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Identification of Candida species in faeces of children with Autism Spectrum Disorder (ASD) carbohydrate and noncarbohydrates diets

Identifikasi spesies candida dalam faeces anak dengan Autism Spectrum Disorder (ASD) yang diet dan tidak diet karbohidrat

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Abstract

Increased colonization of *Candida*, especially *C. albicans* can occur in children with ASD and cause the severity of symptoms. One of the triggers for increased colonization in ASD children is the consumption of carbohydrates. This study aimed to identify *Candida* species in the faeces of ASD children who diet and nondiet carbohydrates from schools with special needs in Jember, East Java. The research design is a true experimental laboratory. The sample consisted of 26 ASD children and 13 healthy children as controls, with different subjects calculated using the Krejcie and Morgan formula. The test samples were identified for fungi by examining germ tube morphology, chlamydospores, and *Candida* species and compared with controls. The results showed differences in *Candida* species in the two test groups. Species *C. albicans, C. dubliniensis*, and *C. tropicalis* were found in almost all the faeces of ASD children with noncarbohydrates diet. The species *C. parapsilosis*, and *C. albicans* were found in a small proportion of the faeces of ASD children's carbohydrate diet. In contrast, the species found in the control group were *C. albicans, C. dubliniensis, C. parapsilosis,* and *C. glabrata*. In conclusion, carbohydrate consumption can affect the *Candida* species in the faeces of children with ASD.

Keywords: *Candida* species, Candidiasis, carbohydrate diets

Abstrak

Peningkatan kolonisasi *Candida* terutama *C. albicans* dapat terjadi pada anak *Autism Spectrum Disorder* (ASD) dan menyebabkan keparahan gejalanya. Salah satu pemicu peningkatan kolonisasi pada anak ASD adalah konsumsi karbohidrat. Tujuan penelitian untuk melakukan identifikasi spesies *Candida* pada feces anak ASD yang diet dan tidak diet karbohidrat yang berasal dari beberapa sekolah berkebutuhan khusus di Jember Jawa Timur. Desain penelitian adalah *True Eksperimental Laboratory*. Subjek terdiri dari 26 anak ASD dan 13 anak sehat sebagai kontrol dengan subyek yang berbeda dihitung dengan rumus *Krejcie* dan *Morgan*. Sampel uji diidentifikasi jamurnya melalui pemeriksaan morfologi germ tube, klamidospora, serta spesies *Candida* dan dibandikan dengan kontrol. Hasil penelitian menunjukkan perbedaan spesies candida pada kedua kelompok uji. Spesies C. *albicans, C. dubliniensis*, dan *C. tropicalis* ditemukan pada hampir semua feses anak ASD yang tidak diet karbohidrat. Spesies *C. parapsilosis, C. albicans* ditemukan pada sebagian kecil feses anak ASD yang diet karbohidrat, sedangkan spesies yang ditemukan pada kelompok kontrol adalah *C. albicans, C. dubliniensis, C. parapsilosis,* dan *C. glabrata*. Kesimpulan, konsumsi karbohidrat dapat mempengaruhi spesies candida yang ada pada faeces anak dengan ASD.

Kata Kunci: spesies *Candida*, kandidiasis, diet karbohidrat

Introduction

Autism spectrum disorder (ASD) is a heterogeneous brain nerve disorder that clinically manifests as a persistent disorder resulting in problems in communication and social interaction with repetitive behaviours, from mild to severe range. These problems lead to abnormal development of communication and socialization in children (Courchesne et al., 2019; Van Rooij et al., 2018). According to the data, the global prevalence of ASD in 2000 was about 1/160 (Hossain et al., 2017), and so far, about 1,5% of the world population has ASD, and the ratio of males to females is 2.5:1 (Lyall et al., 2017).

Previous research showed that children with autism have a disorder in the gastrointestinal system more frequently and severely than children without autism. One of the possible causes of the GI system disorder in children with ASD is the disruption of the normal flora in the intestines which can trigger the overgrowth of potentially pathogenic microorganisms (Hughes et al., 2018; Srikantha & Mohajeri, 2019). One of the most common pathogenic microorganisms associated with autism is *Candida*, especially *Candida albicans*. Therefore, the rapid and aggressive growth of *Candida albicans* in children with ASD will cause GI disturbances and behavioural symptoms.

