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Gut Flora in Autistic Children as Biomarker for Autism in Thi-Qar Province/Iraq

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***Abstract*— This is the first study that investigated the microbial factor as biomarker in autistic children and discuss roles of this factor in the pathogenesis of autism. The participants in current study were 145 persons, only 50 sample of stool could collected (35 autistic children and 15 healthy children). Autistic children were attended to autism unit at Disabled Hospital in Thi-Qar province, Iraq during the period from January to November 2016.**

The results showed males (81%) more than female (19%) with ratio 4:1 and also results explain the age group of 3-5 years recorded the highest percentage (41.05%). Distribution of autistic children according to sibling showed six were brotherly with occurrence rate 6.3%. Stool samples were subjected to examination and culture.

The total aerobic count of isolated bacteria was 140 isolates. Gram-negative isolates were identified by API Enterobacteriaceae system. The results were Escherichia coli, Enterobacter cloacae, Klebsiella pneumonia, Pseudomonas aeruginosa and Proteus mirabilis with percentage 38.5%, 19.23%, 11.53%,7.69%, and 3.84% respectively. On the other hand, gram positive cocci isolates included Enterococcus faecalis, Staphylococcus aureus and Staph. epidermidis with percentage 11.53%, 4.80% and 2.88% respectively. A significant difference (P⩽0.05) was recorded between bacterial isolates.

Quantity and quality of isolated bacteria (colony/g *104) were done. E.coli isolates were the highest count with 261*104 colony/g while, Staphylococci epidermidis were recorded the worse colony count with 30*104 colony/g. The quality results showed Escherichia coli the most common gram negative bacterial isolates (38.46%). On the other hand, the highest gram positive cocci isolates were included Enterococcus faecalis (11.53%), with significant difference (P⩽0.05) between bacterial isolates.

The ability of bacterial isolates to produce histidine decarboxylase was examined on Niven medium. The positive result include colonies with purple halo around them. Only 10 isolates (25%) from all isolates were produce histidine belong to E.coli. On other hand, result of parasite examination explain no parasite in all samples.

From this study can conclusion the altered gut microflora may play an essential role in the pathogenesis of autism. Despite the accurate evidence, this etiological heterogeneity is still not recognized by autism researchers, and most studies fail to take it into account.

Keywords— Autism ,Biomarker , pathogenesis of autism, microbial factor

I. INTRODUCTION

Autism spectrum disorders (ASDs) are linked to developmental and functional abnormalities of the brain appearing before 36 months of age. They are characterized by impairments in reciprocal social interactions, impairments in verbal and non-verbal communication skills, stereotyped behavior and interests (Li et al., 2015). Often, autistic children suffer gastrointestinal difficulties consistent with altered composition of gut microbial inhabitants (Grimaldi et al., 2017). A gastro- intestinal (GI) symptoms, such as abdominal pain, gaseousness, diarrhea, constipation and flatulence are common in autism patients (Chaidez et al., 2014). The prevalence of GI symptoms in autistic children ranges from 23 to 70% (Chaidez et al., 2014). Moreover, the observed GI symptoms are associated with the severity of autism (Adams et al., 2011; Gorrindo et al., 2012). Although, many studies did not show a relationship between GI symptoms and autism. But, other studies suggested imbalances in the gut microbial plays an important role in the etiology of autism (Buie et al., 2010; Kang et al., 2013; Grimaldi et al., 2017). The gut microbiota have main role in neurodevelopment and mental health (Foster and McVey Neufeld, 2013) as well as, many behavior that seem in autistic children such as irritability, sleeplessness and posturing may be belong to gut flora (Van De Sande et al., 2014). The microbiota and its metabolites might be involved in the pathophysiology of ASD (Li et al., 2017).

