Brief communication

Molecular typing of fecal eukaryotic microbiota of human infants and their respective mothers

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The micro-eukaryotic diversity from the human gut was investigated using universal primers directed towards *18S rRNA* gene, fecal samples being the source of DNA. The subjects in this study included two breast-fed and two formula-milk-fed infants and their mothers. The study revealed that the infants did not seem to harbour any micro-eukaryotes in their gut. In contrast, there were distinct eukaryotic microbiota present in the mothers. The investigation is the first of its kind in the comparative study of the human feces to reveal the presence of micro-eukaryotic diversity variance in infants and adults from the Indian subcontinent. The micro-eukaryotes encountered during the investigation include known gut colonizers like *Blastocystis* and some fungi species. Some of these micro-eukaryotes have been speculated to be involved in clinical manifestations of various diseases. The study is an attempt to highlight the importance of micro-eukaryotes in the human gut.

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1. Introduction

The human gut is a natural habitat for a large community of microorganisms that have co-evolved with humans. This large community of microorganisms is now known to be composed of a vast number of bacteria, fungi and protozoa (Finegold *et al.* 1983). Some microbes are known to be closely associated with the human gut, while others are transient. The microbes present in the gut form a large percentage of fecal mass, sometimes as much as 60% of the total fecal mass. This amounts to a cell density of 10^{11} to 10^{12} cells per gram of luminal contents (Suau *et al.* 1999). It is obvious that such large number warrant a closer look into the interaction that these microbes have with their human host. Recent studies have shown that a plethora of important factors are controlled directly or indirectly by the microbes resident or transient within the human gut (Francisco and Juan 2003). The resident microbes, for example, are known to modulate the immune system by interactions with the Peyer's patches (Francisco and Juan 2003). Gut bacteria are known to compete for space and food and thus prevent colonization of harmful or pathogenic bacteria. It has also been observed that there is a disturbed microflora in case of diseases of the intestine such as irritable bowel syndrome or Crohn's disease (Duchmann *et al.* 1995; Macpherson *et al*. 1996). Recently microbes have also been implicated in the development of colon cancer (Stevens and Hume 1998). This is due to the fact that not all microbes in the gut are beneficial in nature, performing routine functions like conversion of carbohydrates into short-chain fatty acid (SCFA) or conversion of dietary and endogenous nitrogenous products to ammonia or production of vitamins. Some microbes are capable of breaking down dietary products into toxic substances like phenols, thiols and indols (Stevens and Hume 1998). These observations have led to recent attempts in introducing beneficial microbiota into the human gut through consumption of probiotic foods.

Keywords. 18S rRNA library; gastrointestinal tract; micro-eukaryotic diversity

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In natural ecosystems, bacteria are abundant and it is widely believed that they have an indefinite lifespan. Their numbers are controlled either by competition, lysis by phages and finally predation or inhibition by eukaryotes like amoeba and fungi respectively. In light of this fact, it is of paramount importance to study microbiome of the gut. This is because the establishment and dynamics of micro-eukaryotes in the human gut could be the major modulators of bacterial numbers and types. This has major implications in the health and well-being of the human host. To the best of our knowledge, there are very few studies to understand the gut micro-eukaryotic diversity using molecular methods (Kuhbacher *et al.* 2006; Scanlan PD and Marchesi JR 2008). In the present study the human gut eukaryotic microbiota from fecal samples obtained from four infants and their respective mothers was investigated using the culture-independent molecular method.