The results of some studies revealed that there is a relationship between autism and candidiasis. Horvath and Perman reported an increased rate of a positive fungal culture for yeast in the duodenal fluid (57%) of autistic children undergoing endoscopy compared with the age-matched control group (Iovene et al., 2017). Candida albicans was the most represented species Candida albicans (Lasheras et al., 2020). A separate study found Candida in nearly 60% of ASD samples, with none in controls. They also identified Hyphae formation, suggesting that the dimorphic yeast had switched to its invasive and adhesive form (Hughes et al., 2018).

The findings of the past studies discovered that there was a decrease in autistic symptoms after patients were given a gluten-free diet and casein; this was presumably for the reason that gluten and casein both could increase the amount of yeast in the gastrointestinal tract of the patients, thereby it could enhance the symptoms in children with ASD (Tas, 2018).

Carbohydrate consumption on candidiasis in children with ASD can be proven by checking out the growth of *Candida* in the faeces. In addition, the identification of candidiasis in the laboratory generally only reaches the genus. At the same time, the examination of the species level has not been widely carried out even though the determination of *Candida* species is important because of the high phenotypic similarity between *Candida* species. This study aimed to identify what *Candida* species were present in the faeces of ASD children on a carbohydrate diet. The results of this study can later be used as a basis for managing diet and therapy in children with ASD to provide more specific therapy to reduce the rate of death due to resistance to antifungals.

Method

This research uses the true experiment laboratory method. The sample is the faeces of ASD children from special schools in Jember district, East Java. The number of samples of ASD children analyzed was 26 and 13 healthy samples with different subjects calculated by Krejcie and Morgan formulas. Samples were taken by purposive sampling. This research was conducted in 2018-2019 and has received ethical approval from The Ethical Committee of Medical Research Faculty of Dentistry Jember University, with Number: 092/UN25.8/KEPK/DL/2018.

The faeces used in this study were sterile plastic pots, test tubes, micropipettes, serological tubes, slide glass and lid glass, Petri dishes, beaker glass, measuring cups, ose, spreaders, and light microscopes. The materials used in this study were Sabouraud Dextrose Agar (SDA), physiological salt, Agar Rice-cream Tween 80 (RCT) medium, *Candida* bromocresol green (BCG) medium Agar base, chloramphenicol, neomycin, and egg white.

Samples

Faeces of Autism Spectrum Disorder (ASD) children referred to in this study are faeces samples of ASD children aged two years to 12 years who do not receive diet, antibiotics, and antifungals, without distinguishing their gender and the diagnosis has been determined based on Diagnostic and Statistical Manual IV Task Force (DSM IV-TR).

Samples were obtained from schools for children with special needs in Jember Regency (school identity is not mentioned here). In contrast, the faeces samples for healthy children referred to in this study were for children aged 2 to 12 years who did not receive antibiotics or antifungals by not distinguishing between types of gender. The samples used were faeces no more than 24 hours after being excreted and stored at ±4^{0}C.

Preparation Sample

The faeces samples were dissolved in physiological saline: 5 tubes of physiological saline solution were prepared for 1 sample, each containing 9,0 mL of physiological saline solution. First, the faeces sample was dissolved in the first tube and vortex until dissolved and homogeneous. Then, it was taken using a 1000 µL micropipette, put in the second tube, and so on until the fourth tube. The samples cultured in the SDA media were only in tube 4 (dilution factor 10-4).

Seeding Faeces Specimens

Each faeces sample was cultured in Sabouraud Dextrose Agar (SDA) medium, which had been added with 0,5 mg/ml chloramphenicol antibiotic. The sample used was a dilution factor of 10-4. Planting was done by taking a small sample with a 100,0 µl micropipette and then removing or spreading it to the medium's entire surface. The culture was incubated at room temperature for 7-10 days.

Morphological Identification of Fungi

After incubation, the morphology of culture colonies was observed. *Candida's* results were positive when the fungus colony grew yellowish or creamy with a convex surface. The results were negative if the colony did not grow with *Candida's* characteristics or until ten days or more

Results and Discussion

Morphological Identification of the Mushroom Colonies.

The morphological identification of fungal colonies was carried out to determine whether *Candida* fungi were present in the sample. The morphology of the *Candida* colony was circular in size (3,5-6) x (6-10) µm with a slightly convex, smooth, sometimes slightly folded surface, especially in older colonies. The *Candida* colony was yellowish-white (soft cream) and had a characteristic smelt (Jacobsen & Hube, 2017; Kurtzman et al., 2011; Staniszewska et al., 2013).

