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A Comparative Study on an Anaerobic Oral Microbiota among Autistic and Non-autistic Children

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Authors' contributions

This work was carried out in collaboration between all authors. Author ARC designed the study, protocol and wrote first draft of manuscript. Author FARR conducted experiment and managed literature searches. Author AP contributed to collect plaque samples and author AB performed the statistical analysis. All authors contributed equally in this research and approved the final manuscript.

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ABSTRACT

Aim: An evidence suggests that microbiota plays an important role in health and diseases. Studies also suggested that microbiota may be different in children with an autism spectrum disorder (ASD) than typically developing children. The aim of the study was to examine the oral bacterial strain/s commonly found in children with autism.
Study Design: Forty-three (43) children with ASD and forty-three (43) non-autistic children from the same age group were recruited from one of the autistic society in Malaysia from three centres

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in Klang Valley, Malaysia.
Results: Total *e*ighty- six oral samples were tested using biochemical tests from autistic and non-autistic individuals. Eleven different bacterial species were identified. *Actinomyces naeslundii* was found in eight autistic samples whereas *Prevotella intermedia* and *Porphyromonas gingivalis* were not detected in any of the samples.
Conclusion: The study found that *Actinomyces naeslundii* was significantly present in autistic group.

Keywords: Actinomyces naeslundii; Prevotella intermedia; Porphyromonas gingivalis; oral microbiota; autism.

1. INTRODUCTION

Autistic Spectrum Disorder (ASD) or Pervasive Developmental Disorder (PDD) is a continuum of disorders which varies in severity between individuals. Prevalence and epidemiology of autism was reported from World Health Organization (WHO) that 1 in 110 children worldwide and Kementerian Kesihatan Malaysia (KKM) reported 1 in 600 children in Malaysia. Recent studies by the Centers for Disease Control and Prevention (CDC) confirmed several medical conditions which are predominant in autistic children compared to healthy children including dental issues.

Studies have been conducted on human microbiome project with the objective to gnomically characterize the total of human associated microorganism [1,2] and concluded that microorganisms plays an important role in human health [3,4]. Millions of microbes present in gut lives symbiotically in the human body and share their genome with human. Collection of microorganisms present in the distal gut of human body are sensitive and vary on the basis of diet, age, sex, genome etc of the individual [5]. Gut microbiome are involved in many pathological conditions including autism [6]. Researchers suggested the possibility in future where modification of the microbiome of the built environment (MoBE) would result in improved mental health conditions including autism [7]. There are some evidences found which shows link between gut microbiome with obesity and Type 1 & Type 2 diabetes [8] however, there is no confirmed link found so far between the oral and gut microbiome responsible for autism. Further studies needed to confirm the link between oral microbiome and autism.

The microorganisms found in the human oral cavity have been referred to as an oral micro flora. Researchers reported that more than seven hundred bacterial species, of which over 50% have not been cultivated, have been detected in the oral cavity. There is a distinctive predominant bacterial flora of the healthy oral cavity that is highly diverse, site and subject specific [9]. Microorganisms from the oral cavity have been shown to cause a number of oral infectious diseases, including caries, periodontitis, endodontic infections, alveolar osteitis, and tonsillitis [10]. Specific oral bacterial species have been implicated in several systemic diseases, such as bacterial endocarditis [11], aspiration pneumonia [11,12], osteomyelitis in children [13], preterm low birth weight [14,15], and cardiovascular disease [16].

The objective of this study is to assess the oral bacterial strain/s commonly found in children with autism and to compare the oral bacterial species found in autistic children with healthy children.

2. MATERIALS AND METHODS

The present study was independently reviewed and received Institutional Review Board approval. The parents of autistic and non-autistic children were informed and gave written concern prior taking sample.

