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Quantitative Stool Culture of Candida in Egyptian Children with Autism Spectrum Disorder

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ABSTRACT

Background: A group of neurodevelopmental diseases known as autism spectrum disorder (ASD) is characterized by difficulties with social interaction and communication as well as limited or repetitive activities.

Objective: To estimate the quantity of Candida in stool of autism spectrum disorder (ASD) patients compared to normal children and to find the association between Candida colony count and severity of ASD.

Patients and Methods: The study involved 40 children with autism and 40 typically developing children who were recruited from the pediatrics and adolescent psychiatric clinic at the pediatric hospitals of Ain Shams University. The research included participants who ranged in age from 3 to 14 years, (mean age of 6.30±2.40 years**)**. Stool sample was collected from each patient in a sterile container, cultured on Sabouraud Dextrose Agar (SDA) and colony count was determined. Identification of isolated Candida species was done using chromogenic media**.**

Results: The study revealed statistically significant difference in Candida isolation rate among patient and control group with p-value (p=0.006). Candida species were isolated from 23 patients (57.5%) and from 10 children in control group (25%), but there was statistical insignificant difference in Candida colony count between severe autistic group compared to mild to moderate autistic group. There were 14 patients (60.9%) with C. Albicans; 2 patients (8.7%) C. Glabrata; 6 patients (26.1%) C. Krusei and one patient (4.3%) C. Utilis**.**

Conclusion: Children with ASD had increased rates of intestinal Candida species colonization, which may be a symptom of a condition associated to immune system abnormalities that may contribute to the etiology of ASD. In ASD, C. albicans was the most common isolate.

Keywords: Quantitative Stool Culture, Candida, Autism Spectrum Disorder.

INTRODUCTION

A variety of neurodevelopmental diseases collectively known as autism spectrum disorder (ASD) are largely distinguished by difficulty in social interactions, verbal and nonverbal communication, and stereotypical or repetitive behaviors [(1)].

The etiological pathway and pathogenesis of ASD is still controversial. However, maternal infection during pregnancy, maternal obesity and diabetes, excessive early childhood vaccination, heavy metal toxicity, severe infection in the first 2 years of life, involvement of bacteria, viruses and fungi, long-term exposure to antibiotics in early life [intestinal microbial dysbiosis, gastrointestinal (GI) problems; leaky gut syndrome; allergies, immune dysfunction, neuroinflammation, developmental abnormalities of the nervous system, neurotransmitter imbalances (serotonin, dopamine, γ-aminobutyric acid (GABA), noradrenaline), and metabolic factors deficiency. Autism development may be also influenced by oxidative stress, environmental variables, and genetics[(2)]**.**

Children with ASD have been found to have a high prevalence of gastrointestinal discomfort, including bloating, diarrhea, constipation, and abdominal pain, suggesting that the gut microbiota may play a role in the pathogenesis of gastrointestinal symptoms associated with ASD [(3)]. The so-called "microbiome-gut-brain axis" is a significant relationship between the CNS and the gut microbiota [(4)]**.**

A fungus that resembles yeast called Candida albicans lives in practically every human. It feeds on the dark, wet mucous membranes that border the digestive system, vagina, and mouth. It can be found in little colonies. Candida albicans can infect people, especially those with weak immune systems [(5)].

C. albicans generates ammonia (NH3) as a metabolite, which has been believed to be associated with autism when present in excess. Propionic acid might be converted to beta-alanine, which shares structural similarities with the inhibitory neurotransmitter GABA (gamma, aminobutyric acid), in the presence of ammonia metabolites in the gastrointestinal system. It is a neurotransmitter found in the mammalian central nervous system. Its main functions include controlling muscle tone by inhibiting receptors and lowering neuronal excitability throughout the nervous system [(6)]. Children with suspected or identified ASD have had several Candida species isolated from their feces [(7)].

Aim of the work was to estimate the quantity of Candida in stool of autism spectrum disorder (ASD) patients compared to normal children and to find the association between Candida colony count and severity of ASD.

