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RESEARCH ARTICLE

EXPLORATION OF GASTROINTESTINAL MICROFLORA IN FECES OF TWO MOROCCAN AUTISTIC CHILDREN: 2 CASE STUDIES.

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Abstract

The autism may be involved in microbiology disturbance and gastrointestinal health of autistic patients. In this study, the analysis of the stool specimen of the Moroccan individual with autism provides fundamental information about the overall gastrointestinal health of the patient. Abnormal intestinal microorganisms in the gastrointestinal tract are widely known to cause disease. The stool analysis included parameters for digestion absorption, cultures for bacteria and yeast, parasite testing, an inflammatory marker, short fatty acids, PH, blood presence. In addition, a questionnaire E 2 is used as to confirm the autism and to elicit intestinal problems during the first months.The gastrointestinal health status of these children is not widely disturbed. Abnormal microflora or significant aberrations in intestinal health markers are detected in the patient with autism. The information about the gastrointestinal health status leads to select an appropriate antibiotic therapy in order to decrease the autistic symptoms. The activity of antimicrobial and antifungal drugs against respectively dysbiotic flora and yeast involved namely *Citrobacter freundii* and *Candida albicans* were presented.

Introduction:-

A large number of microorganisms accommodates in human intestinal tracts which can play a crucial role in the regulation of multiple host metabolic pathways in both health and disease (Sikrov et al ,2010.clemente,2012). Intestinal bacteria secrete both detrimental as well as beneficial compounds and overgrowth of certain species of bacteria or other changes to the normal intestinal microbiota have frequently been reported in association with autism (Parracho,1997). Autism is a spectrum of developmental disorders, with onset in early childhood, affecting social, communicative and imaginative development(Wing,1995). Numerous hypothesis have been proposed regarding the etiology of autistic children (including pathogenesis), still the condition remains unclear.

Recent studies have correlated gut dysfunction with Autism spectrum disorder (ASD) group and suggested a possible role of the gastrointestinal (GI) microflora in symptomatology and/or severity of symptoms in autistic children (Shaw,1995.Bolte,1997) . Of the GI problems reported in subsets of autistic individuals, the most common

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are chronic constipation, diarrhea, foul-smelling stools, gaseousness, changes in normal microbiota, abdominal bloating and abdominal pain (Buie, 2010. Coury, 2012. horbaht, 1999) but the underlying cause of GI symptoms remains unclear.

The treatment of gastrointestinal dysfunction in ASD with antibiotics or probiotics has been proposed as a way to regulate intestinal micobiota and subsequently improve symptom severity in ASD(Rachel et al ,2013.Tuohyet al ,2003) .However, repeated antimicrobial use may disrupt the protective commensal microflora and create an environment more favorable to colonization by one or more toxic producing species (Bolte et al ,1998). One group of bacteria known to produce powerful neurotoxins is clostridia(Brook,1994).Cultivation studies by Finegold et al. (2002) revealed that children with autism harbor certain clostridia species in their feces that non-autistic children do not (Fingeoldet al,2002)

In Morocco, very little research is interested in autism in Moroccan children. The only existing data are collected from some associative centers who care of children suffering from autism and or neurobehavioral impairments. In our previous study (harchaoui et al,2013) , we evaluated the biochemical and neurobiological parameters of some Moroccan autistic children. In the present study, and with the objective of contributing to enrich this field and to understand the different factors that influence this disorder, we explore the gastrointestinal microflora profile of a case of two Moroccan autistic children.

Patients and Methods:-

Ethical clearance and informed consent:-

This study was approved by the GDRI of Neurosciences France-Morocco Ethics Committee. The recruitment of patients is based on the following criteria:

1. Written consent of the children's parents,
2. Children physically healthy and not previously undergone any antibiotic, antifungal or any medication that can influence the study, within the last month.

Patients:-

Two children (1 boy and 1 girl) are involved in this study. They are aged 17 and 18 years old respectively. The children are diagnosed for autism and they are inserted into an associative structure in Rabat, Morocco, that cares for children suffering from neurobehavioral impairments.

Patient (A):-

Female child of 18 years is diagnosed with autism. She is the youngest child of her family, she is physically normal and had a difficulty of sucking and diarrhea. She learned to walk alone between 8 and 12 months. Between 2cd and 4th year, she suffers from PICA syndrome. At the age of 3 or 4 years, she is "going into a shell"; she became so distant and lost in thought. Moreover, she was indifferent to any affection and seems to be happier when we do not take care of her. Before 5 years, she was able to speak but not to answer. Her abnormal behavior has been discovered between 13 and 24 months.

