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Detection of *Sutterella* in the Stool of Egyptian Children with Autism Spectrum Disorders

Nancy Younis Omar[1], Shwikar M. Abdelsalam Ahmed[1], Hanan Azouz[2], Sarah Magdy Abdelhamid[3*] and Naglaa Moustafa Abdelaziz[4]

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Alexandria University, Egypt.
[2]Department of Pediatrics, Faculty of Medicine, Alexandria University, Egypt.
[3]Department of Microbiology and Immunology, Faculty of Pharmacy, Damanhour University, Egypt.
[4]Ministry of Health Hospital, Alexandria, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Authors NYO and SMAA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors HA, SMA and NMA managed the analyses of the study, managed the literature searches and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Background: Autism spectrum disorder (ASD) is a multifaceted group of neurodevelopmental disorders. Gastrointestinal problems are commonly reported in children with autism and may correlate with autism severity. A recent study reported that *Sutterella* species were frequently found in individuals with autism that was not found in controls.
Aim: The aim of the present study was to study the role of *Sutterella* species in Egyptian children with Autism Spectrum Disorders (ASDs).
Methods: Thirty children diagnosed with ASDs according to DMS-V criteria aged between 2.5-8 years old together with a cross matching control group of 30 healthy neurotypical children were included in the present study. Gastrointestinal symptoms were assessed with a modified six-item GI

**Corresponding author: E-mail: Sara.magdy@pharm.dmu.edu.eg, Sarah_magdy@yahoo.com;*

Severity Index (6-GSI) questionnaire. Stool specimens were taken for detection of *Sutterella* by the Conventional PCR using primers that amplify a 260-bp region spanning the variable regions from V6 to V8 of the 16S rRNA gene.
Results: *Sutterella* species was detected in 24 cases out of 30 ASD patients (80%), and in 11 (36.7%) out of the control cases. There was no significant correlation between the presence of *Sutterella* and the severity of ASD or GSI.
Conclusion: These results indicate that *Sutterella* species may have a role in ASD.

Keywords: Autism; Sutterella; PCR; 16S rRNA; ASD; stool.

1. INTRODUCTION

Autism spectrum disorder (ASD) is a multifaceted group of neurodevelopmental disorders. After the publication in May 2013 of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V), autism disorders were merged into one umbrella for diagnosis of ASD [1]. Previously, they were recognized as distinct subtypes, including the severe form of autistic disorder, childhood disintegrative disorder, pervasive developmental disorder-not otherwise specified (PDD-NOS) and the milder form of Asperger syndrome. Delay in language and age of onset are not emphasized in DSM-V diagnostic criteria [1].

Since autism was first explained in the 1940s, it has been looked upon as a rare disorder, occurring at a rate of 4-5 every 10,000 children [2]. Since then, however, incidences have been on the rise. Now autism is generally accepted to be a grand challenge to global mental health. [3,4]. The prevalence of ASD among children with developmental disorders reached 33.6% and 11.5% in Egypt and Tunisia respectively [5].

The gastrointestinal microbiota (GM) is known to play an essential role in physiological homeostasis in the intestine and periphery, including brain development and behavior [6,7]. For the last several years several clinical studies have shown altered gut microbiota composition in patients with neurodevelopmental disorders [8].

During the recent years, a substantial amount of new data has underlined the importance of the gut as a triggering place for autism [9]. In children with ASD, the presence of gastrointestinal (GI) dysfunction is often associated with increased irritability, tantrums, aggressive behaviour, and sleep disturbances. Moreover, modulating gut bacteria with short-term antibiotic treatment can lead to temporary improvement in behavioral symptoms in some individuals with ASD [10,11].

Members of the genus *Sutterella* were first described in 1996 by Wexler et al. [12]. They are anaerobic or microaerophilic, short rods gram-negative, non-spore-forming bacteria from the family of *Sutterella*ceae which belongs to the class of *Betaproteobacteria* of the order *Burkholderiale* and were isolated from human faeces [13].

There is evidence that *Sutterella* occurs in human faeces as a common member of the human indigenous microflora [14]. Studies have, however, linked *Sutterella* to the pathogenesis of GI disturbances in children with autism [15], though the studies are not sufficient to ensure that there is a definite relationship.

