Gut Microbiota in Type 2 Diabetes Individuals and Correlation with Monocyte Chemoattractant Protein1 and Interferon Gamma from Patients Attending a Tertiary Care Centre in Chennai, India

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) and obesity are associated with changes in gut microbiota and characterized by chronic low-grade inflammation. Monocyte chemoattractant protein-1 (MCP-1) and interferon gamma (IFNγ) are proinflammatory cytokines which play an important role in the development of T2DM. We undertook this study to analyze the gut microbiota of T2DM and nondiabetic subjects and to determine the profile of MCP 1 and IFNγ in the same subjects attending a tertiary care center in Chennai, Tamil Nadu, India. Methods: The study included 30 subjects with clinical details. Stool and blood samples were collected from all the subjects. DNA was extracted from fecal samples and polymerase chain reaction was done using fusion primers. Metagenomic analysis was performed using ion torrent sequencing. The reads obtained were in FASTA format and reported as operational taxonomic units. Human MCP 1 and IFNγ enzyme linked immunosorbent assay (ELISA) were performed for 23 serum samples. Results: The study consisted of 30 subjects; 17 were T2DM and 13 were nondiabetics. The gut microbiota among T2DM consisted predominantly of Gram negative bacteria; Escherichia and Prevotella, when compared with the nondiabetic group with predominantly Gram positive organisms such as Faecalibacterium, Eubacterium, and Bifidobacterium. The mean MCP-1 values in the diabetic group were 232.8 pg/ml and in the nondiabetic group 170.84 pg/ml. IFNγ (mean 385.5 pg/ml) was raised in glycated hemoglobin (HbA1c) group of 6.5–7.5% which was statistically significant. Association of Escherichia with T2DM and association of Bifidobacteria in the nondiabetics were also statistically significant. Conclusion: Escherichia counts were elevated in T2DM with HbA1c of 6.5–8.5% which was statistically significant suggesting that lipopolysaccharides present in the cell wall of Gram-negative bacteria may be responsible for low-grade inflammation as evidenced by elevated MCP-1 and IFNγ levels in T2DM with the same HbA1c levels.

Key words: Gut microbiota, glycated hemoglobin, interferon gamma, monocyte chemoattractant protein-1, metagenomic sequencing, nondiabetes, type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder caused by a combination of hereditary and environmental

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Obesity is the primary cause for obesity-linked insulin resistance. Obesity and diabetes mellitus are characterized by a state of low grade inflammation. As of 2014, 387 million people globally are diabetic and more than 1.9 billion are overweight of which 600 million are obese. India accounts for 66.84 million diabetics and stands second to China which accounts for 96.28 million. Recently, changes in gut microbiota have been implicated in the development of T2DM and obesity. Gut microbiota affects energy balance, glucose metabolism, and low-grade inflammation associated with obesity and T2DM.

We undertook this study to determine the composition of gut microbiota in T2DM and nondiabetic subjects and to identify alterations in gut microbiota using metagenomic sequencing as well as to explore the role of monocyte chemoattractant protein-1 (MCP-1) and interferon gamma (IFNγ) in contributing to low-grade chronic inflammation in T2DM.

**METHODS**

**Subjects and sample collection**

This study protocol was approved by the Ethical Committee of Sri Ramachandra University. Informed written consent was obtained from all the subjects enrolled in the study. The study included 17 subjects diagnosed with T2DM and 13 nondiabetics as confirmed by the physician and glycated hemoglobin (HbA1c).

Details such as past history, personal and treatment history, HbA1c, fasting blood sugar, postprandial blood sugar, body mass index (BMI), and diet of the participants were recorded in a proforma. The inclusion criteria for the study were subjects with T2DM whose HbA1c was > 6.5% and nondiabetics willing to participate with HbA1c < 6.5%. Exclusion criteria included type 1 diabetes mellitus, personal history of smoking and alcohol consumption, treatment with antibiotics in the preceding 3 months, chronic inflammatory diseases, use of corticosteroids, breastfeeding or pregnancy.

**Sample collection**

A total of 30 fecal samples (17 T2DM and 13 nondiabetics) were collected in a sterile container, brought to the laboratory, and kept at +4°C until processing. In addition, 23 (12 T2DM and 11 nondiabetics) serum samples were collected from the same subjects in gel-based, yellow-capped vacutainer tubes (Becton and Dickinson, USA). All samples were coded giving a specific laboratory coding.

T2DM subjects were categorized according to HbA1c as (6.5–7.5% n = 7, 7.6–8.5% n = 5, and >8.5% n = 5). BMI was also used to categorize the T2DM subjects as normal BMI (18–23) n = 1, overweight (23–28) n = 5, and obese (>28) n = 11.

Nondiabetic subjects with normal BMI were n = 4, overweight n = 5, and obese n = 4.

