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Study of Candida Species in Stool of Children with Autism Spectrum Disorders in Alexandria, Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. Author SASA designed the study protocol. Author MAM, the corresponding author performed the laboratory work and wrote the manuscript. Author HGA provided the clinical data of the patients. Author MHA managed the statistical analyses of the data. Author YSR assisted in the laboratory work. Author HAG collected the samples. Author AEI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aims: The pathogenesis of Autism spectrum disorders (ASDs) is still controversial. The aim of the present study was to investigate the presence and to identify the species and the antimicrobial sensitivity of Candida present in stool of autistic children.
Subjects and Methods: A total of 50 children with ASD, 36 siblings controls (brother or sister) and 50 healthy controls were enrolled in the study. Stool samples were subjected to examination and culture on Sabouraud dextrose (SDA) and Brilliance Candida selective agars (BCA) (Oxoid, UK)

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followed by complete identification and antifungal susceptibility disc diffusion testing. DNA extraction was performed for all stool samples and then subjected to multiplex PCR for identification of Candida species.

Results: Out of the 50 ASD stool samples, 23 (46%) were culture positive and 33 (66%) were PCR positive. Of which, 51 different Candida species were isolated. *C. glabrata* (43.1%) was the most commonly isolated, followed by *C. parapsilosis* (19.6%), *C. tropicalis* (17.7%), then *C. albicans* (9.8%), and *C. krusei* (9.8%). All *C. albicans* isolates were sensitive to nystatin, fluconazole and voriconazole. When controls (siblings and healthy) were compared with ASD patients, the rate of Candida isolation was found significantly lower in controls (p value = .019 & p value = .046 respectively). No significant difference was found between Candida positive and negative ASD groups regarding the severity of autism and GIT manifestations.

Conclusion: The present work found that the colonization with Candida spp. did not affect the severity of symptoms in ASD children. However, Future studies should verify whether Candida elimination therapy is useful to manage ASDs symptoms.

Keywords: Autism; Candida; nystatin; multiplex PCR.

1. INTRODUCTION

Autism spectrum disorders (ASDs) are complex neurodevelopmental conditions characterized by cognitive impairments, social interaction skills defects and communication, language and behavioral problems [1]. ASDs arise from a combination of genetic and environmental factors [2]. Although, their pathogenesis is still controversy; however, several biochemical and immune events as well as many gastrointestinal tract (GIT) dysfunctions have been described [3,4].

A strong correlation between GIT symptoms and autism severity, altered intestinal permeability, intestinal dysbiosis together with microbiota changes were described [5]. Dysbiosis could be related to yeast infection and overgrowth. Several clinicians report the presence of Candida-related symptoms in autistic children and various hypotheses on the biochemical cascade following such an infection have been postulated [6,7].

It is evident that autistic children suffering of Candida overgrowth shows increased irritability, aggressive behavior, and sleep disturbances. In addition to other GIT manifestations like diarrhea, constipation, vomiting, abdominal pain/ discomfort, gaseousness, and foul-smelling stools [4,8].

C. albicans, when present in excess has been hypothesized to be correlated with autism, it produces ammonia (NH3) as a metabolite. Propionic acid is in the presence of ammonia metabolites in the GIT, could be converted to beta-alanine, which is structurally comparable to the inhibitory neurotransmitter GABA. [9]. Similarly, there are many reports showing a decrease of autistic symptoms after the patient is placed on a gluten and/or casein free diet. Both gluten and casein can increase quantities of yeast in the GIT of patients [10].

The aim of the present study was to investigate the presence and to identify the species of Candida present in stool of autistic children and to determine their in vitro susceptibility to selected antifungal drugs, in order to provide data for a possible specific therapeutic intervention. The study was also conducted to find any association between ASD severity, GIT disturbance and intestinal colonization with Candida.

2. SUBJECTS AND METHODS

A total of 50 children with ASD, 36 children constituting their siblings (brother or sister) and 50 neurotypical children (healthy controls) were enrolled in a prospective observational study. ASD Subjects were recruited from Al-Shatby Pediatric University Hospital. Healthy controls were recruited from school districts and community centers.

Inclusion criteria were as follows:

1) Age between 2 to 18 years.
2) No usage of any type of antibiotic or antifungal medications within the last month.
3) Diagnosis of ASD according to CARS (Childhood Autism Rating Scale). The total CARS score was used to evaluate the global degree of ASD disease; 30 serving as a cut off for a diagnosis of autism, values ≤38 were considered mild-moderate

degree and values >38 were considered of severe degree of the disease [11].