2. Material and methods

2.1 *PCR and library construction*

The fresh stool samples from four infants and their respective mothers on days 0, 7 and 30 after delivery were collected and immediately transported to the laboratory in anaerobic transport medium on ice. Among these four infants, two were full-term, vaginally delivered and breast-fed, while other two were full-term cesarean-section-delivered. The cesarean-section-delivered babies had received initial formula milk supplementation along with breast milk. All the subjects were healthy at the time of the sample collection and were not on any antibiotics. Upon arrival, the samples were subjected to DNA extraction using Qiagen stool DNA extraction kit. A slight modification that employed bead beating was used to ensure the complete lysis of fungi and protozoa if present. The resultant DNA was checked for purity and quality on an agarose gel and was subsequently quantified. The DNA was used as template for PCR using eukaryotic 18S rRNA gene-specific primers: Euk1AF (5′- CTG GTT GAT CCT GCC AG −3′) and EUK516R (5′- ACC AGA CTT GCC CTC C −3′) (Wilms *et al.* 2006). All samples were taken in triplicates in order to minimize PCR bias. PCR was performed as described earlier (Wilms *et al.* 2006). The PCR products were verified through 1 % agarose gel electrophoresis. It was observed that the infant samples did not yield any visible PCR product on the agarose gel. In order to check the DNA for PCR quality, universal 16S rDNA primers were used (Weisburg *et al.* 1991). This resulted in a positive amplification, which meant the DNA was of PCR quality but could be devoid of micro-eukaryotic DNA. In order to check for DNA extraction efficiency, a known amount of fungal and yeast culture was inoculated in the feces. The DNA extraction and PCR amplification were repeated, which yielded a positive result; thus it was inferred that the infant feces were devoid of any PCR-detectable micro-eukaryotes. The triplicates of each positive sample of the mothers were then pooled and subsequently ligated in pGEM-T vector as per the manufacturer's protocol. The ligated PCR product was used to transform *Escherichia coli* JM-109 chemical competent cells. The transformed cells were plated on LB plates containing ampicillin, X-Gal and IPTG. The resultant clone library was screened for positive clones using vector-specific primers. The screened clones that were positive for the insert were inoculated in LB containing ampicillin, and plasmid extraction was carried out using MONTAGE mini prep kit as per the manufacturer's instructions. Sequencing was performed on an ABI 3730 using Big Dye chemistry version 3.1. The inserts were sequenced from both ends using vector-specific primers. The resultant sequences were then assembled using Chromas Pro software and a chimera check was performed using publicly available software, Check Chimera and Bellerophone. After excluding the two detected chimeras the remaining 158 sequences of approximately 560 base pair length were subsequently used for statistical and phylogenetic analysis.

2.2 *Accession numbers*

All the sequences obtained in the present study were deposited to NCBI sequence data base with accession numbers EU326530 to EU326687.

3. Results

3.1 *Phylogenetic analysis of micro-eukaryotes*

The nucleotide sequences generated from the clone library were analysed using DOTUR (Schloss and Handelsman 2005), for unique OTU's (operational taxonomic units), where a sequence similarity of less than 97% between clones was used as a cut-off value. Based on these conditions 10 unique phylotypes were observed. Upon increasing the cut-off value to 98%, 12 distinct phylotypes were observed. In order to define a rigorous parameter the unique phylotypes resulting from the 97% cut-off limit were used. A representative sequence from each phylotype was used to query for similar sequences in public databases, using the NCBI BLAST algorithm. Initial results using BLAST with default parameters revealed that the library mostly consisted of *Blastocystis*, *Saccharomyces* and *Candida sp*. However, there were representation of 18S rDNA sequence of some plant species like *Nicotiana tabacum* and *Hedychium spicatum* (table 1). The plant species that were represented in the library were inferred to be from the remnants of the undigested plants ingested by the

Table 1. Summary of micro-eukaryotic organisms present in feces of healthy mother

Taxonomic Group	Accession Number	Group	Number Of Clones
Blastocystis sp.	AB107965.1	Stramenopile	64
Saccharomyces sp.	Z75578.1	Fungi	58
C.albicans	DQ515959	Fungi	17
Nicotiana tabacum	AY079155.1	Plantae	7
Cicer arietinum	AJ577394.1	Plantae	5
Hedychium spicatum	AB047728.1	Viridiplantae	2
Sporobolomyces ogasawarensis	AB035718	Fungi	1
Kluyveromyces hubeiensis	AY325966.1	Fungi	1
Uncultured Banisveld	EU091876.1	Fungi	1
M. racemosus	X54863.1	Fungi	1
Lycogala flavofuscum	X54863	Fungi	1

mothers. For further phylogenetic analysis the representative clone from each phylotypes were used to construct a phylogenetic tree using PHYLIP (Felsenstein 1989), with 1000 replicates (figure 1). The resultant tree revealed the