**Fecal DNA extraction**

DNA extraction was done using QIAGEN Fast DNA Stool Mini Kit (Qiagen, Germany) as per the manufacturer’s protocol.

Briefly, 220 mg (approximately) of stool was taken in a microcentrifuge tube to which 1 ml of InhibitEX buffer was added and vortexed until samples were thoroughly homogenized and centrifuged at 14,000 rotations per minute (rpm) for 1 min. Proteinase K (25 μl) was added in a new centrifuge tube to which 600 μl of the supernatant from the previous step was added. Lysis buffer (600 μl) was added to the above mixture, vortexed for 15 s, and incubated at 70°C for 10 min. Ethanol (600 μl) was added to the lysate, vortexed, and centrifuged at 14,000 rpm for 1 min. The supernatant was transferred to spin column, centrifuged until all the lysate was loaded on the column. Wash buffer (500 μl) 1 and 2 were added subsequently, centrifuged at 14,000 rpm for 1 and 3 min, respectively. The spin column was transferred to a new collection tube and spun at 14,000 rpm for 2 min. Spin column was then transferred to a new microcentrifuge tube, and 200 μl of elution buffer was added, incubated at room temperature for 3 min, and centrifuged at 14,000 rpm for 1 min for obtaining DNA. The eluted DNA was aliquoted and stored immediately at −20°C until polymerase chain reaction (PCR) was performed.

**Polymerase chain reaction amplification**

PCR was performed for the extracted DNA using Qiagen multiplex PCR mastermix (Qiagen) with Fusion primers [Tables 1], and PCR carried out [Table 2a and 2b].

**Polymerase chain reaction product purification**

PCR product purification was done by carboxylic coated magnetic beads. The purified PCR product size and quantity were verified by Agilent bioanalyzer 2100. Based on the result obtained the PCR product was diluted to 26 pmol/μl and used for metagenomic sequencing.

**Metagenomic sequencing**

Metagenomic sequencing was performed for all the fecal sample’s PCR products using Ion Torrent PGM (Ion Torrent Personal Genome Machine) sequencing platform.
Methodology
Adapters were ligated to the ends of DNA fragments during PCR. The fragments were clonally amplified onto ion sphere particles. The ion sphere particle coated with the template was applied to Ion chip and deposited in chip wells which were then loaded into PGM machine and sequencing run setup.

Analysis
NCBI 16S database was chosen for analysis of the reads. Reads <60 bases were excluded, and percentage of the match for grouping similar sequence levels was set at 90%. The reads were clustered into operational taxonomic units at 90% level. The results were displayed in the form of a pie chart and used for further analysis.

Enzyme linked immunosorbent assay
Enzyme-linked immunosorbent assay (ELISA) was performed for MCP-1 and IFNγ using Peprotech mini ELISA development kit (Peprotech, NJ, USA) as per manufacturer’s protocol for all the available 23 serum samples. Readings for MCP-1 and IFNγ were taken using an ELISA plate reader (Thermoscientific Multiskan EX) monitored at 5 min interval for 20 min and 10 min, respectively, at 405 nm with reference range at 650 nm. The quantity of the test sample was measured using the curve generated by the standard concentrations of each calibrator using the graph provided by the manufacturer.

Statistical analysis
Statistical analysis was done using SPSS software version 16.0 (IBM SPSS Pvt. Ltd.,) and R software 3.0.2. (The R foundation).

Results
Among the subjects enrolled 14 were male and 16 were female, the age ranged from 22 to 70 years. T2DM subjects were grouped based on HbA1c into three categories - 6.5–7.5% (n = 7), 7.6–8.5% (n = 5), and >8.5% (n = 5); the BMI ranged from 20.2 to 38.8.

Nondiabetics (HbA1c < 6.5%) were categorized based on BMI into normal weight (n = 4), overweight (n = 5), and obese (n = 4) [Tables 3a and b].

Most of the subjects were from Chennai (67%), and neighboring districts in Tamil Nadu (17%), three from Andhra Pradesh (10%), and two from North-East India (6%). All the subjects were nonsmokers and nonalcoholics, with no history of intake of antibiotics/probiotics in the preceding 3 months and gave no history of steroid therapy.
Analysis of gut microbiota at the phyla level showed Firmicutes (49%), Proteobacteria (32%), Bacteroidetes (17%), and Actinobacteria (2%) among T2DM and Firmicutes (49%) Proteobacteria (26%), Bacteroidetes (18%), and Actinobacteria (7%) among nondiabetics [Figure 1a and b]. However, there were differences observed between T2DM and nondiabetics at the genus level. The predominant phyla encountered in T2DM was Proteobacteria, composed of Escherichia, Citrobacter, and Acinetobacter. Gram positive bacteria such as Firmicutes which comprises of Faecalibacterium, Eubacterium, Clostridium, and Bifidobacterium of Actinobacteria were encountered in nondiabetics. Among the T2DM Escherichia (Gram-negative bacteria) was predominant and in the nondiabetics Faecalibacterium followed by Eubacterium and Bifidobacterium were predominant [Table 4]. Escherichia was part of gut microbiota in 58.8% of T2DM subjects and 7.7% of nondiabetics (P = 0.007, odds ratio = 7.6). Apart from Escherichia, Lactobacillus was also found to be 35.3% in T2DM and 7.7% in nondiabetics. Bifidobacterium was found predominantly among the nondiabetic 38.5% (P = 0.04 using Fisher’s Exact Chi square test) [Table 5].

