STUDY OF THE GUT ENTEROTYPES IN SOME EGYPTIAN PATIENTS WITH REMITTING RELAPSING MULTIPLE SCLEROSIS

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Abstract

Background: Gut microbiota cluster into three enterotypes named the Bacteroides, Prevotella and Ruminococcus. While each person's microbial "fingerprint" is unique, there are specific patterns seen in those that are healthy and those that have specific illnesses. The aim of the present study is to identify the enterotypes that are likely related to Multiple Sclerosis Egyptian patients as well as their possible role in the course of the disease. Subjects & Methods: Thirty patients with remitting relapsing multiple sclerosis, who presented to the MS Clinic of Alexandria University Hospital were enrolled in our study. These were diagnosed according to according to McDonnald 2017 criteria. A cross matching control group of 20 healthy subjects of similar age and sex were included. Stool specimens were taken from each. Quantitative SYBR Green Real-Time PCR was done for the identification and quantitation of Bacteroides, Prevotella and Ruminococcus which constitute the core of the three major enterotypes. Results: Enterotype 1 is the most common enterotype detected in MS and control cases (80% versus 65%). For Enterotype 3, it was not detected in any of the 20 control cases while detected in multiple sclerosis case (16.7%). However, by comparing the multiple sclerosis and control cases Enterotype 2 is significantly less in multiple sclerosis than control (3.3% versus 35%). Conclusion: Although Enterotype 2 is significantly less in multiple sclerosis patients, collapsing the whole microbiome variations into dominant enterotypes was not appropriate to identify disease association or to be used as a disease biomarker.

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Background : Gut microbiota cluster into three enterotypes named the *Bacteroides* , *Prevotella* and *Ruminococcus* . While each person's microbial "fingerprint" is unique, there are specific patterns seen in those that are healthy and those that have specific illnesses. The aim of the present study is to identify

the enterotypes that are likely related to Multiple Sclerosis Egyptian patients as well as their possible role in the course of the disease. **Subjects & Methods:** Thirty patients with remitting relapsing multiple sclerosis, who presented to the MS Clinic of Alexandria University Hospital were enrolled in our study. These were diagnosed according to according to McDonnald 2017 criteria. A cross matching control group of 20 healthy subjects of similar age and sex were included. Stool specimens were taken from each. Quantitative SYBR Green Real-Time PCR was done for the identification and quantitation of *Bacteroides, Prevotella* and*Ruminococcus* which constitute the core of the three major enterotypes. **Results:** Enterotype 1 is the most common enterotype detected in MS and control cases (80% versus 65%). For Enterotype 3, it was not detected in any of the 20 control cases while detected in multiple sclerosis case (16.7%). However, by comparing the multiple sclerosis and control cases Enterotype 2 is significantly less in multiple sclerosis than control (3.3% versus 35%).**Conclusion:** Although Enterotype 2 is significantly less in multiple sclerosis patients, collapsing the whole microbiome variations into dominant enterotypes was not appropriate to identify disease association or to be used as a disease biomarker.

Keywords : gut microbiome; multiple sclerosis; Real Time PCR; 16S rRNA; dysbiosis; enterotypes

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What's already known about this topic?

Gut microbiota cluster into three enterotypes. Prevalence of a specific enterotype can depend on long-term dietary habits, high-fat and protein diet enhances the growth of Enterotypes 1, while a diet rich in carbohydrates supports the raise of Enterotype 2 and high fiber diet vegetables with Enterotype 3.

While each person's microbial "fingerprint" is unique, there are specific patterns seen in those that are healthy and those that have specific illnesses.

What does this article add?