Exclusion criteria were: Autism secondary to genetic syndromes; Rett syndrome; childhood disintegrative disorder; epilepsy; neurological syndromes; concomitant condition of known celiac disease; diabetes mellitus type, inflammatory bowel disease or hepatic disorders, as well as known and serologically proven food intolerances.

Matched controls were recruited in good mental and physical health: no stomach/gut problems such as chronic diarrhea, constipation, gas, heartburn, bloating, etc. No Attention Deficit Disorders. Unrelated to an individual with autism (not a brother, sister, parent, aunt, or uncle).

2.1 Data Collection and Clinical Assessment

1. A structured sheet was used to collect administrative and clinical data of the patients.
2. Gastrointestinal symptoms were assessed using a modified version of the GI Severity Index " 6-GI Severity Index" [5].

2.2 Specimen Collection, Preservation and Transport

A single stool sample was collected from each of ASD subjects, siblings and controls. The fresh stool samples were collected in sterile containers, then immediately transferred to Alexandria University Microbiology Research Lab to be processed. Part of stool samples were stored at -80℃ till PCR processing.

2.3 Stool Examination and Culture

2.3.1 Microscopic examination of stool

Microscopic examination of stool was performed using wet smear to examine pus cells, RBCs, mucus, and parasites, and with methylene blue stain to verify the presence of yeast-like organisms by means of light microscope using x10 and x100 oil immersion objectives respectively.

2.3.2 Culture

A weighted quantity of the fecal sample (100 mg) was suspended in 1 ml of sterile physiological solution, homogenized by vortexing and left at room temperature for a few minutes. Ten µL of the suspension was used to inoculate culture media. The samples were inoculated onto Columbia Blood Agar (CBA; Oxoid, UK), Sabouraud Dextrose Agar (SDA; Oxoid, UK) supplemented with 0.5 g/liter Chloramphenicol and 0.5 g/liter Gentamicin, and Brilliance Candida Agar (BCA; Oxoid, UK) for selective isolation of Candida spp. The incubation was carried out aerobically at 37 ℃ for 24-48 hrs in the case of CBA, while at 30℃ for up to 5 days in the case of SDA and BCA. After incubation, the count of colonies grown on culture medium was performed and expressed as CFU/g feces. All cultures were considered positive if colonies count was $\geq 10^3$ [12].

The isolated colonies on CBA and SDA were identified by classical morphological and biochemical tests including gram stain, germ tube formation in human serum, blastoconidia, pseudohyphae, chlamydospores formation on rice extract agar–Tween (BD, Germany). Colonies on BCA were identified by their colors according to the manufacturer's instructions. All isolated colonies were identified to the species level by mass spectrometry MALDI-TOF MS UltraFlex system (BrükerDaltonik) according to the manufacturer's instructions.

2.4 Antifungal Susceptibility Testing

It was performed for ASD Candida isolates only. It was done by disc diffusion method using the following antifungal discs: Amphotericin B (AMB; 100µg) (Bio rad), Voriconazole(VOR; 1µg) (Oxoid), Fluconazole (FCA; 25 µg) (Oxoid) and Nystatin (NS; 100 units) (Oxoid).The results were interpreted according to CLSI criteria M44A [13]. *C. albicans* ATCC 90028 was used as control strain.

2.5 Multiplex PCR for Identification of Candida Species

2.5.1 DNA extraction from stool samples

It was performed using ISOLATE Fecal DNA Kit (Bioline, UK) according to the manufacturer's instructions. Two µL DNA extract was used as template in PCR reaction.

2.5.2 Multiplex PCR amplification

The multiplex PCR protocol was performed as previously described by Chang et al. [14].

Table 1. Primers sequences for multiplex Candida PCR and length of PCR products for different Candida species

Primer name	Sequence
ITS	ITS $_{forward}$ (5'-TCC GTA GGT GAA CCT GCG G-3')
	ITS $_{reverse}$ (5'-GCT GCG TTC TTC ATC GAT GC-3')
CA	CA $_{forward}$ (5'-GGT TTG CTT GAA AGA CGG TAG-3')
	CA $_{reverse}$ (5'-AGT TTG AAG ATA TAC GTG GTA G-3')
Candida species	**PCR product (bp)**
C. krusei	182
C. tropicalis	218
C. albicans	219, 110
C. parapsilosis	229
C. glabrata	482

Fungus-specific universal primers (ITS) were used to amplify a small conserved portion of the 18S rDNA region, the adjacent ITS1, and a small portion of the 28S rDNA region. In addition, *C. albicans*-specific primers (CA) were also included in the same PCR mixture to amplify a portion of the ITS2 region of *C. albicans* (Invitrogen by Life Technologies, Thermo Fisher Scientific Inc., USA) (Table 1 above).