The aim of the present study was to detect the association between *Sutterella* species in stool and Autism Spectrum Disorders (ASDs).

2. MATERIALS AND METHODS

2.1 Subjects

Thirty autistic children with and without gastrointestinal symptoms, who presented to the autism clinic of Alexandria University Children's Hospital were enrolled in our study. These children were diagnosed with ASDs according to DMS-V criteria [1] and CARS (Childhood Autism Rating Scale) was used to assess severity of autism [16].

A cross matching control group of 30 healthy neurotypical children of similar age and sex was also included.

After receiving informed consent from the parent, a clinical history for each case and stool sample were obtained.

In addition gastrointestinal symptoms were assessed in cases with a modified six-item GI

Severity Index (6-GSI) questionnaire. Specifically, it included only six items (constipation, diarrhea, stool consistency, stool smell, flatulence, and abdominal pain). The absence of a symptom was 0, its presence ranged from 1 to 2 depending on the severity of the symptom, thus the score includes the number of symptoms and their severity [17].

2.2 Specimen Collection, Preservation and Transport

Stool specimens were collected by parents, kept in the freezer upon defecation at home, and within the same day delivered to our laboratory frozen, where aliquots of each specimen were frozen at −80℃ until DNA extraction in the same week.

2.3 Polymerase Chain Reaction Assay

DNA was extracted from 150 mg stool samples using ISOLATE Fecal DNA Kit (Bioline, UK) according to the manufacturers' instructions. In brief, fecal samples were added directly to a bashing beads lysis tube and they were rapidly lysed by bead beating in a vortex, without the use of organic denaturants or proteinases. The DNA was then bound, isolated and purified using spin columns. The resulting DNA extracts were stored at -70℃ until PCR assessment.

Conventional PCR for detection of *Sutterella* was carried out using primers SuttFor (5'-CGCGAAAAACCTTACCTAGCC-3') and SuttRev (5'-GACGTGTGAGGCCCTAGCC-3'), that amplify a 260-bp region spanning the variable regions from V6 to V8 of the 16S rRNA gene [15].

Reaction was performed in a final volume of 25 µl of PCR mixture containing 0.8 µM of each primer (Metabion International AG. Germany), 2X MyTaq™ Red PCR Mix (Bioline, UK), and 3 µl of template DNA. DNA amplification was carried out with a thermal cycler (Genius Techne, England) as follow: Denaturation at 95℃ for 15 minutes in the first cycle, followed by annealing for 1 minute at 60℃, extension for 1 minutes at 72℃, and denaturation for 1 minute at 94℃ for a total of 30 PCR cycles. The extension for the last cycle was increased to 5 minutes to ensure complete extension of the amplified fragment. The amplified product was detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide [15].

2.4 Statistical Analysis of the Data

The statistical significance of observed differences was evaluated using independent t test for normally distributed continuous variables and the Mann-Whitney *U* test for non-normally distributed continuous variables and the $\chi 2$ or Fisher's exact test for categorical variables, where appropriate. $P < 0.05$ was considered statistically significant. Data were analyzed with IBM SPSS version 23.0.

3. RESULTS AND DISCUSSION

3.1 Results

Out of the 30 patients, 23 were males and 7 were females with male to female ratio of 3.3:1. The mean age was 4.4 ± 1.5 and their age ranged from 2.5-8 years. Out of the 30 control cases examined there were 21 males and 9 females, with male to female ratio of 2.3:1. The mean age ± SD was 4.3 ± 1.9, and their age ranged from 2-8 years. According to Childhood Autism Rating Scale (CARS), all the ASD cases were mild to moderate with mean CARS 29.8±3.55. Fifteen out of 30 (50%) of ASD patients were mild while the other 15 were moderate ASD. For the male patients 11 out of 23 (47.8%) and 12 out of 23 (52.2%) were mild and moderate ASD respectively. For the female patients 4 out of 7 (57.1%) and 3 out of 7 (42.9%) were mild and moderate ASD respectively, (statistically nonsignificant, *P* value 0.204).

