Microbiology and gastrointestinal health study of Moroccan autists

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**Abstract**

The autism may be involved microbiology disturbance and effect gastrointestinal health of autism. In this study, the analysis of the stool specimen of thre 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, inflammatory marker, short fatty acids, PH, blood presence. In addition, a questionnre 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 lead 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.

**Key Word:** Autism, Gastrointestinal health, Microflora, biomarkers, Antibiotic susceptibility.

**Introduction**

Autism is a complex disorder and probably embraces several differing entities. There are no specific diagnostic tests so the disease is defined by its characteristics cognitive defects, including delays in understanding and use of language; social, communication and behavioral problems; repetitive behaviors; unusual sensitivity to stimuli such as noises; and restricted interests [1]. There are no specific criteria that distinguish the different types of autism from one another. The Autism Research Institute collected data on autism since 1965 and noted that regressive autism, beginning around 18 months of age, was very uncommon until the mid-1980’s and between 1991 and 1995 there were over twice as many with onset at 18 months as at birth [2]. Individuals with autism often suffer from gastrointestinal problems [3]. The prevalence of gastrointestinal problems is different from study to study and dpends on the age of the study population, but there is a general consensus that gastrointestinal problems are common in autism.Existing hypotheses relate autism with several abnormalities, infection and others [4]. Auisitic behavior is often accompanied by numerous symptoms such as abdominal pain, constipation or diarrhea, connected with deregulation of physiological microflora [5,6].non specific symptoms are often connected with intestinal inflamtion and eleveted level of lactoferrin [7,8]. MacFabe et al has pointed to the likely effect of propionic acid and other short-chain fatty acids [9]. The results of a study published in 2000 [10]. Most of autistic children had gastrointestinal problems such as abdominal pain and bloating, and constipation and/or diarrhea; these symptoms improved considerably. Gastrointestinal symptoms that may be very distressing have been noted in some children who have regressive autism and there is speculation that this may represent a specific subset of autism.

The microorganisms may be cotribute considerbaly to autism [11]. The latter studies of fecal flora in such children revealed early studies of fecal flora in such children revealed higher counts of clostridia and more different species of clostridia than are found in stools of age and sex-matched children. Recent studies using the powerful pyrosequencing technique indicates that other bacteria may be more important in inducing the disease in susceptible individuals and that still other organisms, such as *Bifidobacterium* are more prevalent in controls than in autistic children; such organisms might be protective if administered as a specially tailored probiotic. They used basic anaerobic culturing techniques to count and isolate microorganisms, followed by Polymerase Chain Reactio (PCR) targeting the 16 S rDNA to identify the isolates cultivated. The number and type of Clostridium and Ruminococcus species in children with autism differed from the control children. Song et al. 2004 [12].

Gastrointestinal problems in children with autism may contribute to the severity of the disorder. Abdominal pain, constipation, and/or diarrhea are unpleasant and likely to produce frustration, decreased ability to concentrate on tasks, behavior problems, and possibly aggression and self-abuse, especially in children unable to communicate their discomfort. These problems also result in a decreased ability to learn toilet training, leading to increased frustration for the child and their parents/ caregivers [13].

The cause of these gastrointestinal problems is unclear. However, The excssesive use of oral antibiotic can lead to the alteration of gut flora. The treatment of the gut flora by oral vancomycin incompletely absorbed improve temporarly the behavior for children with late- onset autism [14], but the benefits were lost after treatment stopped. This study demonstrated the importance of gastrointestinal flora and the difficulty in permanently normalizing them.

Gastrointestinal problems are common in children with autism and may contribute to autism behavioral symptoms. However, more research is needed.

The aim of this study is to investigate some gut flora and biomarkers of gastrointestinal function in Morrocan children with autism. The gut flora investigated include both beneficial and pathogenic bacteria that are easily cultured, but the culture methods used were able to detect only some of the bacteria.

**Patients**

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

 - Written consent of the children’s parents,

 - Children physically healt

 - No usage of any type of antibiotic or antifungal medications within the last month

Three children (2 girls and 1 boy) are involved in the present study. They are aged between 15 to 18 years old. 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 Aya, female child aged 4 years is diagnosed with autism. Aya is the second of three sisters and she is physically normal. She had frequent infections breathing and constipation during the first months of life. She learned to walk alone between 24 and 36 months. Between 2cd and 4th year, she had an irresistible urge to eat something. 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 7 and 12 months.