The morphological identification results of fungal colonies in the faeces of ASD children with carbohydrate and noncarbohydrate diets and healthy children can be seen in Table 1.

Table 1. The morphology of fungal colonies in the samples of ASD noncarbohydrate diets, ASD with carbohydrate diets, and healthy children (control)

No. of samples	ASD noncarbohydrate diets	ASD carbohydrate diets	Control
1	-	-	+
2	-	-	-
3	+	-	-
4	+	-	+
5	-	-	+
6	+	-	-
7	+	+	-
8	-	-	-
9	-	+	-
10	-	-	+
11	+	-	-
12	-	+	-
13	-	+	-

+ : Mushroom colonies that grow on media are *Candida* colonies.

- : Colonies that grow on media are not *Candida* colonies, or no colonies grow on media

Table 1 is the result of the morphological identification of fungal colonies from 13 samples of ASD noncarbohydrate diets, 13 samples of ASD children with carbohydrate diets, and 13 samples of healthy children were observed for colony morphology to determine whether it was a positive or negative *Candida* fungus. The samples were stated as negative when they grew colonies but not for *Candida* fungus or did not grow colonies on the media. From the analysis results, all positive Candida samples continued to be identified, except for sample number 11 in ASD children, due to the non-existence of colonies growing at the purification stage. The *Candida* colonies that have been incubated on SDA media for 7-10 days at room temperature are shown in figure 1.

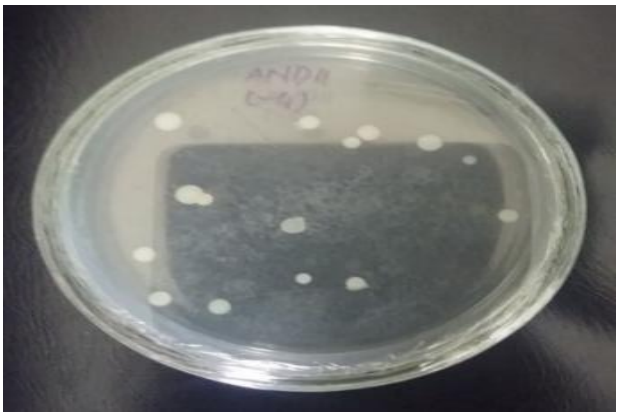

Figure 1. *Candida* Colonies that have been incubated on SDA media for 7-10 days at room temperature

Identification of the Germ Tube

Identifying the germ tube aimed to see the presence or absence of the germ tube in the sample. This germ tube can indicate that the *Candida* contained in the sample is pathogenic (Matare et al., 2017). Only *Candida* can form a germ tube that can be analyzed by this method. The included *Candida* are *C. albicans, C. dubliniensis,* and *C. tropicalis*

Microscopic analysis results were compared with the literature, where the positive germ tube samples were indicated by the presence of the short hyphae (filaments), the lateral extension occurred in yeast cells without narrowing their initial shape (Moya-Salazar & Rojas, 2018; Yazdanpanah & Khaithir, 2014). The results of the germ tube identification in the faeces samples of ASD children and healthy children can be seen in Table 2.

Table 2. The results of the germ tube identification in ASD children samples, ASD children samples on carbohydrate diets, and healthy children (control)

ASD noncarbohydrate diets		ASD carbohydrate diets		Control	
No.	Result	No.	Result	No.	Result
3	+	7	-	1	-
4	+	9	-	4	-
6	+	12	-	5	-
7	-	13	+	10	+

+ : There is the formation of germ tube
- : No formation of germ tube

Of 4 ASD children samples, there were three positive germ tube samples, namely samples 3, 4, and 6, which were marked by the growth of filaments and lateral extension of the yeast cells, as shown in Figure 2. Whereas in the sample of ASD children with a carbohydrate diet, there was only one positive sample of the germ tube, sample 13.