In this study, GI disturbances were scored using a modified version of standardized GI-Severity Index (GSI), which takes into account constipation, diarrhea, stool consistency, stool smell, flatulence, and abdominal pain. The mean of GSI was 3.5±1.8. Out of the 30 ASD cases, 27 (90%) have at least one GI symptom at the time of examination. Only 3 cases (10%) did not have any symptom. As regards bowel movement, out of the 30 ASD cases at the time of 15 (50%), 4 (13.3%) and 11 (36.7%) had constipation, diarrhea, and no constipation/diarrhea respectively. For other symptom, 22 (73.3%), 14 (46.7%), 7 (23.3%) and 2 (6.7%) had abnormal stool smell, flatulence, abdominal pain, and unformed stool respectively. There was negative correlation between the GSI and CARS of the ASD cases (-0.0286) and a significant relation at *P* value <0.001. None of the control cases had GI disturbances (Table 1).

By comparing the GSI and the CARS of ASD cases, the mean GSI for mild was 3.53±1.7, while that for the moderate was 3.47±2.1 (statistically non significant, *P* value 1.96).

Table 1. Characteristics of ASD patients

Characteristics	No (%)
Mean age ± SD	4.5±1.6
Age range	2.5-8
Male: Female ratio	3.3:1
CARS	29.8±3.55
GSI ± SD	3.5±1.8
GI Symptoms	27 (90%)
Abnormal stool smell	17 (70.8%)
Constipation	13 (54.2%)
Flatulence	13 (54.2%)
No constipation/diarrhea	7 (29.2%)
Abdominal pain	5 (20.8%)
Diarrhea	4 (16.7%)
Unformed stool	2 (8.3%)

Sutterella was detected in 24 out of 30 ASD cases (80%), and in the 11 out of 30 (36.7%) in control cases and this is statistically significant with *P*=0.001 (Table 2).

As shown in Table 3, *Sutterella was* detected in 24 ASD cases with mean age ± SD 4.5±1.6, while the remaining 6 cases (20%) with mean age ± SD 4.1 ± 0.5 were negative (statistically nonsignificant t= 0.2, *P* value 0.854) (Fig. 1). Among the male patients, 19 out of 23 cases (82.6%) were positive for *Sutterella.* For the female patients, 5 out of 7 cases (71.4%) were positive. The difference between the percentage of male and female positive cases is statistically nonsignificant (*P* value 0.517). The CARS of the positive cases was 29.8±3.55 while that of the negative cases was 31.66±3.33 (statistically non significant, *P* value 0.489).

The GSI of the positive cases was 3.7±1.8 while that of the negative cases is 2.8±2.0 (statistically non significant, *P* value 0.313).

As shown in Table 3, out of the 24 *Sutterella* positive ASD cases 13 (54.2%), 4 (16.7%) and 7 (29.2%) had constipation, diarrhea, and no constipation/diarrhea respectively. For other symptoms, 17 (70.8%), 13 (54.2%), 5 (20.8%) and 2 (8.3%) had abnormal stool smell, flatulence, abdominal pain, and unformed stool respectively. For the 6 *Sutterella* negative ASD cases 2 (33.3%), 0 (0%) and 4 (66.7%) had constipation, diarrhea, and no constipation/ diarrhea respectively. For other symptoms, 5 (83.3%), 1 (16.7%), 2 (33.3%) and 0 (0%) had abnormal stool smell, flatulence, abdominal pain, and unformed stool respectively, (statistically nonsignificant).

Table 2. Comparison between *Sutterella* positive ASD and control cases

	ASD	Control	P value
Total cases	**24 (80%)**	11 (36.7%)	0.001
Age			
Mean age ± SD	4.5±1.6	4.3 ± 2.2	0.900
Age range	2.5-8	2-8	
Gender			
Male	19 (79.2%)	8 (72.7%)	0.673
Female	5 (20.8%)	3 (27.3%)	
CARS			
Mean ± SD	29.8±3.55	-	
GI Symptoms			
GSI Mean ± SD	3.5±1.8	0	0.116
GSI Range	0-6	0	
Constipation	13 (54.2%)	0	0.002
Diarrhea	4 (16.7%)	0	0.150
No constipation/diarrhea	7 (29.2%)	11 (100%)	0.00009
Abnormal stool smell	17 (70.8%)	0	0.00009
Flatulence	13 (54.2%)	0	0.002
Abdominal pain	5 (20.8%)	0	0.102
Unformed stool	2 (8.3%)	0	0.324