- Globalization has affected people's eating habits, leading them to consume high-fat and high-calorie foods as most of our study participants were Enterotype 1.
- This study confirmed the revised enterotypes classification that removed Enterotype 3, restricted to areas depending mainly on vegetables, from the enterotypes.
- Enterotypes are not appropriate to identify disease association or to be used as a disease biomarker, but it can reflect the dietary pattern of subjects.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (CNS) characterized by demyelinating lesions that are disseminated both in space and time.[(1)]It is one of the most common demyelinating disorder and considered as a major cause of nervous system disability in young adults.[(2)]

The etiology of MS is not fully appreciated, although strong evidence points to genetic and environmental factors. [(3)] As an important environmental factor to our body, the gut microbiota plays a major role in human health and disease. [(4,5)]

Gut microbiota has been classified into three main enterotypes, each one owning specific metabolic features. Each enterotype is characterized by the relative abundance of one of the following genera:*Bacteroides* (more represented in Enterotype 1), *Prevotella*(more abundant in Enterotype 2), *Ruminococcus* (prevalent in Enterotype 3). Prevalence of a specific enterotype can depend on long-term dietary habits, indeed high-fat and protein diet enhances the growth of Enterotypes 1, while a diet rich in carbohydrates supports the raise of Enterotype 2 and high fiber diet vegetables with Enterotype 3. [(6)] Recent findings suggest that both genetic and environmental factors are involved in influencing the inter-individual diversity of gut microbiota. [(7,8)]

Surveys of humans from around the world have revealed differences in gut microbiota composition among geographically separated populations. Globalization has affected people's eating habits, leading many of them to consume high-fat and high-calorie foods. Modern diets have adverse effects on human health and raise global issues, particularly for young generation in developing areas. [(8-10)]

While each person's microbial "fingerprint" is unique, there are specific patterns seen in those that are healthy and those that have specific illnesses. [(6)]Therefore, this study was designed to identify first differences in the gut enterotypes of patients with MS compared with healthy controls, in an attempt to identify the enterotypes that are likely related to MS as well as their possible role in the severity of the disease.

METHODS

Subjects :

The study protocol was approved by the Local Ethics Committee of Faculty of Medicine, University of Alexandria (0201018) that is conformed to the ICH GCP guidelines. All participants provided a written informed consent to inclusion of the clinical and laboratory data for research purposes upon fulfilling the study enrollment criteria.

Thirty MS cases, who presented to the MS Clinic of Alexandria University Hospital, were enrolled in our study. These were diagnosed clinically according to revised McDonald criteria 2017. [(11)] In addition, complete neurological and systemic examination including expanded disability status scale (EDSS). [(12)]

A cross matching control group of twenty healthy subjects of similar age and sex were included.

Specimen collection, preservation and transport

Stool specimens were collected, kept in the freezer upon defecation at home, and within the same day delivered to our laboratory frozen, where aliquots of each specimen were frozen at -80 °C until DNA extraction in the same week.

DNA Extraction

DNA was extracted from 150 mg stool samples using ISOLATE Fecal DNA Kit (Bioline, UK) according to the manufacturers' instructions. In brief, fecal samples were added directly to a bashing beads lysis tube and they were rapidly lysed by bead beating in a vortex, without the use of organic denaturants or proteinases. The DNA was then bound, isolated and purified using spin columns. The resulting DNA extracts were stored at -80°C until PCR assessment.

SYBR Green Real-Time PCR

Primers

Oligonucleotide primers were targeted at the 16S rRNA gene (rDNA) sequences of *Bacteroides* , *Prevotella* and*Ruminococcus* . Primers were also used to amplify a conserved 16S rDNA sequence present in all bacteria (universal primer set, recognizing domain bacteria), the amplification of which served as the denominator against which the amplification of the other bacteria were compared. All of the primer sequences shown in Table 1 were derived from the previously published studies.[(13-15)]Primers were commercially obtained (Metabion International AG. Germany).

Detection and Quantitation :

The real-time PCR protocol was performed as previously described by Tomova et al., 2015. [16] Amplification was performed in a light cycler (Rotor Gene Q, Qiagen, Germany) using a SensiFAST TM SYBR No-ROX PCR kit (Bioline Co. UK). In short, forward and reverse primers (4 pmol each) were used in 20 µl reactions containing 2 µl of the DNA extract.