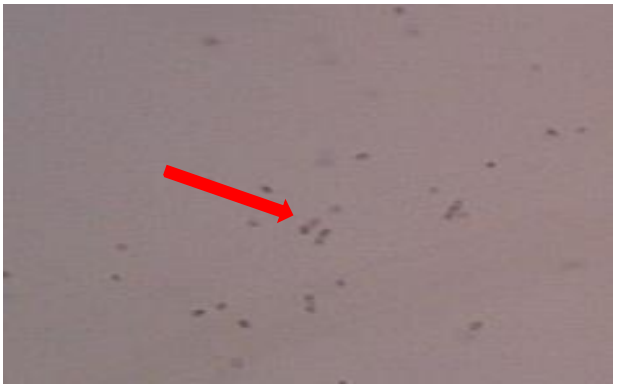

Figure 2. The picture above identifies germ tubes in ASD children samples with a 400x magnification microscope.

The red arrow in the picture shows the extension of hyphae (filaments) in *Candida* cells which can be called a germ tube.

Slide Culture Identification

The slide culture method aims to identify and view Chlamydospores. Chlamydospores are produced by several fungi, one of which is *Candid* (Böttcher et al., 2016; Noble et al., 2017; Palige et al., 2013). In *Candida* mushrooms, there are only certain species that can produce Chlamydospores, namely *C. albicans* and *C. dubliniensis* (Böttcher et al.,

2016). The identification results of the slide culture in the faeces samples of ASD carbohydrate, noncarbohydrate diet, and healthy children can be seen in Figure and Table 3.

Table 3. The results of Chlamydospores identification on the slide culture samples of ASD children and healthy children

ASD noncarbohydrate diets		ASD carbohydrate diets		Control	
No.	Result	No.	Result	No.	Result
3	+	7	+	1	-
4	+	9	+	4	-
6	-	12	+	5	-
7	+	13	+	10	+

+ : There is the formation of Chlamydospores
- : No formation of Chlamydospores

The identification results of 4 samples of ASD noncarbohydrate diets indicated three positive samples containing Chlamydospores, namely samples 3, 4, and 7, on microscopic observation. There was only one negative sample of Chlamydospore revealed in this sample, sample 6. In addition, the ASD samples with a carbohydrate diet showed four positive samples of Chlamydospores. Whereas there was only one positive sample revealed, which was sample 10, in the results of the samples of healthy children, while the other three samples were negative. A positive sample of Chlamydospores can be interpreted as a sample of *C. albicans* or *C.dubliniensis*.

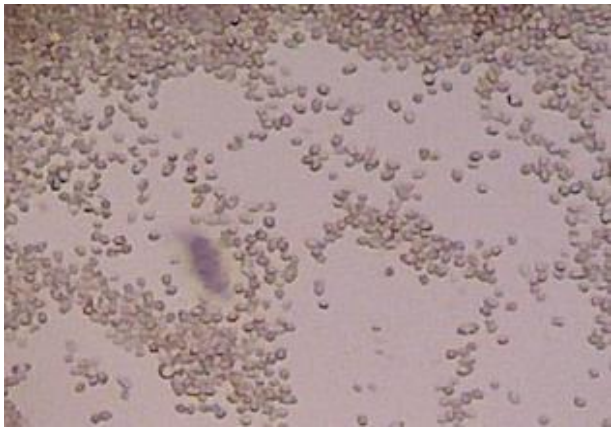

Figure 3. Microscopic analysis of *Candida* Chlamydospores on a slide culture using RCT media with a 100x magnification microscope

***Candida* Species Identification Using Candida Bromcresol Green (BCG) Agar Base**

The identification of BCG aimed to identify several *Candida* species, which are indicated by differences in colour and morphology of the colonies of each *Candida* species (De Angelis et al., 2020; Torosantucci et al., 2017; Zimbro et al., 2009).

The results of identifying *Candida* species in the normal subject group, ASD noncarbohydrate diet, and ASD with carbohydrate diets can be seen in Table 4. Of the four methods used by *Candida* species in ASD noncarbohydrate diets, ASD samples with a carbohydrate diet and normal children can be identified. *Candida* species found in normal children, ASD noncarbohydrate diets, and ASD with carbohydrate diets were C. parapsilosis, C. glabrata, C. tropicalis, and C. albicans.