Table 3. Characteristics of *Sutterella* positive and negative ASD patients

	***Sutterella* Positive**	***Sutterella* Negative**	***P* value**
Total cases	**24 (80%)**	**6 (20%)**	
Age			
Mean age ± SD	4.5±1.6	4.1 ± 0.5	0.556
Age range	2.5-8	3.5-5	
Gender			
Male	19 (79.2%)	4 (66.7%)	0.517
Female	5 (20.8%)	2 (33.3%)	
CARS			
Mean ± SD	29.8±3.55	31.66±3.33	0.489
GI Symptoms			
GSI Mean ± SD	3.5±1.8	2.2±2.2	0.116
GSI Range	0-6	0-6	0.361
Constipation	13 (54.2%)	2 (33.3%)	0.324
Diarrhea	4 (16.7%)	0 (0%)	0.088
No constipation/diarrhea	7 (29.2%)	4 (66.7%)	0.536
Abnormal stool smell	17 (70.8%)	5 (83.3%)	0.361
Flatulence	13 (54.2%)	1 (16.7%)	0.099
Abdominal pain	5 (20.8%)	2 (33.3%)	0.517
Unformed stool	2 (8.3%)	0 (0%)	0.464

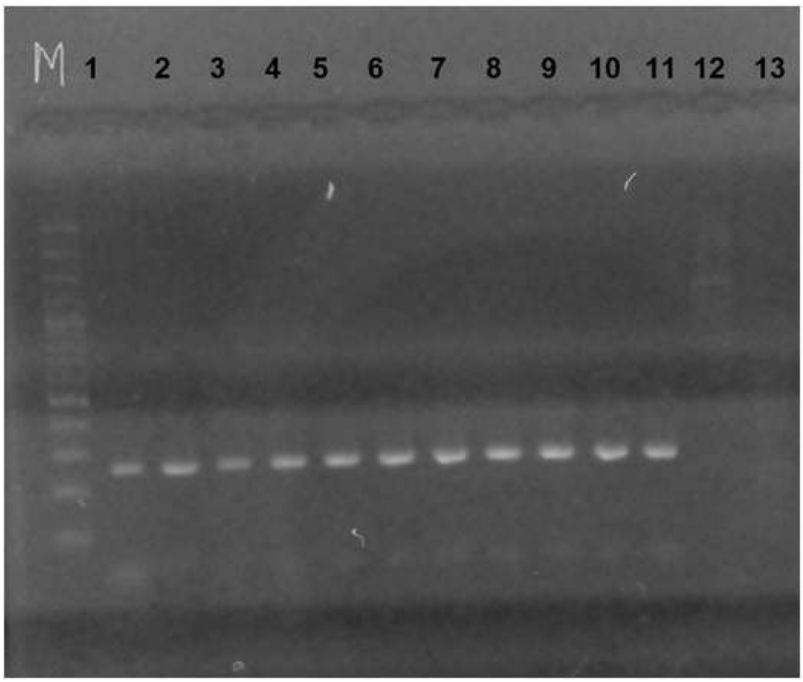

Fig. 1. Agarose gel electrophoresis for detection of *Sutterella* PCR product (v6-v8), lane M: 100 bp ladder, lanes 1-11: Positive cases showing PCR product (260 bp)

As shown in Table 2, *Sutterella was* detected in 11 cases of the control group. The mean age ± SD of the positive cases was 4.3 ± 2.2, while that of the negative cases was 4.3 ± 1.7 (statistically nonsignificant t= 0.11, *P* value 0.897). Among the male patients there were 8 out of 21 cases were positive for *Sutterella* (38.1%). For the female patients, 3 out of 9 cases were positive (33.3%). The difference between the percentage of male and female cases is statistically nonsignificant (*P* value 0.046).

