studies apparent. One can reasonably suggest then, that clinicians should perhaps be extra cautious when managing conditions that are likely to affect microflora in the ASD patient group. For example, reasonable caution should be taken in ASD patients with constipation. Effective treatment of constipation may well carry more substantial benefits to the ASD patient versus their non-ASD counterparts. Equally, the often overlooked and evasive diagnosis of small intestinal bacterial overgrowth (SIBO) could be potentially disastrous to a patient with ASD, assuming of course the microflora abnormalities do affect brain in some way [24-29].

It is therefore useful to serve a reminder that whilst the underlying pathophysiology of ASD remains unknown, clinicians should continue to engage the range of possible responses to different physical health problems in patients with ASD and act accordingly, from a patient-focused perspective.

Conclusion

Quantitative Polymerase Chain Reaction (QPCR) can measure species specific fragments of RNA/DNA in a quantifiable measure. QPCR stool analysis shows promise in monitoring microflora compositions and may have use in early detection of microflora abnormalities in ASD patients. In the present study, the qPCR analysis from 29 patients with ASD was compared with 7 unhealthy non-ASD controls. The mean firmicutes to bacteroidetes ratio was 66:34 (SD 8) in the ASD-cohort versus 54:46 (SD 11) with a significant difference between groups of 8.5% ($P=0.007$, CI=2.4 to 14.5). Further analysis on 159 cases supported these initial findings with the mean level of firmicutes to bacteroidetes in cases ($n=147$) of 63:37 and in controls ($n=12$) of 55:45 (mean difference of 8.6% $P=0.005$, CI=3.1 – 12.9), and this withstood correction for age and sex. There was no significant correlation identified between the ASD group with GI symptoms and without ($n=28$). Taken together with the other studies in the area, further investigations are warranted into the aetio-pathological role of microflora alterations in autism.

Conflict of Interest

The authors declare no conflict of interest.

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