Patient (B):-

17 years old, male diagnosed with autism. He is the only child in his family, he is physically normal with an excellent health. He learned to walk alone between 8 and 12 months. Between 2 and 4 years, he sucks often metallic objects. A child at the age of 3 or 4 years was "locked in his shell" or so distant and lost in thought. He is indifferent to any brand of affection and seems being happier when you do not take care of him. Before 5 years, he was able to speak but not to answer. The abnormal behavior of the child has been discovered between 2 and 3 years.

Study Protocol:-

1) The study was explained to participants and informed parent consent/child assent was received.
2) Stool samples were collected and sent by 2-days express shipping to Doctor's Data in a blinded fashion to the Great plain laboratory (USA).

In this study several tests were realized including:-

Lab Methodology:-
All laboratory measurements were conducted by Doctor's Data (St. Charles, IL, USA).

Bacterial/Yeast Culture Susceptibility:-
The process of bacterial cultivation involves the use of optimal artificial media and incubation conditions to isolate and identify the bacterial etiologies of an infection as rapidly and as accurately as possible.

Quantification: The quantification of culture-based methods was done on a scale of 1-4, defined as: 1+ = Rare, 2+ = Few, 3+ = Moderate, and 4+ = Many or Heavy growth of microorganisms. The estimates of recovery are:-
0 = no growth, less than 10^3 colony forming units/ gram of feces = 1+ growth
10^3 - 10^4 colony forming units/gram of feces = 2+ growth
10^5 - 10^6 colony forming units/gram of feces = 3+ growth
> 10^7 colony forming units/gram of feces = 4+ growth
Parasitology: The parasitological tests were were all negative.

Stool Chemistry Testing:-
Lysozyme:-
An enzyme that belongs to the group of alkaline glycosidases. The main source for fecal lysozyme is the intestinal granulocytes. Lysozyme is an antibacterial. It is secreted by recruiting macrophages and monocytes at the site of inflammation. Lysozyme is useful in the diagnosis and monitoring of Crohn's Disease and also in bacterial, viral, allergenic, and autoimmune caused bowel inflammations (Vander et al,1998).

Lactoferrin:-
A biomarker of inflammation caused by diarrhea, fecal leukocytes are found in the stool in large numbers (curront et al,1992). It is very stable and is not degraded during infections by the toxins of pathogens. The assessment of lactoferrin level allows differentiating between inflammatory bowel disease (IBD) and non-inflammatory bowel syndrome (NIBS)(kane et al,20003.kayazawaka,2002) .

Secretory IgA (sIgA) :-
The major immunoglobulin in saliva, tears, colostrum, nasal mucous, mother's milk, tracheobronchial and gastrointestinal secretions (Dugad,2007.Kudoh&wu,1999). It plays a major role in preventing adherence of microorganisms to mucosal sites, in activating the alternative complement pathway, and in activating inflammatory reactions(Nishioet al,1992) . Fecal sIgA is elevated in a response of the mucous immune system, an imbalanced immunological barrier on the intestinal mucosa, and in an autoimmune disease (Stols et al, 1984). It is decreased in children with sIgA deficiencies.

Elastase:-
The Elastase enzyme level can be used for the diagnosis or the exclusion of exocrine pancreatic insufficiency, which may be associated with chronic pancreatitis, cystic fibrosis, carcinoma of the pancreas, Diabetes mellitus Type 1, Shwachman-Diamond syndrome and other etiologies of pancreatic insufficiency.

Short chain fatty acids (SCFA):-
are the end products of anaerobic microbial fermentation of dietary fiber(scerpella et al,1995). Levels thus reflect the concentration of intestinal flora as well as soluble fiber in the diet (Araki et al,2002.Topping et al,2001). The SCFA distribution reflects the relative proportions of the beneficial SCFA (n-butyrate, propionate and acetate), thus providing an indirect measure of balance among the anaerobic organisms in the colon. These beneficial SCFA are crucial to the health of the intestine, serving as sources of fuel for the cells and the rest of the body. Decreased levels may reflect insufficient normal colonic flora, a diet low in soluble fiber, or prolonged intestinal transit time (Toppinget al,2001)**.** Abnormal levels of short chain fatty acids in the stool can indicate malabsorption and are used as metabolic markers.

Results:-
Four types of beneficial bacteria were investigated, including *Bifidobacterium Lactobacillus spp, E. Coli and Enteroccus* (Table 1). Healthy levels of each of the beneficial bacteria are indicated by either a 3+ or 4+ (0 to 4 scale). Abnormal levels of bifidobacterium, E.coli, enterococcus spp were observed in patient A feces. However patient B stool showed abnormal levels of E.coli. Simultaneously, imbalanced and dysbiotic microflora was

observed in patient B stool (Bacillus spp, klebsiella oxytoca and citrobacter freundii). The presence of the candida albicans was identified in stool culture of both children, whereas we noted the absence of any parasites in the microscopic examination of their stool samples.