Table 4. The results of BCG identification in the samples of ASD noncarbohydrate diets, ASD carbohydrate diets, and control

ASD noncarbohydrate diet		ASD carbohydrate diets		Control	
No.	Result	No.	Result	No.	Result
3	*C. abicans*	7	*C. parapsilosis*	1	*C. parapsilosis*
4	*C. abicans*	9	*C. parapsilosis*	4	*C. glabrata*
6	*C. tropicalis*	12	*C. parapsilosis*	5	*C. albicans*
7	*C. abicans*	13	*C. abicans*	10	*C. albicans*

Candida is a normal body flora, meaning it exists in every human being. *Candida* is known as a dimorphic fungus that normally exists in the digestive tract, upper respiratory tract, and genital mucosa in mammals. However, the increasing population of this flora can cause problems (Erdogan & Rao, 2015) (Singh et al., 2020). The difference in the growth of fungal colonies between ASD children and healthy children samples might be in numbers, yet this still needs further research. The increasing number of *Candida* mushroom colonies can also be supported or made worse by consuming foods that contain lots of carbohydrates (Doreswamy et

al., 2020; Nitschke et al., 2020; Tas, 2018). This might happened to the ASD samples used in this study. As explained earlier, the samples of ASD children used in this study were samples of ASD children who did not undergo dietary therapy, so that the possibility of the growth of *Candida* colonies with greater numbers can occur.

From the results of the identification of all methods performed, several species of Candida were found in the ASD children with noncarbohydrate diet samples, namely *C. albicans, C. dubliniensis,* and *C. tropicalis. C. parapsilosis* and *C. albicans* were found in the samples of ASD children who undergo carbohydrate diets. Whereas in the healthy children samples, there were *C. parapsilosis species, C. glabrata, C. dubliniensis,* and *C. albicans* found*. C. dubliniensis* in this identification is limited to the possibility of the growing number in the sample since *C. dubliniensis* cannot be identified by the BCG method. *C. dubliniensis* can be identified by the germ tube and slide culture methods. However, the germ tube method cannot distinguish between *C. albicans, C. dubliniensis,* and *C. tropicalis* species because theoretically there is no difference between the germ tubes produced by the three species. In the slide culture method, *C. albicans* and *C. dubliniensis,* which should be distinguished by looking at the differences in Chlamydospores, cannot be distinguished in this study because the incubation time was too long. Some of these things caused *C. dubliniensis* not to be identified with certainty in this study, so there were only possible samples containing *C. dubliniensis*. The sample was a positive sample of germ tube or slide culture.

From the results of the species analysis found in ASD children samples, the samples were dominated by pathogenic *Candida* species. This study's results differ from those of other studies that identify fungi in ASD children on a carbohydrate diet. In the sample of ASD children who undergo a carbohydrate diet, several species of *Candida* were discovered from 4 samples that grew *Candida* colonies: 3 positive samples of *C. parapsilosis* and one positive sample of *C. albicans*. Compared to the two studies, *Candida* pathogens in ASD children have more potential to grow due to the imperfect immune formation of ASD children. ASD children had low duodenal enzyme activity (58%). It leads to reduced digestion and low carbohydrate reduction, resulting in saccharide accumulation in the intestinal lumen. Saccharides are food for *Candida* pathogens, so the higher the number of carbohydrates consumed, the *Candida* will grow faster and more aggressively. It leads to a condition that triggers *Candida* to be pathogenic. This situation can occur in patients suffering from various systemic disorders (diabetes, leukaemia, and lymphoma) and with immunodeficiency, one of whom has ASD. In ASD children, developing *Candida* tends to be pathogenic because the immune system of ASD children is lower than healthy children, resulting in an increase of *Candida* fungus colonies in ASD children (Doreswamy et al., 2020).

The presence of *C. albicans* in a sample of healthy children is not dangerous as long as the amount does not exceed normal limits. Previous research stated that *C. albicans* grew more in ASD children than healthy children (Doreswamy et al., 2020; Strati et al., 2017). Therefore, it is also important to determine the number of colonies of *Candida* species. This research is still qualitative identification with a limited number of samples, so further research is needed to find out how the influence of carbohydrate consumption in ASD children on the growth of Candida species quantitatively. Identifying *Candida* species in ASD children samples can also help the selection of therapy for ASD children. However, antifungal sensitivity to *Candida* species has not been much studied, and further research is still needed.

Conclusions

The study results show that the *Candida* species found in faeces samples of ASD children's carbohydrate, and noncarbohydrate diets and controls are different. *C. albicans* species is more commonly found in ASD children's noncarbohydrates diet.

Some suggestions from this research are the need to increase the number of samples to improve the accuracy of the study, the need to identify *Candida* species using other methods such as the chromagar method there is a need for further quantitative research related to the number of colonies found in ASD children on carbohydrate diets to confirm the influence of their diets.

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