PATIENTS AND METHODS

The study involved 40 children, diagnosed as autism spectrum disorder; all of them fulfilled DSM -V

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criteria of ASD [1]. Patients were recruited from Pediatric and Adolescent Psychiatric Clinic and 40 normal children as control group. The research included participants ranging in age from 3 to 14 years (mean age of 6.30±2.40 years). Males predominated, with an about 4:1 male to female ratio.

Children undergoing antifungal therapy, those on cytotoxic or immunosuppressive medications, and those whose routine laboratory tests—such as those for blood sugar, renal function, or liver function—were abnormally high or low were excluded from the research. The kids in this study underwent CARS (Childhood Autism Rating Scale) [8]. Patient interviews were conducted with all patients (personal as well as family history including consanguinity, delay in language development, and hearing loss,), developmental history throughout pregnancy, labor, delivery, and postpartum, evaluation of language (response, eye contact as well as head coordination), E.N.T examination, vocabulary evaluation for passive and active words, (CARS) and the threshold for an autism diagnosis was set at 30, with mild-moderate autism (30–37) and severe autism (> 37) [9].

Additionally, a neurological exam and audiological evaluations, such as tympanometery and acoustic reflex testing and auditory brain stem evoked potentials, were performed.

Stool Culture:

Stool sample was collected from each patient in a sterile container, cultured on Sabouraud Dextrose Agar (SDA) and colony count was determined. Identification of isolated Candida species was done using chromogenic media.

The samples were added to a 500 ml plate of Sabouraud Dextrose Agar (SDA; Oxoid, UK) and infected onto it. At 37°C, the incubation took place aerobically for 24-48 hours. Colonies growing on culture media were counted after incubation and expressed as colony-forming unit (CFU)/g feces. Quantitative culture was done according to method of **Iovene and his colleagues** [10] with modification as follows:

A clean plastic tube was used to weigh 200 mg of stool sample then three hundred microliter of sterile saline was added to the weighed sample, using a sterile loop, combine, then wait a few minutes at room temperature. After mixing, 50 microliter of the suspension was used to culture the feces onto the Sabouraud dextrose agar that had been produced. Plates were incubated aerobically at 37°C for 48 hours After incubation, the number of Candida colonies was counted, multiplied by 50 and expressed as CFU/g feces finally gram stain was done from growing colonies suspected as Candida species to confirm.

Insufficient or sparsely populated colonies in the stool samples (like those of the control group) were regarded as evidence of negative growth. Subculture was done (from colonies of each plate) on HiCrome Candida Differential Agar (Himedia, India) to identify candida species. Cultural characteristics observed after an incubation at 30-35°C for 40-48 hours. Then identification was according colour of each species.

Ethical consent:

Ain Shams University Faculty of Medicine's Ethics Committee gave the study the thumbs up. Before enrolling any children in the study, parents of all potential participants were given the work's goals and asked for their signed agreement. The database's handling was promised to be confidential. The Declaration of Helsinki, the code of ethics of the World Medical Association, was followed when conducting this research on humans.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 18 for Windows was used to code, process, and analyze the obtained data (IBM SPSS Inc, Chicago, IL, USA). Using the Shapiro Wilk test, the distribution of the data was examined for normality. Frequencies and relative percentages were used to depict qualitative data, which were compared by chi square test. Quantitative information was presented as mean SD (Standard deviation). Two independent groups of normally distributed variables were compared using the independent samples t-test (parametric data). P value less than 0.05 was regarded as significant.

RESULTS

The study was conducted on a wide age group ranging from 3 to 14 years, (mean age of 6.30±2.40 years). There was male predominance with male to female ratio about 4:1. According to demographic statistics on age and sex, there was no group that varied significantly from one another.

The study showed statistically significant higher mean of stool count in severe of autistic group compared to mild to moderate of autistic group, with p-value (p=0.041). According to the candida species, the difference from mild to moderate and severe autism was not statistically significant.

Table (1) shows a statistically significant difference in Candida isolation rate among patient and control group.