Table 1:-Microflora found in stool analysis.

Patients	**A**	**B**
Bacteriology culture		
Benefecial flora		
Bifidobacteruim	4+	0+
E.coli spp	4+	4+
Lactobacillus spp	0+	1+
Enterococcus spp	4+	2+
Imbalances flora		
Bacillus spp	0+	2+
Klebsiella oxytoca	0+	2+
Alpha haemolytic strep	0+	0+
Dysbiotic flora		
Citrobacter freundii	0+	1+
Mycology (Yeast) culture		
Candida albicans	1+	1+
Campylobacter jejuni	Neg	Neg
Parasitology/microscopy		
Giardia lamblia	Neg	Neg
Cryptosporiduim	Neg	Neg

Digestion/ Absorption markers:-

Elastase, fat stains, muscle fibers, vegetable fibers, carbohydrates were measured. The levels of the digestion and absorption markers are within the normal range in these patients (Table 2).

Table 2:-Digestion and Absorption markers.

Patient	**A**	**B**	**Ref.range**
Elastase	> 500	278	> 200 µg /ml
Fat stain	None	None	None-Mod
Muscle fibers	None	None	None-rare
Vegetable fibers	Rare	Rare	None-Few
Carbohydrates	Neg	Neg	Neg

Inflammatory markers:-

High levels of inflammatory markers were observed in both children (table3). A great level was noted in the patient B (Lysozyme= 1470 ng/ml) whereas a slight enhancement in Lactoferrin levels observed in patient A stool (7,5 µg/ml). However, we did not show any variation regarding the two other markers (WBC and Mucus) for both of them.

Table 3:-Inflammatory markers revealed in stool analysis.

Patients	**A**	**B**	**Ref.range**
Lysozyme	740	1470	< 600 ng /ml
Lactoferrin	7,5	0,5	< 7,3µg/ml
WBC	None	None	None-Rare
Mucus	Neg	Neg	Neg

Secretory IgA:-
Elevated levels of SIgA were observed in patient B (223 mg/ml) compared to the patient A who kept a normal levels (< 204 mg/ml) (table 4).

Table 4:- Secretory IgA in stool IgA (mg/ml).

Patients	A	B	Ref.range
SIg A	95	223	51-204/ml

Short Chain Fatty Acids:-
The total amount of SCFA was slightly higher in the patient B compared to the patient A who presented normal values. He has a total SCFA concentration up to the normal value range (16,3 mg/ml versus 14 mg/ml). Similarly, the level of butyrate was also higher in this child B (6,4mg/ml instead 3,8 mg/ml) while other short chain fatty acid levels remain normal.(see table 5).

Table 5:-Short chain fatty acids.

patients	**A**	**B**	**Ref.range**
Acetate	55	37	36-74%
Propionate	19	22	9-32%
Butyrate	22	39	16-39%
Valerate	4	2	1-8%
Butyrate	2,9	6,4	0,8 -3,8mg/ml
Total SCFA	13	16, 3	4-14mg/ml

Intestinal Health Markers:-
The results showed that both children presented in their stool: a negative RBC, the absence of blood in the feces and rare growing fecal yeast. However, the patient B has an acidic stool (fecal PH=5, 4) with PH value below to the normal range (see table6).

Table 6:- Intestinal health markers.

	A	**B**	**Ref.Range**
RBC	None	None	None-Rare
PH	6,2	5,4	6-7,8
Occult blood	Neg	Neg	Neg
Yeast	Rare	Rare	None-Rare

Yeast and bacterial susceptibilities:-
The result of antimicrobial and antifungal susceptibility testing is shown in table 7. The antibiotic that will be most effective against citrobacter freundii is Ciprofloxacin in the case of patient B. Whereas, many antifungal are likely to cure patient A infections depending on the diagnosis of her status are successively fluconazole, Itraconazole, ketoconazole, Nystatin, these two latest antifungal are effectiveness against Candida albicans in patient B case.

Table 7:-Citrobacter Freundii and Candida albicans susceptibilities.