Table 2 shows the comparison between the *Sutterella* positive ASD and neurotypical control cases as regards age, gender and GI symptoms (statistically nonsignificant, *P* value 0.771, 0.842, and 0.313 respectively).

3.2 Discussion

Autism spectrum disorders are a diverse group of disorders caused by a complex interplay between genetic and environmental components. [18,19], There is some preliminary evidence suggesting that exposure to viral or bacterial pathogens may play a role in triggering the disorder in a small subgroup of individuals with autism [20,21].

Of the many medical co-morbidities associated with ASD, gastrointestinal (GI) distress has gained significant attention because of its reported prevalence and association with symptom severity [22,23]. The propensity for associated GI issues in many ASD children has led some researchers to hypothesize a gut microbial involvement in disease [24,25].

Whether microbiota changes can contribute to the development or progression of autism symptoms is unknown. Attempts have been made to define and isolate the possible, specific intestinal bacterial species that may play some role in this disorder. Recently, the focus has been on the presence of the genus *Sutterella*. [15,25].

The present study was conducted on 30 ASD and 30 neurotypical control children. The ASD patients were 23 males and 7 females with a ratio 3.3:1, their age range 2.5-8 years and mean age 4.4 ± 1.5. GI disturbances have been apparent in 90% of our patients, in the form of abnormal stool smell (73.3%), constipation (50%), flatulence (46.7%), abdominal pain (23.3%), diarrhea (13.3%), and unformed stool (6.7%).

By using CARS for the assessment of the severity of autism, all of ASD patients were mild to moderate. There was no significant difference between male and female patients as regards the severity of autism. Also there was no significant difference between the GSI and the CARS of ASD cases, the GSI for the mild was 5.3, while for the moderate was 5.2.

By PCR *Sutterella* DNA has been detected in 80% of ASD cases, and 36.7% of control cases. There was no significant correlation between the presence of *Sutterella* and the severity of ASD or GSI.

Regarding the sex distribution, the present study agrees with studies reporting that autism is more common in boys than girls while it disagrees as regards autism is more severe in girls than boys, but this may be explained by the small sample size of the females to allow for comparisons in our study [4,26].

As regards the GI disturbances in the present study our results agree with many studies reporting GI disturbances as a common co-morbidity in ASD patients as the case with 90% of our ASD patients. However, it disagrees with the positive correlation of GSI with ASD symptom severity [27–30]. Comparison between studies is quite difficult due to the fact that different methodologies for assessment of ASD severity and GI symptoms severity. Moreover, our study may not be comparable due to the small sample size and limited diversity of cases, all are mild to moderate.

As regards *Sutterella*, our results demonstrated that *Sutterella* is detected much more in the feces of children with ASD relative to the controls. This confirms the findings of Williams et al [15], who were the first to develop *Sutterella*-specific PCR assays for detecting *Sutterella* species in biological and environmental samples, used in our study. They demonstrated increased levels of *Sutterella* in intestinal biopsy samples from 12 of 23 (52%) ASD children with GI disturbance and its absence in the 9 control children with GI symptoms only, further emphasizing that such species might play an important role in the microbiota gut-brain axis [15].

However, a study done by Kang et al. [31], have found no increase in *Sutterella* in their ASD cases compared to the controls. The association of *Sutterella* with ASD was reported in other studies that found differences in *Sutterella* detected from mucosal-biopsies as compared to those fecal samples, which may explain the different prevalences of *Sutterella* between various studies [15].

In contrast Wang et al. [25] observed higher levels of *Sutterella* and *Ruminococcus* spp. in stool samples of individuals with ASD compared to controls. Their results thus indicate that fecal samples used in molecular techniques are

sufficient for the detection and quantification of *Sutterella* in the human gut, including children with ASD.

4. CONCLUSION

The role that *Sutterellae* play in ASD is not yet apparent but they may relate to bigger scale shifts in the microbial populations as a consequence of the condition. The outcome of this study offer strong grounds to carry out further investigations to ascertain its possible role - if ever – in the pathogenesis of ASD patients.

DISCLAIMER

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Authors have declared that no competing interests exist.

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