Pooled plaque samples were collected in stuart transport medium and cultured on blood agar plate. All cultures were processed with in 4 hours of collection and diluted by 25 folds. 100 µl aliquot were cultured on Columbia agar with 5% sheep blood. The plates were incubated in an anaerobic environment in anaerobic jars at 37℃ for 7 days [9,17,18]. For morphological identification, pure colony was stained by Gram stain and followed by confirmatory bacterial identification using API Rapid ID 32 A Kit (bioMérieux® SA, Marcy-l'Etoile, France), an identification system for anaerobes in 4 hours using 29 miniaturized enzymatic tests and a database. The control group consisted of 43 non-autistic individuals of the same age group solicited from school of non-autistic children.

Study was conducted from July to Dec 2013. Mild and moderate cases of autism from age group of 4 to 16 years will be considered for study whereas severe cases of autism with comorbidity were excluded from study.

2.1 Statistical Analysis

Considering a proportion of 10% oral bacterial assessment with a precision of 9%, 43 subjects (sample size) were required for the study. Sample size was calculated for a confidence coefficient of 95%. Data was collected after bacterial analysis for *Actinomyces, Provetella* and *Porphyromonas gingivalis*. Statistical tests (chi- square test and Mann-Whitney U analyses) were completed by using SPSS.

3. RESULTS AND DISCUSSION

Studies have been conducted on beneficial microorganism in animals and humans on mental health. Results showed that there are microorganisms which have relevance with mental health. In mouse model studies, it was found that *Bacteroides fragilis,* which found as human commensal mainly present in the human, animal, waste water and waste water treatment plants [19] are involved in the developmental protection from some of the behavioural symptoms associated with autism spectrum disorder [20]. Besides this, *Mycobacterium vaccae* was studied in human model research and found relevant with the increased cognitive function, decreased pain in patients with advanced non-small-cell lung cancer [21] whereas *Bifidobacterium longum* was studied in human model and found more relevant with the decreased anxiety and depressive symptoms in healthy volunteers (administered with *L. helveticus)* [22,23].

Research have also been conducted to compare the oral health of healthy and special need population. Research findings, highlighted that persons with developmental disease have significantly higher rates of poor oral hygiene and need periodontal treatment than the general population. Overall, individuals with disabilities appear to have a higher prevalence than individuals without disabilities [24]. Present research findings also support the earlier findings especially for few specific bacteria which were observed specifically in autistic group. During our research, baseline demographic characteristics of participants were also analysed as stated in Table 1.

Preliminary examination of *Actinomyces naeslundii* was completed by differentiating colonies of *Actinomyces naeslundii* on Columbia blood agar macroscopically with white and smooth colonies and microscopically at 400X magnification as showed in Fig. 1 and Fig. 2.

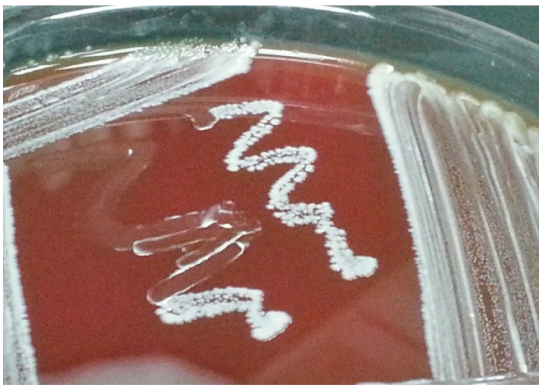

Fig. 1. White and smooth colonies of *Actinomyces naeslundii* on Columbia blood agar

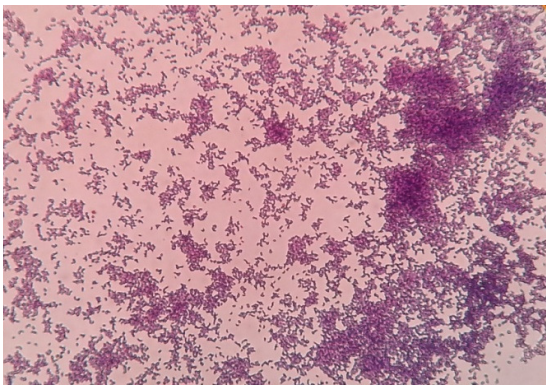

Fig. 2. *Actinomyces naeslundii* bacilli as seen at 400X magnification

A total of eighty-six samples were tested with the rapid ID 32A kit, 6 of which consisted of mixed bacterial colonies and were re-tested to obtain identification of bacterial species in pure colonies. A total of 11 different bacterial species were identified from these samples, as illustrated in Fig. 3.