	A	**B**
Citrobacter Freundii		
Amoxicilin	-	R
Ampcilin	-	R
Augmentin	-	R
Ciprofloxacin	-	S
Trimeth-sulfa	-	R
Candida albicans		
Fluconazole	S	R
Itraconazole	S	R
ketoconazole	S	S
Nystatin	S	S

Discussion:-

A growing number of data indicate that many factors, including diet, age and gastrointestinal agents (e.g., microbiota), may play an important role in the autism spectrum disorders (ASD)(Macktabe,2011) . Even though variation on the composition of the microbiota were described for ASD patients compared to healthy individuals, the trend and importance are not already defined (Gondalia,2012).

Our results showed that fecal composition of ASD children showed many species such as *bifidobacteria, Lactobacillus spp E. Coli, Enteroccus* and *Bifidobacterium*, but a decreased Bifidobacterium content, was observed in fecal samples of the patient B. In accordance with our study, other data demonstrate the content of Bifidobacterium species- and strain-specific decreased in fecal samples of ASD and they might influence the immunity (De angles et al,2013) , thus because they are able to synthesize exopolysaccharides, which act as fermentable substrates for human intestinal bacteria. However, the Secretion and excretion of immunoglobulin A to feces differ with type of indigestible saccharides(kudohet al,1999) . In our case, we didn't establish any correlation between the immune system and autism, but we found that autistic patient B possesses a high level of S'IgA and inflammatory markers which may plead in favor of a clear interaction between microbiota, immune system and disease etiology (Adams et al,2011).

SCFAs represent a group of compounds derived from the host microbiome that are plausibly linked to ASDs and can induce widespread effects on gut, brain, and behavior. Along with acetate and butyrate, PPA is known to reduce gastric motility and increase the frequency of contractions, presumably via a reflex that involves direct contact of these short chain fatty acids with the terminal ileum . In contrast to animal studies, Adams et al. reported lower levels of several SCFA in autistic subjects. But, in our study, the SFCA levels were higher in autistic patient B. The Differences might be explained by an alteration of some amino acids 32 (Roy et al,2006), fermentation of dietary carbohydrates, with consequent changes in systemic metabolites.

The fecal pH of one of autistic child (B) was lower, which may disturb colon function, especially carbohydrate absorption and could also cause changes in the microbiome that corresponded with alterations in fecal pH and fecal consistency. Furthermore, some studies reported that pH was strongly negatively correlated with total SCFA, presumably because SCFA's contribute to colonic pH (Adams et al,2011). In that study, they demonstrated that pH was also negatively correlated with lysozyme, suggesting that higher pH is associated with lower levels of lysozyme and vice versa. It seems that we have similar case because we observed that the autistic child had a low pH, increased levels in SCFA's and inflammatory markers, but we cannot support this data for instance, especially many variables can affect this study including the diet component (Mediterranean diet) and the size of samples that we worked with.

It has been a great deal of speculation that yeast infections are a major problem in autism. Our results revealed positive fungal cultures for yeast in autistic children feces (i.e Candida albicans). In another study, Adam et al showed that yeast is present at normal levels in the stool of both normal and autistic children.

The treatment of gastrointestinal dysfunction in ASD with antibiotics or probiotics has been proposed as a way to regulate intestinal micobiota and subsequently improve symptom severity in ASD (Sandler et al,2000). In our study, the *Citrobacter Freundii* and *Candida albicans* was found in the stool of autistic children, the sensitivity of some antibiotics were tested against the bacterial and fungal flora in the aim to limit or annihilate their action on gastrointestinal status, some of them revealed a positive activity against citrobacter freundi (such as ciproflaxin) and Candida albicans (such as ketoconazole and Nystatin) particularly in patient B. However, the key issues such as the prevalence and best treatment of the gastrointestinal disorders in individuals with ASD are incompletely understood (Galiatsatos et al ,2009).

Study Limitations:-

There are several important limitations to this study that should be acknowledged. One is the small sample size, and the resultant limitations are ought to generalize the findings and statistical power. Also, we did not utilize a control group because we don't have any clinical reference ranges for normal control populations compared with autistic populations.

Finally, we did not control for dietary differences amongst the participants. Evidently, it would be ideal to manipulate dietary intake to remove its influence on GI microbiology and to fulfill the gap between microflora population levels and autism severity in Moroccan context.

Conclusion:-

In conclusion, our study showed that children with autism had gastrointestinal (GI) problems and its severity differ between the two child (the boy has a severe GI problem more than the girl), which in accordance with many studies showing that ASDs is a male-typical disorder. These disturbaces were characterized by: imbalance in beneficial bacteria, Low pH, increased levels of SCFA , lysozyme and inflammatory markers. In fact, interaction between microbiota, immune system and disease etiology is possible, the use of probiotics in this case is beneficial but we don't know if the treatment can affect the studied parameters (pH, lysozyme, SFCA…) or its effects are limited to defend against pathogenic strains.

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