Clinical and experimental articles have reviewed the influence of the gut microbiota on the animal central nervous system (CNS) and suggested the existence of a microbiota gut-brain axis (Bienenstock et al., 2015; Mayer et al., 2015). The microbiota gut-brain axis is likely the

method of communication between the brain and the gut microb
iota through neural, endocrine and immune pathways influence on brain function and behavior features (Cryan and Dinan, 2012; Carabottia et al., 2015) by direct connections of the intestinal epithelium to the brain stem and secondary projection sites through the nerve, and by indirect connections of the gut to the brain by alterations in immunity and metabolism (Hsiao, 2014).

According to previous studies have provided evidence about the association of gut flora with autism. This is the first study that investigated the association between normal flora as a biomarker for autism in Thi-Qar province.

II. Methodology

1- participants

In present study, 145children (95 autistic children and 55 healthy children), for both the sexes were examined. The diagnosed children with an autism were submitted by the child's physician (Dr. Naama Glud Kazar Al-Tamimi) in autism unit of disabled Hospital in Thi-Qar province/Iraq from July to November, 2016. The diagnosis of ASD was made in accordance with the standardized criteria provided in the American Psychiatric Association's Diagnostic and Statistical Manual-IV.

2- Samples collection:

Only 50 fresh fecal samples were collected in sterile plastic containers at the homes of the patients and controls. Then, immediately transferred to the Microbiology Laboratory in the same day , fresh bulk-stool that has not been mixed with urine and place in a clean container. If there is a delay in obtaining the preservatives, refrigerate untreated stool specimens at 4°C (Mawgoud et al., 2016).

3- Bacterial Identification

A) Culture: To quantify aerobic bacteria, a weighted quantity of the fecal sample (1g) was suspended in 10 ml of sterile physiological solution, homogenized by vortex and left at room temperature for a few minutes. 10-fold serial dilutions were prepared, each dilution was seeded on the MacConkey agar and Blood agar, then incubated in aerobic conditions. After incubation, the count of colonies grown on culture media was performed and expressed as CFU/g feces. All cultures were considered positive if colonies count was $\geqslant$103 (Iovene et al., 2016).

B) Microscopic examination: Gram stain was performed to detect response of bacteria for stain then study shape and their arrangement.

C) Diagnosis by API E20: The final diagnosis of bacterial isolates performed by using: API 20E system, for the diagnosis of Enterobacteriaceae according to the manufacturer's instructions.

D) Histidine producing: To detect ability of bacteria to produce decarboxylase, used Niven's medium that prepare in Lab according to (Niven et al., 1981). A loop of each culture was spread on the NA then incubated aerobically at 25 and 5° C for 48 h and 10 days, respectively. A purple colonies with halo around them on Niven's medium consider positive result.

4-Statistical Analysis: The results of the study were statistically analyzed by using SPSS statistics program version 16. A P value P$\leqslant$ 0.05 was considered significant.

III. Results

Distribution of autistic children according to gender, age and sibling

The present study were included 95autistic children involved 77males (81%) and 18 females (19%) and 50 healthy children as control (28 males and 22 female). Statistically, there were significant differences (P$\leqslant$ 0.05) among children according to gender distribution, table (1)

Table (1): Distribution and percentages of autistic patients and control according to gender.

Gender	Patient		Control	
	No.	%	No.	%
Male	77	81	28	56
Female	18	19	22	44
Total	95	100	50	100

P.value $\leqslant$ 0.05

All the children whom infected with autism divided according to age as shown in table (2). The age group of 3-5 years recorded the highest percentage (41.05%), followed by age group of 6-8 years (35.78%) when compared with the other age groups that record lower percentage shown in 12-14 years (4.21%), 9-11 years (7.36%) and in less than 3 years group (11.57%). The results showed significant differences (P$\leqslant$ 0.05) between the age groups.

Table (2): Distribution and percentages of autistic children and control according to age group.

Age group (Years	Patients No.(%)	Control No.(%)
Less than 3	(11.6)11(11,6)	1(2)
3 – 5	39(41,05)	11(22)
6 – 8	34(35,78)	13(26)
9 – 11	7(7,36)	13(26)
12 – 14	4(4,21)	12(26)
Total	95(100)	50(100)

P. value $\leqslant$ P$\leqslant$ 0.05

Identification of Bacterial isolates

1. Microscopic examination

Microscopic examination was applied to all isolates after having stained by Gram stain to detect their response to stain, the cell was taken counter stain of Gram reaction consider negative bacilli but, the cell that stained with primary stain have been positive cocci bacteria. The results showed 80.77% from isolates were negative and 19.23% were positive for Gram stain.