Actinomyces meyeri, Actinomyces viscosus, Propionibacterium acnes, and *Gemella morbillorum* were found in both autistic and non-autistic groups, whereas *Actinomyces israelii* and *Propionibacterium propionicus* were only found in the non-autistic population. *Actinomyces naeslundii, Propionibacterium granulosum,*

Clostridium difficile, Capnocytophaga spp and *Bacteroides ureolyticus* were only found in the autistic samples though *Prevotella intermedia* and *Porphyromonas gingivalis* were not detected in any of the samples.

Actinomyces naeslundii was present in eight (18.6%) samples from the autistic group however, it was absent in thirty-five (81.4%) samples from the autistic group and in all samples of the non-autistic group. A highly significant association was observed between the presence of *Actinomyces naeslundi*i and autism. However, gender, ethnicity, gingivitis, plaque and calculus were not found to be significantly associated with *Actinomyces naeslundii*. The chi-square analysis revealed that there was a significant difference in the prevalence of *Actinomyces naeslundii* between the autistic and non-autistic groups as shown in Table 2.

Mann-Whitney U analyses on age and dental indices was also completed with regards to *Actinomyces naeslundii. Age, Gingival index, plaque Index and calculi index were found insignificant to be associated with Actinomyces naeslundii* as shown in Table 3.

Table 1. Baseline demographic characteristics of the participants

Demographics	Autistic (N_1 = 43) n_1 (%)	Non-autistic (N_2 = 43) n_2 (%)	Total (N = 86) n (%)
Gender			
Male	40 (93.0)	22 (51.2)	62 (72.1)
Female	3 (7.0)	21 (48.8)	24 (27.9)
Age group (years)			
4–8	18 (41.9)	18 (41.9)	36 (41.9)
9–12	19 (44.2)	13 (30.2)	32 (37.2)
13–16	6 (13.9)	12 (27.9)	18 (20.9)
Ethnicity			
Malay	28 (65.1)	30 (69.8)	58 (67.4)
Chinese	8 (18.6)	2 (4.6)	10 (11.6)
Indian	6 (14.0)	6 (14.0)	12 (14.0)
Others	1 (2.3)	5 (11.6)	6 (7.0)

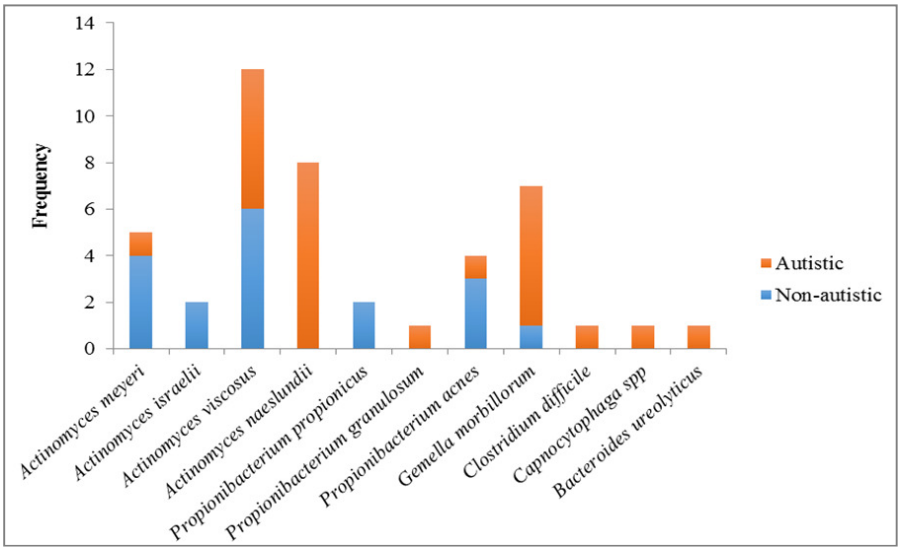

Fig. 3. Distribution of eleven bacterial species found in forty-three autistic and non-autistic samples tested using the rapid ID 32 A kit