2.6 Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA). Statistical analysis of quantitative and qualitative data, including descriptive statistics, was performed. Continuous data are expressed as mean ± standard deviation (SD), unless otherwise specified. Frequency analysis was performed with Chi-square test. The intergroup differences were assessed by one-way analysis of variance test for parametric variables.

3. RESULTS

3.1 Microscopic Examination Results

Microscopic examination of the stool samples revealed no blood, mucus, pus, or parasites in the three groups, thus no significant differences among the investigated groups. Yeast-like cells were detected in the majority of ASD stools as well as in the siblings and healthy controls.

3.2 Culture Results

Out of the 50 ASD stool samples, 23 (46%) were culture positive by both SDA and BSA. Of these, 34 different Candida spp. were isolated; *C. glabrata* (15 isolates; 44.1%), *C. parapsilosis* (six isolates; 17.7%), *C. albicans* (five isolates; 14.7%), *C. tropicalis* (five isolates; 14.7%), and *C. krusei* (three isolates; 8.8%). Eight ASD cases had mixed Candida growth. The results of culture positive cases were in accordance with that of PCR for the 23 cases. Ten ASD stool samples were culture negative although PCR positive.

While, out of 36 ASD siblings controls, 14 (38.9%) were culture positive, of which 23 Candida isolates were identified; ten (43.5%)*C. glabrata*, four (17.5%) *C. parapsilosis*, three (13%) for each of *C. albicans*, *C. tropicalis* and *C. krusei*. Nine siblings had double Candida spp. growth. Regarding the healthy controls, 10(20%) were culture positive and 18 Candida isolates were isolated (seven *C. glabrata*, four for each of *C. tropicalis* and *C. krusei*, two *C. parapsilosis*, and one *C. albicans* isolate). Eight controls showed growth of two Candida spp. Fig. 1(a), 1(b).

3.3 Antifungal Susceptibility Results

The results of disc diffusion testing of the 34 ASD Candida isolates are mentioned in Table 2.

3.4 PCR Results

Candida spp. was detected in the stool of 33/50 (66%) of ASD children, 25/36 (69.4%) of their siblings. From a total of 50 ASD stool samples, 51 Candida of different species were isolated. *C. glabrata* (22/51; 43.1%) was the most commonly isolated one, followed by *C. parapsilosis* (10/51; 19.6%), *C. tropicalis* (9/51; 17.7%), then *C. albicans* (5/51; 9.8%), and *C. krusei* (5/51; 9.8%). Ten ASD cases had simultaneously two Candida spp. while four cases had mixed three Candida species Fig. 2.

Table 2. Antifungal susceptibility of the 34 Candida species isolated from ASD cases

Organism	AMB			FCA			VOR			NS		
Total no of isolates n=34	**S**	**S-DD**	**R**	**S**	**S-DD**	**R**	**S**	**S-DD**	**R**	**S**	**S-DD**	**R**
C. albicans (N=5)	5(100%)	0	0	5(100%)	0	0	5(100%)	0	0	5(100%)	0	0
C. glabrata (N=15)	14(93.3%)	0	1(6.7%)	0	0	15(100%)	7(46.7%)	0	8(53.3%)	0	7(46.7%)	8(53.3%)
C. krusei (N=3)	3(100%)	0	0	0	0	3(100%)	1(33.3%)	0	2(66.7%)	0	1(33.3%)	2(66.7%)
C. parapsilosis (N=6)	6(100%)	0	0	4(66.7%)	0	2(33.3%)	6(100%)	0	0	3(50%)	1(16.7%)	2(33.3%)
C. tropicalis (N=5)	4(80%)	0	1(20%)	1(20%)	1(20%)	3(60%)	2(40%)	1(20%)	2(40%)	5(100%)	0	0

AMB: Amphotericin B, FCA: Fluconazole, VOR: Voriconazole, NS:Nystatin

S: Susceptible, SDD: Susceptible-dose dependent (Candida susceptibility is dependent on achieving maximum blood levels), R: Resistant

Table 3. The comparison between the three investigated groups according to the rate of Candida identified by culture and by multiplex PCR