PCR amplification was performed with initial denaturation at 95 @C for 10 minutes, followed by 40 cycles of denaturation at 95 @C for 30 seconds, annealing at 60 @C for 30 seconds, and extension at 72 @C for 30 seconds. Melting curve analysis was performed from 40 to 95 @C with a plate reading step after every 1 @C and held at a particular temperature for 10 seconds to check the specificity of the product formed.

Quantitation of specific bacterial DNA was not expressed as absolute number but expressed relative to total (universal) bacteria DNA present in a stool sample by the RQ software (Qiagen).

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test or Monte Carlo correction. Variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agostino test. If it reveals normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations were done using independent t-test. For Quantitative variables comparison between groups were done using Mann Whitney test and Kruskal Wallis test. Significance of the obtained results was judged at the 5% level.[17]

RESULTS

Out of the 30 RRMS patients, 13 (43.3%) were males and 17 (56.6%) were females with female to male ratio of 1.3:1. Their mean age ± SD was 31.43±4.21 years, and their age ranged from 25 to 40 years. Mean age of onset was 26.02±4.79 years.

Out of the 20 control subjects examined there were 10 males (45.5%) and 12 females (54.5%), with female to male ratio of 1.2:1. The mean age ± SD of the cases was 32.3 ±5.57, and their age ranged from 23-40 years.

Clinical Characteristics of MS Patients:

All patients enrolled in the present study were RRMS. The mean duration of illness was 5.4 years, with mean relapse rate (relapse number/disease years) 1.4. The mean of EDSS was 3 and the most common presenting symptom was sensory (83.3%), followed by motor (66.7%), optic neuritis (63.3%) and cognitive (20%) symptoms. Three (10%) cases were positive for CSF oligoclonal band. By asking patients for gastrointestinal symptoms only 3 (10%) cases complaint of constipation. All patients were on disease modifying therapy. Five (9.1%) cases have positive family history of MS (Table 2).

The Enterotypes in the Study Participants

Profiling of the gut microbiome of the study groups was done to characterize their enterotypes which are dominated by *Bacteroides*(Enterotype 1), *Prevotella* (Enterotype 2) or *Ruminococcus*(Enterotype 3).

As shown in table (3), Enterotype 1 is the most common enterotype detected in MS and control group. Twenty-four (80%) of the 30 MS patients were assigned to Enterotype 1, 1 (3.33%) were assigned to Enterotype 2 and 5 (16.67%) to Enterotype 3. Fifteen (68.18%) of the 22 control subjects were assigned to Enterotype 1, 7 (31.82%) were assigned to Enterotype 2 and none were Enterotype 3. Statistically significant difference was detected between the 2 groups regarding the enterotype distribution. (Monte Carlo X^2= 10.597, P value 0.002).

Table (4) shows the relation between enterotypes of MS patients and different variables which are; age, gender, disease onset and duration, EDSS, sensory, motor optic neuritis and cognitive symptoms, CSF oligoclonal band, therapy and constipation. There is no statistically significant difference between the different enterotypes and the different variables.

Table (5) shows the relation between enterotypes of the control group and different variables; age and gender. There is no statistically significant difference between the different enterotypes and the different variables.

DISCUSSION

In our study the profiling of the gut microbiome revealed that Enterotype 1 was the most common enterotype detected in MS and control cases (80% versus 65%). For Enterotype 3, it was not detected in any of the 20 control subjects, while it was detected in five multiple sclerosis cases (16.7%). The difference between the MS and control groups in these two enterotypes was not significant. However, as regards Enterotype 2, it was significantly less in multiple sclerosis than control group (3.3% versus 35%).