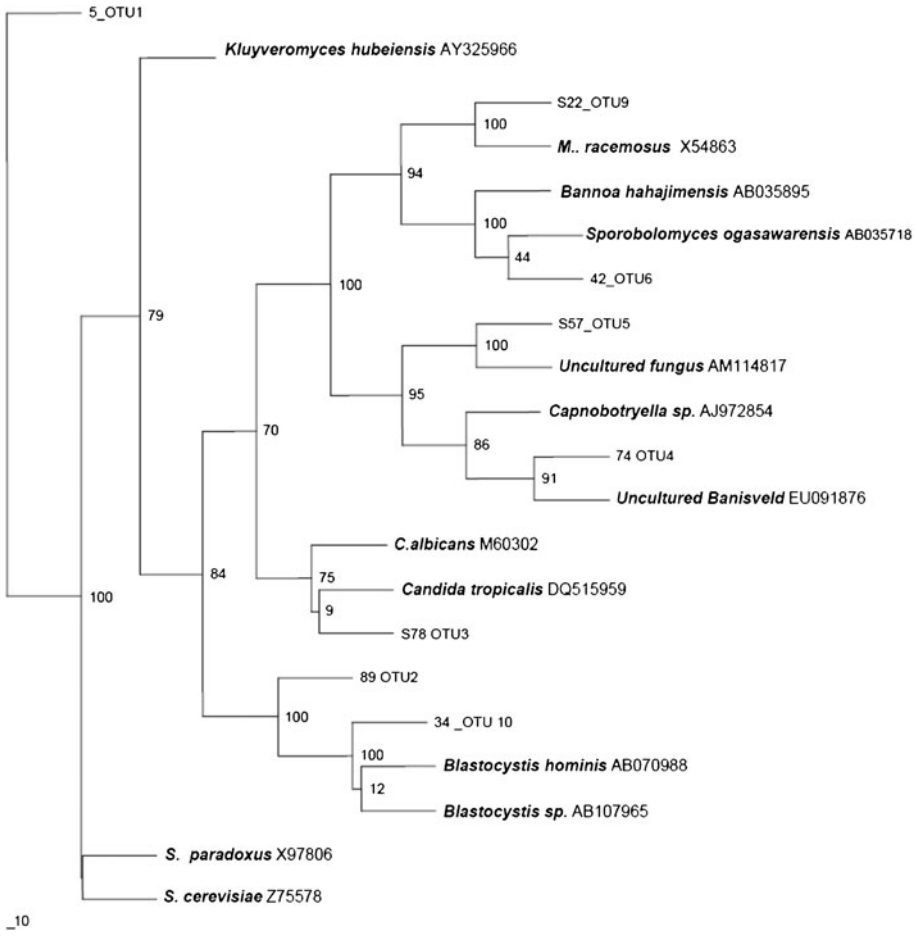

Figure 1. Un-rooted phylogenetic tree of 18S rDNA sequences obtained from the healthy mother feces 18S rDNA library with their Related Established Species. The tree was constructed in PHYLIP software version (Felsentein1993) using maximum parsimony method. The scale bar represents 10 substitutions per nucleotide position. Bootstrap values (1000 replicates) are shown at the nodes.

presence of *Blastocystis* in the library to be distinct from the ones deposited at NCBI GeneBank.

3.2 *Statistical analysis of library clones*

The coverage of clone libraries was calculated to be 96.8% with the formula [1−(n/N)] as described by Goods, where n is the number of phylotypes represented by one clone and N is the total number of clones (Goods 1953). In order to enumerate library coverage, rarefaction analysis (Krebs 1998), Shannon index, the reciprocal of Simpson's index (Magurran 1996) and Chao-1 (Chao 1987) estimator were chosen to characterize the microbial diversity of the sample. Rarefaction compares observed species richness between sites, treatments or habitats that have been unequally sampled (Hughes *et al.* 2001). The rarefaction curve was produced using the software program, Analytic Rarefaction1.3 (*http://www.uga.edu/~strata/software/index.html*). It was observed the sequences generated by 158 clones sufficiently represented the diversity in the samples.

The Shannon index (H^0), a general diversity index, which is positively correlated with species richness and evenness, and is more sensitive to change in abundance of rare species, was calculated to be 1.3851 (Magurran 1996; Hughes *et al.* 2001).

The Simpson's index (D) is a dominance measure and shows the probability that two clones chosen at random are from the same species. The value of this has come to be 0.312183. The index is weighted towards the abundance of the commonest species (Hughes *et al.* 2001; Singleton *et al.* 2001).

4. Discussion

The investigation reveals that there is a marked difference in the micro-eukaryotic diversity in feces obtained from infants and adults. This has major implications in terms of the study of microbiome of the human gut, its establishment and dynamics. Previous studies on the human gut flora have reported that, during the first week after birth, the fore stomach is colonized by a variety of bacteria. These include species of *Escherichia coli*, *Lactobacilli* and *Streptococci* when the infant is breast-fed. Once the infant in weaned, there is establishment of an extremely complex microbiota that comprises transient and resident bacteria. The population dynamics is controlled by variety of factors, which includes host diet and host immune response, among others. However, most of these studies have been limited to study of bacteria and not to micro-eukaryotes. However, the role and establishment of micro-eukaryotes has been well studied in the case of ruminant cattle (Hobson and Wallace 1982). Here it has been noted that resident protozoa are much less in number compared to the bacterial flora. Typically they are at densities of 10^4 to 10^5/g and occupy an equal volume of the fore stomach contents. The predominant species were anaerobic ciliates that are capable of fermenting carbohydrates, storing starch, digesting protein, hydrogenating fatty acids and regulating the numbers of bacteria (Ogimoto and Imai 1981; Hobson and Wallace 1982; Prins 1991; Forsburg *et al.* 1997). They are known to contribute relatively little towards carbohydrate fermentation, but are capable of storing starch, which is converted to protein for subsequent digestion during passage through the abdomen and mid-gut. The uptake of starch and sugars by protozoa is known to have a stabilizing effect in ruminants fed high-grain diets. The rapid production of SCFA and lactic acid in such ruminants could result in the ulceration of the fore stomach and systemic acidosis and dehydration. Another important observation is the defaunation of the rumen impaired the absorption of calcium, magnesium and phosphorus and changed the peptide patterns of duodenal digests. The protozoa were shown to play an integral role in digestion in ruminants, and thus would definitely have a role to play in the case of humans. One of the important roles could be to control bacterial numbers by predation or inhibition in the human gut.