Candida isolated by culture	ASD group		Siblings control group		Healthy control group		Test of significance
	N	**%**	**N**	**%**	**N**	**%**	
Negative	27	54	22	61.1	40	80	Chi-Square test
Positive	23	46	14	38.9	10	20	X^2=7.879
Total	50	100	36	100	50	100	P=**.019**
Candida identified by multiplex PCR							
Negative	17	34	11	30.6	27	54	Chi-Square test
Positive	33	66	25	69.4	23	46	X^2=6.138
Total	50	100	36	100	50	100	P=**.046**

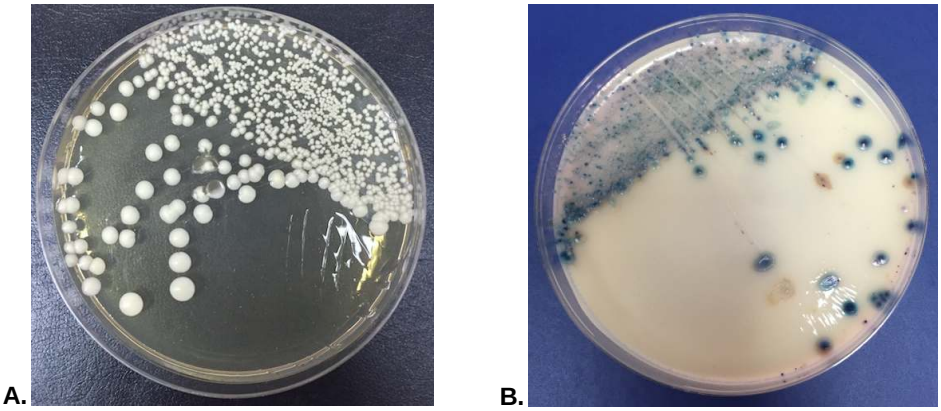

Fig. 1. Results of Candida culture

A. Candida on Sabouraud dextrose agar. B. Brilliance Candida Agar (Oxoid) showing dark blue colonies of C. tropicalis and irregular pink brown colonies of C. krusei

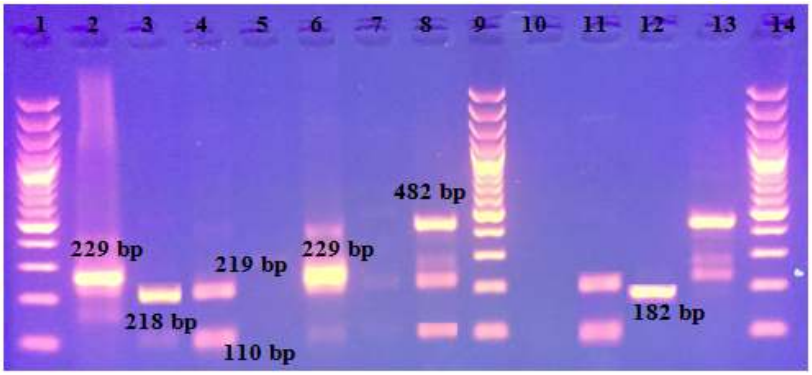

Fig. 2. Agarose gel stained with ethidium bromide of Candida multiplex PCR

Lane 1, 9 & 14: 100-1000 bp DNA ladder. Lane 2 & 6: show C. parapsilosis (229 bp). Lane 3: shows C. troplicalis(218 bp). Lane 4: shows C. albicans (219, 110 bp). Lane 12: shows C. krusei (182 bp). Lane 13: shows C. glabrata (482 bp). Lane 8: shows C. albicans and C. glabrata. Lane 5 & 7: show negative samples. Lane 10: Negative control. Lane 11: C. albicans ATCC 90028 positive control

Regarding ASD siblings controls, the order of frequency of the 38 isolated Candida spp. was as follows; *C. glabrata* (17/38; 44.7%), followed by *C. parapsilosis* (7/38; 18.4%), *C. tropicalis* (5/38; 13.2%), and *C. krusei* (5/38; 13.2%), then *C. albicans* (4/38; 10.5%).Thirteen ASD siblings showed a mixture of two Candida spp. Candida isolates were recovered from 23/50 healthy controls (46%), where 34 isolates were identified; *C. glabrata* (16/34; 47.1%), *C. krusei* (6/34; 17.6%), *C. tropicalis* (6/34; 17.6%), *C. parapsilosis* (5/34; 14.8%) and only one *C. albicans* isolate (2.9%). Two different Candida spp. were identified in each of 11 controls.