We then stratified the gut microbiota based on HbA1c as 6.5–7.5%, 7.6–8.5% and > 8.5%. Analysis of genera based on HbA1c showed that Gram-negative bacteria were predominant among T2DM with HbA1c between 6.5%–7.5% and 7.6%–8.5% [Figure 2].

Chi-square test has shown a statistical significance for the genera Escherichia among T2DM and Bifidobacteria among nondiabetics.

We also applied principal component analysis (PCA) using R software (version 3.0.2) to observe the intercorrelation among bacterial genera across diabetics and nondiabetics. The biplot of 1st PCA (27% variation explained) and 2nd PCA (18% variation explained) generated is shown in Figure 3a and b. The biplot indicates that Faecalibacterium and Bifidobacterium are very strongly associated and form a cluster along with Eubacterium. On the other hand,
Gut microbiota and their role in obesity and T2DM are being extensively studied.\[2,12\] There have been attempts to develop identity marker for gut microbiota among subjects with T2DM or other metabolic disorders. However, the role of gut microbiota in T2DM, especially in relation to HbA1c, has not been well-studied. This is a preliminary study conducted using fecal samples obtained from subjects with HbA1c ranging from 6.5–7.5% and > 8.5%. MCP-1 values were higher in both HbA1c groups, but this was not statistically significant (Wilcoxon rank sum test) [Figure 4b].

Analysis of MCP-1 [Figure 5] in T2DM subjects with HbA1c of 6.5–7.5% showed a mean of 170.08 pg/ml with a range of 56.3–319.3 pg/ml in subjects with HbA1c of 7.6–8.5% and > 8.5%. MCP-1 values ranged from 87.4 to 140.9 pg/ml and 155–241.2 pg/ml with a mean of 115.2 and 198.35 pg/ml, respectively. In the nondiabetics, MCP-1 value ranged from 66.9 to 236.8 pg/ml with a mean of 132.3 pg/ml. Although MCP-1 values were higher in both HbA1c groups, it was not statistically significant ($P = 0.16$ and 0.07, respectively).

We then compared IFN$\gamma$ with HbA1c and found that IFN$\gamma$ value, mean, and range of 385.5 pg/ml and 414.8–984.4 were higher in T2DM group of 6.5–7.5% which was statistically significant using Student’s $t$-test ($P = 0.013$). IFN$\gamma$ values across the other two HbA1c groups and nondiabetics were below detectable level [Figure 6].

**DISCUSSION**

Gut microbiota and their role in obesity and T2DM are being extensively studied.\[2,12\] There have been attempts to develop identity marker for gut microbiota among subjects with T2DM or other metabolic disorders. However, the role of gut microbiota in T2DM, especially in relation to HbA1c, has not been well-studied. This is a preliminary study conducted using fecal samples obtained from subjects with HbA1c ranging from 6.5 to > 8.5% and nondiabetics with HbA1c of < 6.5%. The gut microbial composition

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**Table 5: Correlation of *Escherichia* and *Bifidobacterium* among type 2 diabetes mellitus and nondiabetics**

| Bacteria       | T2DM (%) | Nondiabetics (%) | $P$  |
|----------------|----------|------------------|------|
| *Escherichia*+ | 10 (58.8)| 1 (7.7)          | 0.007|
| *Escherichia*− | 7 (41.2) | 12 (92.3)        |      |
| *Bifidobacteria*+ | 1 (5.9) | 5 (38.5)         | 0.04 |
| *Bifidobacteria*− | 16 (94.1)| 8 (61.5)         |      |

T2DM: Type 2 diabetes mellitus
Pushpanathan, et al.: Gut microbiota in T2DM and correlation with MCP-1 and IFN-γ

of the subjects was studied by metagenomic sequencing targeting the V6 region of the 16S rRNA gene. The gut microbiota has also been correlated with BMI and inflammatory markers such as MCP-1 and IFNγ.