Patient Salwa, female child of 18 years is diagnosed with autism. Salwa is the younger child of her family, she is physically normal. Salwa 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 Ismail 17 years old, male diagnosed with autism. Ismail 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) Parents filled out a questionnaire E2 on their child’s gastrointestinal status.

3) Stool samples were collected and sent by 2-day express shipping to Doctor’s Data in a blinded fashion to the Great plain laborotory (USA).

In this study several tests were realized including :

Bacterial/Yeast Culture, ID and 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. 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 103 colony forming units/ gram of feces = 1+ growth 103 - 104 colony forming units/gram of feces = 2+ growth 105 - 106 colony forming units/gram of feces = 3+ growth > 107 colony forming units/gram of feces = 4+ growth Colony-forming unit (CFU or cfu) is a measure of viable bacterial or fungal numbers. Unlike direct microscopic counts where all cells, dead and living, are counted, CFU measures viable cells.The parasitology test was used and were all negative. It was then decided to do no additional testing on other samples.

Chemistry analysis including :

Lysozyme is a 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 recruited 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 [15,16]. An Enzyme- Linked-Immuno-Sorbent-Assay (ELISA) was used for the quantitative determination of lysozyme in stool, using the Lysozyme ELISA Kit (ALPCO Diagnostics Cat. No. 30-6900) [17].

Lactoferrin: A biomarker of inflammation caused by diarrhea, fecal leukocytes are found in the stool in large numbers [18].. It is very stable and is not degraded during infections by the toxins of pathogens. The assssement of lactoferrin level allow to differentiate between inflammatory bowel disease (IBD) and non-inflammatory bowel syndrome (NIBS) [19-20]. The measurement was performed with IBD-Scan® Kit (Tech- Lab®Blackburg, VA) [21].

Secretory IgA (sIgA) is the major immunoglobulin in saliva, tears, colostrum, nasal mucous, mother’s milk, tracheobronchial and gastrointestinal secretions [22,23]. It plays a major role in preventing adherence of microorganisms to mucosal sites, in activating the alternative complement pathway, and in activating inflammatory reactions [24]. Fecal sIgA is elevated in a response of the mucosa immune system, an imbalanced immunological barrier on the intestinal mucosa, and in an autoimmune disease [25]. It is decreased in children with sIgA deficiencies. The test was performed with the Secretory IgA ELISA Kit (ALPCO Diagnostics Cat. No. 30-8870).

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. The test was performed with the Elastase ELISA BIOSERV Kit (Joli Medical Products Inc.).

Short chain fatty acids (SCFA) are the end products of anaerobic microbial fermentation of dietary fiber [26]. Levels thus reflect the concentration of intestinal flora as well as soluble fiber in the diet [28,29]. 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 [29]. Abnormal level of short chain fatty acids in stool can indicate malabsorption and are used as metabolic markers.

Levels of butyrate and Total SCFA’s in mg/mL are important for assessing overall SCFA production, and are reflective of beneficial flora levels and/or fiber intake [30]. The “volatile” fatty acids in fecal samples were extracted into an HCl solution and quantified using aflame ionization detector (FID) following separation by gas chromatography(GC) [31,32]. The SCFAs that were measured include acetate, proprionate, butyrate, and valerate. Results were verified to be accurate and precise using quality control.

RESULTS

Four types of beneficial bacteria were investigated, including *bifidobacteria, Lactobacillus* *spp E. Coli, and Enteroccus and Bifidobacterium* – see Table I. The patient Aya had much lower levels of *Enterococcus* and much higher levels of *Bifdobacterium*, *Lactobacillus* , E.coli Bacteriology values ranged from 0 to 4.

Dysbiotic flora including *Citrobacter freundii* was observed in the patients Aya and Ismail but rarely not observed in Salwa.

The commensal bacteria observed in few amounts in Ismail was *Bacillus spp,Klebsiella* *oxytoca* and in higher level was *alpha hemolytic strep* in Aya.No commensl bacteria had been observed in Salwa.