None of the ASD *C. albicans* positive cases had concomitant *C. albicans* positivity in their corresponding siblings and vice versa.

When controls (siblings and healthy) were compared with patients with ASD, the rate of Candida isolation, whether by culture or by multiplex PCR, was found significantly lower in controls (p value = .019 & p value = .046 respectively) (Table 3). While when comparing the three groups regarding the type of Candida spp. identified, no statistical significant difference was observed (Table 4).

Concerning the severity of autism by CARS score and the severity of GIT symptoms by 6-GI Severity Index, no significant difference was found between Candida positive and Candida negative ASD groups. i.e. The colonization with Candida spp. did not affect the severity of symptoms in ASD children (Table 5).

Table 4. The comparison between the three investigated groups according to the type of Candida spp. identified

Candida spp. by culture	ASD group		Siblings control group		Healthy control group		Test of significance
	N	%	N	%	N	%	
C. albicans	3	13	2	14.3	0	0	Monte Carlo test
C. glabrata	8	**34.8**	2	14.3	1	10	X^2=10.077
C. Krusei	2	8.7	0	0	1	10	P=.456
C. parapsilosis	1	4.3	1	7.1	0	0	
C. tropicals	1	4.3	0	0	0	0	
Mixed Candida spp.	8	**34.8**	9	**64.1**	8	80	
Total	23	100	14	100	10	100	
Candida spp. by multiplex PCR							
C. albicans	3	9.1	2	8	0	0	Monte Carlo test
C. glabrata	8	**24.2**	6	**24**	7	30.4	X^2=5.023
C. Krusei	3	9.1	0	0	2	8.7	P=.916
C. parapsilosis	4	12.1	3	12	2	8.7	
C. tropicals	1	3	1	4	1	3.7	
Mixed Candida spp.	14	**42.4**	13	**52**	11	47.8	
Total	33	100	25	100	23	100	

Table 5. Results of statistical comparison of clinical scores (CARS and GI severity score) among Candida positive and negative ASD groups

	ASD group Candida by culture		ASD group Candida by PCR	
	Positive n=23	Negative n=27	Positive n=33	Negative n=17
CARS score				
Min-Max	24-41	25-45	24-41	25-45
Median	32	32	32	33
Mean ± SD	31.17±4.6	31.67±5.1	30.69±4.19	32.88±5.71
Mann- Whitney U	Z=-0.333	P=.74	Z=-1.534	P=.125
GI severity score				
Min-Max	1-8	0-9	0-9	0-6
Median	4	3	4	4
Mean ± SD	3.67±1.77	3.04±2.26	3.55±2.15	3.18±1.94
Mann- Whitney U	Z=-1.425	P=.154	Z=-0.26	P=.795

4. DISCUSSION

There are only few reports investigating the fecal microbiota of children with ASD mainly focusing on bacteria [15,16]. Recently, scientists suggest that Candida, particularly *C. albicans* growth in intestines may cause lower absorption of carbohydrates and minerals and higher toxin levels which are thought to contribute to autistic behaviors [6,17]. However, little is known about the presence of Candida spp. in gut of this pediatric patient population.

Recently, Iovene et al. [12] demonstrated the presence of high counts of Candida spp. in more than half of the investigated stool samples from ASDs patients. Similarly El-Shouny et al. [18], found that there was increase in cases of heavy growth of yeast in autistic group compared with the control group. Ekiel et al. [19] also reported quantitative differences. Kantarcioglu et al. [20] in their retrospective study investigating a large number of ASDs subjects reported the presence of Candida spp. in a large percentage of patients (81.4%) compared to controls (19.6%). Emam et al. [21] also reported that there was increased rate of infection by yeast in autism (81.9%) versus control group (28%). Horvath et al. [22], showed that there was increased rate of positive fungal culture for yeast in the duodenal juice (43%) of children with autism undergoing endoscopies more than had the age matched controls with other gastrointestinal problems requiring endoscopies (23%). The results of these studies were in agreement with the results of the present study when controls were

compared with ASD patients, the rate of Candida isolation, was found significantly lower in controls (p value = <0.05). On the contrary, Adams et al. [5] showed no differences of yeast infection among stools from ASDs and healthy controls. However, it should be noted that detection of yeast strains by culture is technically challenging, potentially resulting in a high false negative rate.