Table (1): Comparison between patient and control groups regarding positive candida spp. distribution

Candida	Groups				Chi-square test	
	Patients		Control			
	No.	%	No.	%	X^2	p-value
Negative	17	42.5%	30	75.0%	7.427	0.006*
Positive	23	57.5%	10	25.0%		
Total	40	100.0%	40	100.0%		

*: Significant

Table (2) shows that C. albicans was the most common spp. of candida detected in stool specimens of patient group (14 patient) and control group samples were 5 cases.

Table (2): Comparison between patients and control groups regarding type of Candida species

Candida species	Groups			
	Patients		Control	
	No.	%	No.	%
C. albicans	14	60.9%	5	50.0%
C. glabrata	2	8.7%	3	30.0%
C. krusei	6	26.1%	2	20.0%
C. utilis	1	4.3%	0	0.0%
Total	23	100.0%	10	100.0%

Table (3) shows Candida colony count in both patient and control groups. In patient group the lowest positive count was 20-50 CFU/g stool while the highest positive count was more than 350 CFU/g in patient group. But in control group, the lowest positive count was 20-50 CFU/g stool while the highest positive count was 150-200C FU/g.

Table (3): Comparison between patient and control group Candida stool count

Stool count CFU/g	Patients		Controls	
	no.	%	no.	%
Negative	17	42.5%	30	75%
20-50	4	10%	2	5%
50-100	1	2.5%	2	5%
100-150	3	7.5%	3	7.5%
150-200	0	0.0%	3	7.5%
200-250	1	2.5%	0	0%
250-300	2	5%	0	0%
300-350	6	15%	0	0%
More than350	6	15%	0	0%
Total	40	100%	40	100%

Table (4) shows that there was no statistically significant association between severity of cases and Candida distribution among patient group.

Table (4): Association between severity of CARS and candida distribution among patients group

Candida	Mild to moderate CARS	Severe CARS	X^2	p-value
Positive	19 (59.4%)	4 (50%)	0.006	0.936
Negative	13 (40.6%)	4 (50%)		

Using X^2: Chi-square test

There were 32 patients (80%) had mild to moderate severity of CARS and 8 patients (20%) had severe CARS. There was no statistical significant difference between severity of CARS and candida stool count (Table 5).

Table (5): Association between severity of CARS and candida stool count

Stool Count CFU/g	Severity of CARS				Total	
	Mild to moderate		Severe			
	No.	%	No.	%	No.	%
Negative	13	40.6%	4	50.0%	17	42.5%
50	4	12.5%	0	0.0%	4	10.0%
100	1	3.1%	0	0.0%	1	2.5%
150	3	9.4%	0	0.0%	3	7.5%
250	1	3.1%	0	0.0%	1	2.5%
300	2	6.3%	0	0.0%	2	5.0%
350	5	15.6%	1	12.5%	6	15.0%
More than 350	3	9.4%	3	37.5%	6	15.0%
Total	32	100.0%	8	100.0%	40	100.0%
Chi-square test	6.299					
p-value	0.505					

DISCUSSION

Children with ASD have been found to have a significant prevalence of gastrointestinal discomfort, including bloating, constipation, diarrhea, and stomach pain [(4)]. It has been postulated that excess C. albicans is associated with autism [(6)]. Children with suspected or identified ASD have had several candida species isolated from their feces [(7)].

Our study was conducted on 40 autistic patients diagnosed as autism spectrum disorder according DSM-5 2013. The age ranged from 3 to 14 years. Males predominated, with an about 4:1 male to female ratio. This is in accordance with **Emam *et al.*** [(11)], **Burgha *et al.*** [(12)] and **Loomes and his colleagues** [(13)], who reported that autism affects men more frequently than women, with rates varying from 2:1 to 5: 1. Multiple factors with many genetic variants, environmental factors, sex differential and hormonal factors have been postulated to potentiate ASD risk to male or attenuate such risk in female [(14)].

In our study, there was a statistically significant difference in rate of isolation of Candida species in patient group compared to control group. Candida species have been isolated from 23 patients stool specimens; 23 (57.5%) and ten (25%) in control group.