The presence of yeast was detrmined by culture were only observed rarely in the patient Ismail and Salwa and no yesat were isolted in the patient Aya.The *campylobacter jejuni* and parasites had not been observed in this patients.

Table I. microflora found in stool analysis

|  |  |  |  |
| --- | --- | --- | --- |
|  |   Aya  |  Salwa  |   Ismail |
| Benefecial flora *Befidobacteruim**E.coli spp**Lactobacillus spp**Enterococcus spp*Imbalances flora*Bacillus spp**Klebsiella oxytoca**Alpha haemolytic strep* Dysbiotic flora *Citrobacter freundii* Yeast *Candida albicans* Campylobacter jejuni Parasites *Giardia lamblia* *Cryptosporiduim*  | 4+4+4+0+ 0+ 0+ 4+ 1+ Neg  Neg Neg  |  4+ 4+ 0+ 4+ 0+ 0+ 0+ 0+ 1+NegNegNeg | 0+4+1+2+2+2+0+1+1+NegNegNeg |

Table II : digestion and absorption markers

Elastase, fat stains, muscle fibers vegetables fibers, carbohydrates were measured.The fat stains, muscle fibers vegetables fibers, carbohydrates are rated from 0 to 4.The levels of the digestion and absorption markers are within the normal range in this patients.carbohydrates were listed either negative or postive.

|  |  |
| --- | --- |
|  | Aya Salwa Ismail Ref.range  |
| Elastase Fat stainMuscle fibers Vegetables fibers Carbohydrates  | > 500 > 500 278 > 200 µg /mlNone None None None-Mod None None None None-rareNone Rare None None-Few Neg Rare Neg   |

Possible markers of inflammation, including lysozyme, lactoferrin, white blood cells, and mucus, were investigated. A higher level of lysosyme was observed in childrens Ismail and Salwa.A slight higher level of lactofferrin was only observed in children Salwa.Mucus were listed either negative or positive.

Table III : Inflammtory markers

|  |  |
| --- | --- |
|  | Aya Salwa Ismail Ref.range  |
| LysosymeLactoferrin WBCMucus  | 453 740 1470 < 600 ng /ml4,2 7,5 0,5 < 7,3µg/mlNone None None None-RareNeg Neg Neg Neg |

Levels of SIgA were measured.A hiegher levels were observed both in chlidren Aya and Imsail.whereas, Salwa had a normal levels of IgA.

**Table IV : secrotory IgA in stool IgA (mg/ml).**

|  |  |
| --- | --- |
|  | Aya Salwa Ismail Ref.range |
| SIg A  | 361 95 223 51-204 mg/ml |

The presence of several short chain fatty acids (SCFA), including acetate, proprionate, butyrate, and valerate were measured. A slight higher levels of total (SCFA) and Butyrate were obsreved in pattient Ismail.

Table V : Short chain fatty acids

|  |  |
| --- | --- |
|  |  Aya Salwa Ismail Ref.range |
| Acetate PropionateButyrateValerateButyrateTotal SCFA  | 58 55 37 36-74%21 19 22 9-32%17 22 39 16-39%4 4 2 1-8%1,6 2,9 6,4 0,8 -3,8mg/ml9,4 13 16, 3 4-14mg/ml  |

The presence of RBC, or occult blood were very rare in this patients.The fecal PH is somewhat elevated in patient Ismail. The microscpic yeast observed were rare.

Table VI : Intestinal health markers

|  |  |
| --- | --- |
|  | Aya Salwa Ismail Ref.Range |
| RBC PHOccult bloodYeast  | None None None None-Rare6,8 6,2 5,4 6-7,8Neg Neg Neg Neg None Rare Rare None-Rare  |

Table VII : *Citrobacter* *Freundii* susceptibilities

Ciprofloxacin is not active against *citrobacter freundii* in Ismail and salwa.Trimeth-sulfa is inactive merely in Aya.

|  |  |
| --- | --- |
|  | Aya Salwa Ismail  |
| AmoxicilnAmpcilinAugmentinCiprofloxacin Trimeth-sulfa  | R RR RR RS SS R |

Table VIII : *Candida* susceptibilities

Fluconzole and Itraconazole are inactive against *candida* in Ismail

|  |  |
| --- | --- |
|  | Aya Salwa Ismail  |
| Fluconazole ItraconazoleketoconazoleNystatin  |  S R  S R S S S S |