No significant difference in bacterial phyla was observed in T2DM and nondiabetics. Firmicutes predominated in both groups 50% T2DM and 53% nondiabetics followed by Proteobacteria (33 and 28%) and Bacteroidetes (17 and 19%), respectively.

However, the presence of Proteobacteria is different from previous reports. Phylum Proteobacteria was slightly abundant in T2DM than in nondiabetics, and this is a potential diagnostic marker of dysbiosis and risk of disease.[13]

*Escherichia* was predominant in T2DM patients and positively correlated with HbA1c and BMI. Enterobacteriaceae are reported to be a major cause of mortality and morbidity in T2DM.[14] In addition, a threefold increased risk of complications due to *Escherichia* and other Enterobacteriaceae has been observed.[15] Furthermore, intake of artificial sweeteners impairs the glucose control and can elevate the Proteobacteria levels.[16,17]

Certain species of *Lactobacillus* may contribute to chronic inflammation in T2DM. We observed 35.3% subjects of T2DM had *Lactobacillus*. Several strains of *Lactobacillus* are administered as probiotics, for example, *Lactobacillus paracasei* ssp. paracasei F19 and *Lactobacillus gasseri* SBT2055 are known to reduce visceral and subcutaneous fat. Certain species may contribute to inflammation. A limitation of the study was that we were unable to speciate *Lactobacillus*.

*Bifidobacterium*, a dominant member of the intestinal microbiota and probiotic strain of the phylum Actinobacteria, was increased in the nondiabetics than in T2DM. It has been reported that endotoxaemia negatively correlates with *Bifidobacterium* and positively correlates with improved glucose tolerance, glucose-induced insulin secretion, decreased endotoxemia, and adipose tissue proinflammatory cytokines.[18] This is because *Bifidobacterium* improves mucosal barrier function thereby decreasing the endotoxin levels.[19,20]
In our study, Gram-negative bacteria were found to be higher than Gram-positive bacteria in T2DM when compared with nondiabetics. Lipopolysaccharide (LPS) is a major component of the Gram-negative cell wall, and the human gut serves as a reservoir of LPS. LPS levels were low in healthy subjects suggesting that it is absorbed at a low rate in the gut. Increased level of Gram-negative gut bacteria correlates with high circulating LPS and high insulin resistance in T2DM. Studies have shown that high fat feeding augments LPS concentrations sufficient to increase body weight, fasting glycemia, and inflammation. Further, LPS was identified as a triggering factor in the early development of metabolic diseases and is also a strong stimulator of several cytokines that cause insulin resistance and metabolic endotoxemia. Normally, the intestinal epithelium acts as a continuous barrier to avoid LPS translocation; yet some endogenous or exogenous events may alter this protective function.

When we analyzed the correlation of gut microbiota with BMI alone, we found that Proteobacteria was increased in obese subjects and absent in subjects with normal BMI among T2DM. Escherichia coli positively correlated with obese T2DM subjects.

In our study, we found that MCP-1 was elevated across HbA1c of 6.5–7.5% and > 8.5% when compared with nondiabetics. We have previously reported MCP-1 increases by 37.3 pg/ml for every 1% increase in HbA1c among patients with periodontal disease and T2DM. IFNγ is another proinflammatory cytokine which plays a crucial role in inflammatory disease. The glucotoxic microenvironment created in diabetes may influence the cell to cell signaling capabilities of cytokines. IFNγ may contribute to complications occurring in T2DM and also play a protective role in healthy subjects. Furthermore, increased IFNγ concentrations delay the development of microvascular complications in T2DM. Al-Shukaili et al. reported an increase in IFNγ in T2DM patients compared with normal patients. In our study, IFNγ levels were significantly increased in T2DM subjects with HbA1c of 6.5–7.5% indicating that it rises early in the disease-causing inflammation.

The composition of gut microbiota in our study subjects differed from those previously reported in the West. These differences may be due to the influence of diet and need to be studied prospectively.

We found similar genera between three subjects, two of whom were nondiabetic and one T2DM. All the subjects were unrelated to each other and came from different background. Twins and their families have reported an identical gut microbiota. In our study since all were unrelated, probably similarities in diet may have resulted in an identical gut microbiota.

To the best of our knowledge, this is the first study to profile gut microbiota in T2DM subjects and to compare them with nondiabetics as well as correlate with MCP-1 and IFNγ.

**Conclusion**

*Escherichia* counts were elevated in T2DM with HbA1c of 6.5–8.5% which was statistically significant suggesting that lipopolysaccharides present in the cell wall of Gram-negative bacteria may be responsible for low-grade inflammation as evidenced by elevated MCP-1 and IFNγ levels in T2DM with the same HbA1c levels. The gut microbiota of nondiabetics is characterized by predominance of *Bifidobacteria* and other Gram-positive bacteria.

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**Conflicts of interest**

There are no conflicts of interest.

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