Previous studies were based on cultural approaches for Candida detection given that molecular biology methods were not available until very recently [23]. In the present study we investigated a molecular qualitative multiplex PCR, previously tested for blood cultures, to identify Candida spp. in stool. The method proved to be more sensitive than culture based approach; 33/50 versus 23/50, 25/36 versus 14/36, and 23/50 versus 10/50 positive Candida results for ASD, siblings and healthy controls respectively.

Microscopic examination of stool samples showed no significant differences among the investigated groups, however, it should be considered very useful first step to identify samples rich of yeast-like cells as previously reported [12].

The most common Candida isolated from autistic children during the present study was *C. glabrata*, which is not in accordance with previous studies; that showed that *C.albicans* was largely the most represented species in ASD children [12,20]. While El-shouny et al. [18], reported that *C. krusei* was the most commonly isolated. Colombo et al. [24], stated that the non-albicans Candida species, *C. krusei*, *C. tropicalis* were the recorded yeasts in their ASD cases. Therefore, the correct identification of the species might be a crucial element for efficient therapeutic decisions in patients with ASD, when needed.

Iovene et al. [12] reported that even if GI symptoms were present in 70.2 % of ASDs, their presence does not correlate with the detected counts of Candida spp., meaning that they are not due to its presence. Similar findings were found in the present study where the colonization with Candida spp. did not affect the severity of symptoms in ASD children (p=0.795). However, increased counts of Candida spp., even in the absence of GI symptoms, could be looked at as precocious index of intestinal dysbiosis. Moreover, although *C. albicans* were isolated in low numbers in our study, probably, non-albicans Candida may also cause lower absorption of carbohydrates and minerals in the intestines and may probably take a role in the microbiota–gut–brain axis as reported by Kantarcioglu et al. [20].

In the present study, Candida colonization did not affect the severity of autism (p=0.125), similar findings were reported by the study of El-shouny et al. [18], which indicated that heavy growth of yeast among autistic children is a feature without considering the level of autism severity. Emam et al. [21], reported that number of patients with negative stool culture growth was significantly increased (P = 0.027) in mild-moderate group compared with severe group; while there was statistically insignificant difference in number of minimal and heavy yeast growth.

Children with ASD are treated in clinical practice with antifungal agents despite the lack of scientific evidence of fungal overgrowth. A national survey by the Autism Research Institute reported that antifungal therapy with Diflucan or Nystatin was often beneficial [25]. It is possible that children with autism are more sensitive to even a normal level of yeast. Also, it is possible that antifungal have other effects, such as reducing inflammation [26]. However, it should be noted that antifungal treatments have known toxicity and may require monitoring of liver transaminases for safety.

Many yeasts are susceptible to nystatin, which is active mostly in the intestinal tract and is poorly absorbed systemically. Nystatin tablets or capsules do not dissolve until they reach the stomach or lower; therefore, this is an advantage over other antifungal agents to be limited to the intestinal tract. Fluconazole is another effective candidastatic antifungal agent. Nystatin and/or fluconazole is used to restore the proper balance of microbiota or to treat Candida overgrowth in the intestines of children with ASD [20].

In the present study, only all *C. albicans* isolates were sensitive to nystatin, fluconazole and voriconazole. While other non-albicans Candida showed different sensitivity patterns to the tested antifungals. These results indicate that nystatin, fluconazole or voriconazole cannot be used as empiric treatment for children with Candida overgrowth, but instead culture of stool samples followed by identification and sensitivity testing is mandatory for treatment of those patients. Although the majority of all Candida spp was inhibited by amphotericin B, this drug cannot be

used for treatment of Candida overgrowth in children with ASD due to its high toxicity and the requirement of a parenteral route for administration.

5. CONCLUSION

This study provides significant baseline data for the future research about the Candida– gut–brain axis associated with ASD patients. Future studies could be conducted to verify whether Candida elimination therapy is useful to manage ASD symptoms.

It would also be beneficial to include a simple screening test based on stool culture for diagnosis of Candida overgrowth in ASD children so that parents of infants with yeast sensitivity would know to avoid including products in their children's diets that could produce an overgrowth of gastrointestinal yeast. In addition, our results suggest that, if antifungal treatment would be necessary for those patients, the species identification and antifungal susceptibility tests have to be performed using the strain isolated from stool sample, to select the appropriate antifungal agent.

ETHICAL APPROVAL

An informed consent for specimen use was obtained from each child's parents or legal representative and the study protocol was approved by the ethics review committee of Alexandria University Hospitals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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