2- Diagnosis by API E20

Quality of isolated bacteria were done by API Enterobacteriaceae system. The results showed Escherichia coli the most bacterial isolates with 54 isolates (38.5%) from the total gram negative isolates. Followed by isolates of Enterobacter cloacae with 20 isolates (19.23%), Klebsiella pneumonia with 12 isolates (11.53%), Pseudomonas aeruginosa with 8 isolates (7.69%) then, Proteus mirabilis with 4 isolates (3.84%). On the other hand, gram positive cocci isolates were included Enterococcus faecalis with 12 isolates (11.53%), Staphylococcus aureus with 5 isolates(4.80%) then, Staph. epidermidis with 3 isolates (2.88%) as showed in fig.(4). A significant difference ($P \leq 0.05$) was recorded between bacterial isolates.

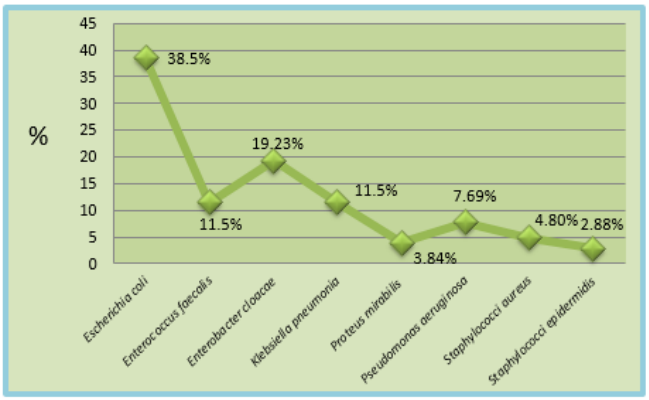

Fig. (1): Bacterial species isolated from stool of autistic children.

3- Quantity count of isolates

Table (3) show quantity and quality of isolated bacteria from stool of autistic children (colony/g *104). E.coli isolates were the highest count with 261*104 colony/g while, Staphylococci epidermidis were recorded the worse colony count with 30*104 colony/g, statistical analysis explain significant difference ($P \leq 0.05$) between isolates.

Table (3): Quantity of isolated bacteria from stool of autistic children and control (colony/g *104).

Isolated bacteria	Colony/g *10 ^{4} (%)	
	Patients	Control
Escherichia coli	261(35.22)	189(33.4)
Enterobacter cloacae	180(24.3)	173(30.57)
Klebsiella pneumonia	47(6.34)	20(3.53)
Pseudomonas aeruginosa	70(9.44)	56(9.9)
Proteus mirabilis	35(4.72)	-
Enterococcus faecalis	85(11.49)	74(13.07)
Staphylococci aureus	33(4.45)	35(6.18)
Staphylococci epidermidis	30(4.04)	19(3.35)
Total count	741(100)	566(100%)

4- Histidine production test

Histidine producing bacteria test was examined ability of bacterial isolates to produce histidine decarboxylase. The positive result include colonies with purple halo around them fig. (2). Only 10 isolates(25%) from all isolates were produce histidine belong to E.coli .

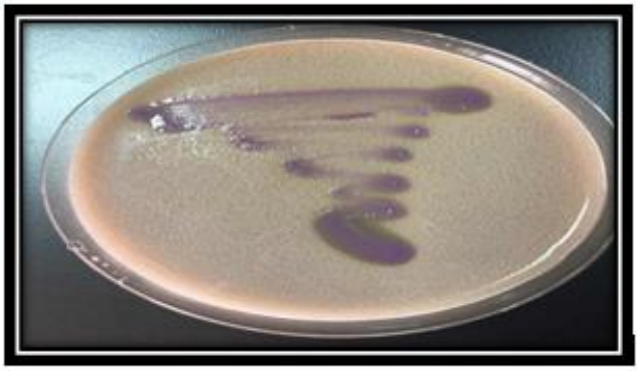

Fig.(2): Histidine producing bacteria (E.coli) on Niven medium.