Horvath and Perman [(15)] observed that children with autism who had endoscopies had a higher rate of positive fungal culture for yeast in the duodenal juice (43 percent) than did children in the control group. Also, **Emam *et al.*** [(11)] reported a higher prevalence of yeast infection in people with autism (81.9%) compared to the control group (28 percent) and **Strati and colleagues** [(4)] detected Candida species in 37.7% of ASD patients in comparison to control group (14.1%). In their retrospective investigation looking into a large number of ASDs, **Kantarcioglu *et al.*** [(7)] revealed that a significant proportion of patients (81.4 percent) compared to controls (19.6 percent) had Candida spp. Similarly, **Adams *et al.*** [(3)]**,** on the other hand, found no differences between healthy controls and ASD patients' feces in terms of yeast infection.

Through dysregulation of cytokines, changes in the intestinal fungi population brought on by an increase in Candida in autistic people's gut microbiota may have a detrimental effect on GI problems. By inducing the immune system, primarily ILC3 cells, to release IL-22, the gut microbiota, in particular some species of Lactobacillus, modifies the immunological responses to Candida in the GI tract [(16)]. Along with IL-17, IL- 22 prevents Candida and other fungal commensals from over proliferating in the gut. Therefore, it's plausible that changes to the gut microbiota in people with ASDs might cause the Candida population to grow, delaying a complete restoration of the bacterial community structure [(4)]**.**

In our work, Candida species isolated from patient group were fourteen C. albicans (60.9%), six C. krusei (26.1%), two C. glabrata (8.7%), and one C. utilis (4.3%). Our control group showed C. albicans (50%), C. glabrata (30%) and C. krusei (20%). This is in concordance with **Falanga *et al.*** [(17)] who reported that infants with ASD tended to be mostly C. albicans species. C. albicans (59.26%), C. glabrata (7.41%), C. tropicalis (7.41%), C. lusitaniae (7.41%), C. parapsilosis (11.11%), and C. krusei (7;41%).

Also, **Emam *et al.*** [(11)] reported (81.9 %) cases positive for C. albicans in their study group. However, **Ahmad *et al.*** [(18)], stated that most common candida spp. in autistic patients was C. glabrata (44.1%), followed by Candida albicans (14.7%), Candida tropicalis (17.7%), and C. parapsilosis (14.7 percent). **Colombo and colleagues** [(19)], claimed that the yeasts found in their instances of ASD were non-albicans Candida species, C. krusei, and C. tropicalis.

In our study, none of the control group had Candida colony count higher than 200 CFU/g stool while in patient group 15 patients (37.5%) showed higher counts. **El-Shouny *et al.*** [(20)] discovered a substantial yeast growth in the autistic group compared to the control group. This is in line with the findings of **Iovene *et al.*** [(10)] who showed that elevated counts were

present in more than half of the stool samples examined from 27 patients with ASDs.

In addition, we demonstrated statistically non-significant difference between stool count in severe CARS cases compared to mild-moderate of cases. In our results, three (9.4%) with mild to moderate CARS severity had candida stool count more than 350 CFU/g in comparison to three (37.5%) with severe CARS and 50% of severe CARS and 40.7% of mild to moderate CARS had culture negative Candida. According to **Ahmad *et al.* [18]** and **Strati *et al.* [4],** candida infection had no impact on the severity of autism (p>0.5).

Similar results were reported by **El-Shouny *et al.*** study **[21],** they showed that excessive yeast growth in autistic children is a characteristic without taking the severity of the autism into account. **Emam *et al.* [11]**, on the other hand, found that patients in the mild-moderate group had substantially more patients with negative stool culture growth than patients in the severe group (P = 0.027).

CONCLUSION

The high rate of intestinal colonization by Candida species in ASD children may represent a part of syndrome related to immune system disorders that may play a role in the pathogenesis of ASD. C. albicans was the predominant isolate than other non-Candida albicans in ASD. The degree of candida colonization, represented as quantitative colony count, does not affect the severity of CARS among ASD patients.

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