DISCUSSION

The gastrointestianl problems may contribute to autistic symptoms in some children. The gastrointestinal issues of studied childres was reported in Questinone E 2, Salwa had a diahrrea problems, Aya besides contipation had a frequent breathing problems during the first months.Ismail had no gastrointestinal healh problems. This suggests that there are either lower amounts of beneficial bacteria as observed in patient Salwa and Ismail whereas which

produce SCFA’s, a lower intake of soluble fiber, a longer transit time, and/ or increased absorption due to increased gut permeability. The latter possible explanation is very intriguing because of work by MacFabe et al. 2007 [33], which demonstrates that SCFA’s can induce autistic-like symptoms when injected into rats. In other words, if lower levels of SCFA’s in the stool are due to increased absorption, then this presumably would lead to higher level of SCFA’s entering the bloodstream, and hence would exacerbate autistic symptoms.

Lysozyme is an important part of the immune system, and protects the gut from pathogenic bacteria by enzymatic attack of their cell walls. It is secreted by recruited macrophages, monocytes, and granulocytes at the site of inflammation. Infants fed formula without lysozyme have three times the rate of diarrheal disease [34]. In this study, lysozyme levels were lower only in children Aya however is elevated in both children Salwa and Ismail. Limited defense against pathogenic bacteria, and thus decrease the need for the immune system to excrete lysozyme. Inflammation.

The unusually lower levels of pH in the autistic children Ismail suggests that there is a general disregulation of pH, which could affect digestion and bacteria. The higher PH is associated with lower levels of lysozyme and vice versa. Also, pH was even more strongly negatively correlated with total SCFA presumably because SCFA’s contribute to colonic pH. The lower amounts of *bifidobacteria* in children with autism is consistent with a pyrosequencing study [35] that also found lower levels of *bifidobacteria* in children with autism, and suggests that supplementation with *Bifidobacteria* is worth investigating. The high levels of *lactobacillus* in

The finding that yeast were existed in few amounts or not existed in the current patient , as there has been a great deal of speculation that yeast infections are a major problem in autism.

While a few or rare amounts of yeast were observed is considered normal.A study by Horvath and Perman [35] reported that majority of children with autism undergoing endoscopies had a positive fungal culture for yeast in their duodenal juice, vs. 23% of age-matched controls with other gastrointestinal problems requiring endoscopies. Since their study involved children with severe enough symptoms to warrant endoscopies,

The presence of parsaites and *campylobacter* *jejuni* were not observed in this children.Aya was presented.

The commensal bacteria observed in few amounts in Ismail was *Bacillus spp,Klebsiella* *oxytoca* and in higher level of *alpha hemolytic strep* in Aya.

It was interesting to note that dysbiotic bacteria such as *Citrobacter Freundii* were present at low levels in both children. Research Institute of parents’ reports that parents find antifungals to be one of the most effective medications for improving behavior [36], It is possible that children with autism are more sensitive to even a normal level of yeast. Also, it is possible that antifungals have other effects, such as reducing inflammation. The treatment of *Citrobacter* *Freundii* could be removed by ciprofloxacin in both Aya and Ismail.only thrimeh\_sulfa in Aya. The antifungal that active against *Candida* *albicans* in Aya are fluconazole, itraconazole, ketoconazole, nystatin. Ismail only ketoconazole and nystatin that are active.howerver, the overuse of drugs contribute in predisposing to autism [37].

CONCLUSION

The overall gastrointstinal status of Morrocan children with autism is different from the patient to other according to several parameters ; dietary intervention, diet caseain-gluten free. The benfecial flora is normal an in adequate amount, the only dysbiotic flora such as citrobacter freundii was involved.Overall, the stool testing was showed that there is a disruption and dysfunction in intestine and there is abnormal biomarkers.

The stool analysis plays a crucial role in pinpointing the dysbiotic bacteria , yeast, digestive dysfunction, and also this approach can selecte the most appropriate antimicrobial therapy.

The ciprofloxacin is active against *citrobacter* *freundii* and ketokonazole and nystatin is active against *candida* *albicans* for the children involved. Treatment of digestive problems appears to have positive effects on autistic behavior.  This approach viewed as complemtery method and is not a treatment in itself.

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