IV. Discussion

Distribution of Autistic children according to gender and age

Differences in type of sample and methodology should be taken into account when comparing recent study with different studies.

In present study, the results had shown that males (81.05%) were more likely to had autism symptoms than females (18.94%) with a prevalence ratio of 4:1 this result agree with many previous studies (Giarelli et al., 2010; Rose'meyer, 2013; Mezzelani et al., 2016), the cause for this difference is not well understood but several theories had been suggested. Molecular evidence confirm of sex-biased genetic effects by displaying highly significant association driven by families with only affected males, and abnormalities of the sex chromosomes are associated with ASD as X-linked intellectual disability (XLID) as etiology of ASD (Betancur, 2011), Study of (Sutcliffe et al., 2005) have replicated evidence for male-biased genetic effects at 17q and they found a significant effect at 17q11.2 near the SLC6A4 locus. Other studies have shown the role of hormonal influences in utero as stimulated factor (Baron-Cohen et al., 2011).

In current study, data associated with distributing of the autistic children according to age documented that the age group 3-5 years had the highest (41.05%), followed by

the age group of 6-8 years (27.05%) when compared with other groups.

Despite, the fact that ASD is a lifelong disorder that starts in early life, it can be recognized for the first time at any age. Some people will not present until later in life. This may be explained by the difficulty or delay of spoken language. The child is not responsive to other people's facial expression or feelings, unable to social play or typical play purposefully near others and impairment in non-verbal communication.

Comparison the present study with other studies was difficult because varied in design and circumstance-ascertainment strategies, but data from a CDC pilot project, suggest that progress has been made in identifying autistic children at younger ages. Preschool-aged children identified with ASD were more likely to have an intellectual disability than school-aged children with ASD (Christensen et al., 2015) this agree with the present study result that explains age group (3-5) the highest percentage (41.05%). Following by, the age groups of (6-8) with a percent (35.78%) this agree with (Christensen et al., 2016) study which explains approximately one in 68 children aged 8 years.

Autistic children often suffer gastrointestinal (GI) problems that correlate with autism signs (Kang et al., 2017). GI symptoms: including constipation, diarrhea, abdominal pain and discomfort or nausea (Coury et al., 2012).

The current study was displayed that the autistic children suffer from constipation, this symptom may be related to an interaction of behavioral and physiologic factors that cause constipation (van den Berg et al., 2009). Loss of normal gut flora can result in the overgrowth of pathogenic flora, which can, in chance, cause constipation and other problems(Adam et al., 2011). A study by (Gorrindo et al., 2012) recognized constipation as the most common symptom (85%) in autistic children according to estimations by pediatric gastro- enterologists.

In the present study, the fecal flora in autistic children was studied and compared with control groups (healthy children). The result of stool culture for the constipated children showed a significant increase of E. coli in feces comparison to other genera, non-significantly different from the control group.

A number of studies reported that children with ASD have altered gut bacteria compared with neurotypical children (Tomova et al., 2015). Changes in the gut flora and function are common in autistic patients (Kang et al., 2017) may be resulted from treated with antibiotics during the first 3 years of life this caused less diverse and composition in both bacterial species and strains (Yassour et al., 2016). In this study, occurrence of E. coli correlated with its ability to prevent other types from growth (Al-Hamawandi, 2014). This is may have served as a biomarker for many diseases such as autism (Zhang et al., 2015).