During the analysis the only micro-eukaryote close to a ciliate protozoan was *Blastocystis* species, which was represented in 40% of the total clones used for analysis. The taxonomic classification as well as the pathogenicity of *Blastocystis hominis* are, however, controversial. It has been considered by many as yeast, fungi or protozoon; data from sequence analysis of the complete SSU rRNA gene has placed it within an informal group, the stramenopiles (Silberman *et al.* 1996). This is a heterogeneous group of unicellular and multicellular protists, including brown algae, diatoms, water moulds, etc. The organism is considered by many as capable of causing diarrhea, but a recent case control study of 99 individuals stool-positive for *B. hominis.* and 193 matched controls did not demonstrate pathogenicity based on the association with the development of gastrointestinal symptoms or pathologic findings on endoscopic examination (Chen *et al*. 2003). However, it is associated frequently with diarrhea in immune-suppressed patients with AIDS and organ transplant patients; it has been reported in renal transplant patients in India (Rao *et al.* 2003). In Thailand, 2 of 58 (3.4%) of HIV+children hospitalized for diarrhea were positive for the parasite (Leelayoova *et al.* 2001). *Blastocystis* have been reported to be the most commonly occurring micro-eukaryote in human feces. The presence of *Blastocystis* has been linked to a number of gut-related diseases. It could be possible that some of the diseases could be the outcome of predation of beneficial bacteria by *Blastocystis* in light of the similar observations in ruminant cattle and their communalistic protozoa. A case in point is the decrease in bacterial numbers that has been observed in gut diseases like IBS and pouchitis.

The second most abundant micro-eukaryotes in the library were from the fungal kingdom. The *Saccharomyces sp.* had a representation close to 36% in the library, which is very similar to *Blastocystis* species. From currently available literature, anaerobic fungi have been reported at concentrations of 10^3 to 10^5 zoospores/g of fluid digesta in animals on high-fiber diets (Stevens and Hume 1998). They are known to contain relatively high concentrations of protein with an amino acid composition similar to that of the alfalfa. They are presumed to be an important source of nutrients for ruminants on low-quality diets. They are like protozoa that can store starch and synthesize protein and appear to aid in the absorption of magnesium and phosphorus. Fungi are also known to be capable of synthesizing protein useful to their host. On the other hand, there are several recent reports implicating fungi in the development of allergic and immune responses. In mice, antibiotic perturbations of gastrointestinal Communities in addition to the Candida albicans supplementation had led to an increase in levels of eosinophils, mast cells, interleukin-5, interleukin-13, gamma interferon, immunoglobulin E and mucus-secreting cells in response to exposure to *Aspergillus fumigates* (Scupham *et al*. 2006). The authors of the above study had suggested that this response may be caused by the production of prostanglain-like oxylipins by the fungus. In addition, as fungi are known to produce an assortment of immune-modulating compounds, it has also been suggested that these microbes could be associated with a wide range of immune alterations.

5. Conclusion

It is to be noted that most of the current literature in the area of human gut and disease is limited to bacteria. In fact, there have been just two reports on the uncultured diversity of micro-eukaryotes from the human gut. However, there are specific reports for individual micro-eukaryotes in diseases, as in the case of *Blastocystis* and *Amoeba*. Recent culture-independent studies have revealed the defaunation of the gut to be closely associated with colonic cancer and other gut diseases. In such observations the leading hypotheses have centered on bacterial defaunation due to competition and phages. This bias could be a result of non-availability of studies on micro-eukaryotes and their importance in gut health. This emphasizes the study of micro-eukaryotes to be included in future investigations concerning the gut microbiota and its dynamics especially with respect to defaunation.

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