This is matching with other studies that revealed that Enterotype 1 is most common enterotype in countries consuming Western type diet.[10] The Western diet is typified as rich in salt, saturated fat, protein, sugar, increased calorie load, and is associated with increasing autoimmune disease prevalence. [18,19]The Western diet is linked to increased inflammation and its components, have been shown to increase gut inflammatory cell abundance.[20]

Shridhar et al. (2015), stated that globalization has affected people's eating habits, leading many of them to consume high-fat and high-calorie foods. [9]

Mowry et al. (2012), demonstrated that RRMS patients exhibit gut microbiome dysbiosis compared to the control group. They justified that the diet plays an essential role in shaping the gut microbiome in adults. [21]

De Filippo and colleagues on 2010 identified cases from rural Africa and those from urban Europe have striking differences in the composition of their microbiota between the two groups , they observed increase abundance of *Ruminococcus* in African cases , they were restricted consumed high fiber diet vegetables compared to urban Europe case which consumed Western diet high in animal protein, in addition to the differential microbial composition between the cohorts, the rural African cases also had significantly greater amount of SCFAS than Europe cases. They found that Enterotype 3 is restricted to areas like rural Africa depending mainly on vegetables. Consequently, they revised enterotypes classification and removed Enterotype 3 from the enterotypes which comes in accordance with our study. [10]

For Enterotype 2, our results agree with others that *Prevotella*is much decreased in neurological and autoimmune diseases associated with gut dysbiosis. Miyake et al. (2015) investigated the gut microbiota of Japanese MS and healthy control cohorts and found an overall difference in the gut microbiota structure and greater interindividual gut microbiota variability with MS versus healthy control comparison. They noted that MS subjects harbored lower levels of *Prevotella.*[22] The majority of MS microbiome studies across different geographical regions (USA and Italy) have reported a reduced abundance of *Prevotella* in patients with MS versus healthy controls. [21,23,24]

A recent study analyzing duodenal biopsies from patients with MS reported that patients with active disease showed a lower abundance of*Prevotella* than healthy controls or patients in remission.[25]Two recent studies showing that fecal transfer from patients with MS, but not healthy controls, to mice increased either disease incidence or severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Based on this, they hypothesize that patients with MS exhibit a general increase in proinflammatory bacteria, rather than exhibiting an increase or decrease in a specific set of bacterial genera. There are certain bacterial genera found to be depleted *(Prevotella)* or enriched (*Akkermansia*) in multiple cohorts of MS patients from different continents. [26,27]

On the other hand, some authors debated the term "enterotype" to describe gut communities. They argue that if there is a bacterium whose increased abundance is associated with a given disease and with one enterotype cluster, then relying on the cluster membership for diagnosis may mask potentially important disease-related variation within each cluster. [8,28] Therefore, they proposed to use the term "biomarker" to describe the dominant taxon of the community rather than "enterotypes" to describe gut microbiomes.[28]Thus, further studies of individual bacterial species were still needed to determine if there are any possible correlations between the gut microbiome and the MS patients.

Studies have reported that enterotypes remain unchanged in both short- and long-term dietary studies, even when randomized to a high-fiber diet for 6 months. [7,29-31] Thus, enterotypes can reflect the dietary pattern of the patients and may guide in their management.

As regards the disease manifestations and severity of our patients, there were no significant correlation between it and different bacteria and enterotypes. These results cannot be compared with previous studies as only one stool sample was collected from each patient.

Conclusion: Enterotype 1 was the most common enterotype detected in MS and control cases. Although Enterotype 2 was significantly less in multiple sclerosis patients, collapsing the whole microbiome variations into dominant enterotypes was not appropriate to identify disease association or to be used as a disease biomarker. However, this enterotype can be used as biomarker for dietary pattern of cases.

DISCLOSURE

FINANCIAL DISCLOSURE

No financial support was received for this research.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Sameh Said : Participated in draft writing and critically reviewed the manuscript.

Shwikar Mahmoud Ahmed : Performed the lab work, analysis and interpretation of the gut microbiome PCR results. Participated in draft writing. Critically reviewed the manuscript.

Mona Hamdy : Performed statistical analysis for the results.

Ahmed Elsayed Ibrahim: Research assistant participated in in data collection and statistical analysis for the results.

Richard Wani: Responsible for recruitment and management of patients. Participated in data collection and draft writing.

Jaidaa Mekky : Participated in draft writing and critically reviewed the manuscript.