The results of the present study were incongruity with the other studies

documented different species of bacteria such as study of (Grimaldi et al., 2017) showed the microbiota of autistic children contained a higher amount of Clostridium spp. and a lower amount of BifIdobacter compared with non-autistic children. While, (Adams et al., 2011) showed their children had lower levels of species of Bifidobacter and higher levels of species of Lactobacillus. But, a study of (Mawgoud et al., 2016) indicated that the counts of both Bifidobacter spp. and Lactobacillus spp. were lower in the stool of autistic children than that of their control group as well as, study (Kang et al., 2013) were characterized by distinct and less diverse gut microbial compositions with lower levels of Prevotella, Coprococcus and unclassified Veillonellaceae. On the other hand, the fecal flora of autism patients contained a higher incidence of the Clostridium histolytic than that of healthy children (Parracho et al., 2005).

Many autistic children often undergo increased oral antibiotic treatment during the earliest years of life (Niehus and Lord, 2006), this cause disrupt of the indigenous gut flora (which plays an important role in the breakdown of plant polysaccharides, encouraging gastrointestinal motility, maintaining water balance, producing some vitamins and competing against pathogenic bacteria) (Krajmalnik-Brown et al., 2015),and promoting the overgrowth of potentially pathogenic microorganisms (Parracho et al.,2005; Krajmalnik-Brown, 2015) such as, the treatment with vancomycin (short-term) resulted development in ASD symptoms, supporting a direct role for the antibiotic sensitive gut bacteria in ASD (Sandler et al., 2000). And treatment with clindamycin (long-term) effected on the family Enterobacteriaceae members (Nyberg et al., 2007).

Children with autism have less diversity of microflora, regardless of whether or not they had GI symptoms. Studies of the gut brain axis supported that gastrointestinal abnormalities can influence brain and behavior (Hsiao, 2014). A multiple factors may effect on the diversity and the composition of the gut flora, including use of antibiotics, environmental influences, dietary habits, transit time of intestinal contents, pH, aging, geographically considerations as well as the interactions between the bacteria themselves illness, stress and lifestyle (Macfarlane and Gibson, 1994; Zhang et al., 2015). Also, the early feeding array influences on the gut microbiota of children and is associated with autism. Breast-feeding for more than 6 months is related with a lower risk of developing autism (Schultz et al., 2006). While, formula-fed infants present an increased species richness attended by an over appearance of Clostridium difficile compared with breast-fed infants (Azad et al., 2013; Penn et al., 2016) found that breast-feeding might protect the infants against GI symptoms.

In the current study, using of Niven agar helped in the isolation of the histamine producing bacteria from the stool of autistic children, only ten samples were given typical purple colonies on Niven medium, and these were the E.coli isolates from the stool, because this medium had the low pH this discourage growth some histamine producing bacteria.

The harming occurred after ingestion of foods with elevated levels of biogenic amines (histamine, tyramine, cadaverine, and putrescine). Excess histamine in foods occurs as a result of the activity of amino acid–specific enzymes derived from bacteria and has been associated with fermented foods such as: Cheese, yoghurt and other fermented milk products (Bjornsdottir et al., 2009).

Many enteric bacterial species produce histidine decarboxylase, and therefore can convert histidine in dietary proteins to histamine within the gut. The major histamine-producing bacteria are Gram-negative mesophilic enteric and marine bacteria (Bjornsdottir-Butler et al., 2010). For

example, some strains of Morganella morganii and Enterobacter aerogenes, which can secrete ⩾1000 ppm histamine during optimum in vitro culture conditions. Strains of other species, including Citrobacter freundi, Vibrio alginolyticus and Escherichia coli are weak histamine producers, producing <500 ppm under the similar conditions (Takahashi et al., 2003). Overgrowth of harmful bacteria induce anxiety and abnormal behaviors (Lyte et al., 2006) and exposed to bacterial toxins develop autism signs (El-Ansary et al., 2012).

V. Conclusion

The altered gut microflora may play an essential role in the pathogenesis of autism. Despite the accurate evidence, this etiological heterogeneity is still not recognized by autism researchers, and most studies fail to take it into account. Future research identifying precisely how the intestinal microbiota participate in the modulation of gut physiology and pathophysiology will be beneficial for our understanding of the interactions between the intestinal microbiota and brain function, which is also helpful in initially developing new therapeutic tools for the management of autism behaviors.

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