Table 2. Chi-square (χ^2) analysis, unadjusted odds ratio (OR) and 95% confidence interval (CI) for the OR of several factors associated with status of *Actinomyces naeslundii*

	***Actinomyces naeslundii* status**			χ^2 value	OR (unadjusted)	95% CI	p-value
	Present (N_1 = 8) n_1 (%)	Absent (N_2 =78) n_2 (%)	Total (N = 86) n (%)				
Gender							
Male	7 (11.3)	55 (88.7)	62 (72.1)	1.041	2.927	0.341-25.157	0.433
Female	1 (4.2)	23 (95.8)	24 (27.9)				
Ethnicity							
Malay	5 (8.6)	53 (91.4)	58 (67.4)	0.098	0.786	0.174–3.553	0.712
Non-Malay	3 (10.7)	25 (89.3)	28 (32.6)				
Autism status							
Autistic	8 (18.6)	35 (81.4)	43 (50.0)	8.821	–	–	0.005*
Non-autistic	0 (0)	43 (100)	43 (50.0)				
Gingivitis status							
Present	5 (7.9)	58 (92.1)	63 (73.3)	0.521	0.575	0.126–2.625	0.436
Absent	3 (13.0)	20 (87.0)	23 (26.7)				
Plaque status							
Present	7 (9.0)	71 (91.0)	78 (90.7)	0.107	0.690	0.074–6.447	0.558
Absent	1 (12.5)	7 (87.5)	8 (9.3)				
Calculus status							
Present	5 (12.2)	36 (87.8)	41 (47.7)	0.777	1.944	0.434–8.706	0.470
Absent	3 (6.7)	42 (93.3)	45 (52.3)				

** Significant finding at $p < 0.05$*

Table 3. Mann-Whitney U analyses on age and dental indices with regards to status of *Actinomyces naeslundii*

	Mann-Whitney U value	p-value
Age (years)	304.0	0.905
Gingival index	202.5	0.086
Plaque index	226.0	0.177
Calculus index	263.5	0.428

The autistic group had a male to female ratio of 13.3:1, which reflects a higher prevalence of autism in males. Two studies involving 117 and 61 autistic subjects reported a gender ratio of 3.6:1 and 2.8:1, respectively [25,26]. The findings of our research reported that the prevalence of *Actinomyces naeslundii* was significantly higher in the autistic than control group where as *Prevotella intermedia* and *Porphyromonas gingivalis* were not detected in any of the samples.

Propionibactterium granulosa, Clostridium difficile, Capnocytophaga spp and *Bacterioides ureolyticus* were only reported in autistic group but not in significant amount, whereas *Actinomyces viscosus* were found equally in both groups. *Gemella morbillorum* was also reported in both groups but significantly present in autistic group.

4. CONCLUSION

Human microbiome project studies showed that microorganism plays an important role in human beings. Prebiotics and probiotics are now a days used as a microorganism therapy to improve gut microbiota and health. Present study was started with the objective to assess the oral bacterial strain/s commonly found in children with autism. The findings of our research reported that total eleven anaerobic microbes were reported out of eighty six samples. The prevalence of *Actinomyces naeslundii* was reported significantly higher in the autistic than non-autistic group. However, our studies reported the presence of *Gemella morbillorum* microbiota in autistic group more than in healthy group. Present study have a limitation of small sample size so it is too early to conclude whether *Actinomyces naeslundii* or *Gemella morbillorum* microbiota bacteria can be useful as a biomarker in detection of autism. Larger multi-centric studies are needed to validate the findings from this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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