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Table (1): Primer sequences and different bacteria types

Bacteria	**Primer Name**	**Primer Sequence (5'-3')**
Total bacteria	*UnivF UnivR*	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT
Bacteroides	B3F B3R	CGATGGATAGGGGTTCTGAGAGGA GCTGGCACGGAGTTAGCCGA
Prevotella	*PrevF PrevR*	CACCAAGGCGACGATCA GGATAACGCCYGGACCT
Ruminococcus	*Rflbr730F Clep866mR*	GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA

Table (2): Clinical Characteristics of MS Patients.

MS	**MS Cases (30)**	**MS Cases (30)**
	No.	**%**
Positive Family History	5	9.1
Constipation	3	10
Mean of Disease Duration (years)	5.4	5.4
Mean Relapse Rate	1.4	1.4
EDSS mean	3	3
<1	20	66.7
>=1	10	33.3
Sensory Symptoms	25	83.3
Motor Symptoms	20	66.7
Optic Neuritis	19	63.3
Cognitive	6	20
CSF oligoclonal band	3	10

Table (3): Comparison between the two study groups according to the Enterotypes.

Enterotypes	**MS**	**Control**
Enterotype 1	24 (80%)	13(65%)
Enterotype 2	1 (3.3%)*****	7 (35%)*****
Enterotype 3	5 (16.7%)	0 (0%)

Enterotypes	**MS**	**Control**
Total	**30 (100%)**	**20 (100%)**
Statistical test	**Monte Carlo X^2 = 11.219**	**Monte Carlo X^2 = 11.219**
P value	**0.002***	**0.002***

Table (4): Comparison between the MS patients with different Enterotypes.

RRMS patients	**Enterotype 1**	**Enterotype 2**	**Enterotype 3**	**Statistical test**	**P value**
No.	24	1*	5	X^2= 6.75	0.009*
Male No. (%)	11 (45.83%)	1	1 (20%)	Monte Carlo X^2 = 2.477	0.24
Female No. (%)	13 (54.17%)	0	4 (80%)		
Mean Age (SD)	32 (4.35)	31	28.8 (2.95)	KW X^2 = 2.562	0.266
Age range	25 - 40	31	27 - 34		
Mean Age of onset (SD)	26.27 (5.22)	25	25 (2.74)	KW X^2 = 0.432	0.805
Mean Disease Duration (SD)	5.73 y (3.28)	6 y	3.8 (2.28)	KW X^2 = 1.624	0.444
EDSS					
<1	8 (33.33%)	1	1 (20%)		
>=1	0	0	1 (20%)		
Sensory Symptoms	20 (83.33%)	1	4 (80%)	Monte Carlo X^2 = 0.24	1
Motor Symptoms	14 (58.33%)	1	4 (80%)	Monte Carlo X^2 = 1.435	0.762
Optic neuritis	16 (66.67%)	1	2 (40%)	Monte Carlo X^2 = 1.866	0.58
Cognitive	6 (25%)	0	0	Monte Carlo X^2 = 1.875	0.469
Constipation	2 (8.33%)	0	1 (20%)	Monte Carlo X^2 = 0.741	1
CSF Oligoclonal Band	3 (12.5%)	0	0	Monte Carlo X^2 = 0.833	0.666

KW= Kruskal Wallis test

Table (5): Comparison between the control group with different Enterotypes.

Control	**Enterotype 1**	**Enterotype 2**	**Enterotype 3**	**Statistical test**	**P value**
No.	13(65%)	7 (35%)	0 (0%)		
Mean Age (SD)	31.2 (6.5)	34.14 (3.89)	0	MW Z=-1.075	0.282
Age range	23 - 40	28 - 40	0		

Control	Enterotype 1	Enterotype 2	Enterotype 3	Statistical test	P value
Male No. (%)	5 (38.5%)	4 (57.14%)	0	Fisher's exact	0.642
Female No. (%)	8(61.5%)	3 (42.86%)	0		

MW= Mann-Whitney U test