Characterization of Human Gut Microflora In Enteric Disease Patients Using Molecular and Biochemical Techniques

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Aims and Objectives:

1. To characterize gut microflora of normal individuals in Indian population using molecular and biochemical techniques.

a. a. Culture based methods
b. 16S r DNA profiling using genus specific primers

2. To study differences in microflora, if any, during enteric disease conditions.

3. To characterize the bacterial flora both in fecal as well as pus samples of amebic liver abscess patients.

4. To study the possible changes in cytokine profile between diseased and normal individuals

Use of cultivation methods for characterizing the large number of bacterial species that harbor the human gut is a daunting task. This study concentrated mostly on resident flora was done in two parts. **Part one was biochemical characterization of aerobic fecal flora in a few individuals group comprised of healthy and diseased individuals, as cultivation of aerobic flora is relatively simple.**

Second part was based on characterizing the bacterial flora using molecular tools. Profile of significant bacteria known to predominate in human gut (comprised mainly by anaerobic ones and few subdominant aerobic bacteria) was compared in both healthy and amebiasis patients.

Our main thrust was to develop molecular probes to make this kind of study more rapid and easier. For this, purpose 16S rRNA sequence based primers were designed. Since 16S rRNA sequence is highly conserved so it is advantageous to pick up genus specific primer sets that can amplify most of the species (gut resident ones) of genus. CLUSTAL W alignment of the sequences belonging to the different genera yielded hypervariable regions among them whereas showing conserved regions among different species belonging to the same genus. Such regions were subjected to close analysis for designing genus specific primer sets. However, for few members like *E. coli* belonging to Enterobacteriaceae family, 16S rRNA sequence based suitable primers could not be designed as they showed mismatches

with other nontargeted genera like *Shigella*. Therefore primers for *E. coli* detection were based on maltose B promoter region sequence (Wang 1996). For *Staphylococcus aureus*, methicillin resistant gene sequence based primers were used.

Characterization of aerobic fecal flora: This was carried out with stool samples collected from healthy female, two diarrhea patients (one old age female and one giardiasis adult male) and a diseased infant.

Different dilutions of fecal samples were plated on universal media (nutrient agar) and MacConkey agar media. Morphological and microscopy analysis were performed for the colonies grown on the above media. In order to identify the cultivable colonies on various differential media, specific biochemical tests were carried out. Results showed that *Aeromonas*, *Alcaligenes*,*Pseudomonas*, *E.coli*, *Citrobacter*, *Bacillus*, *Lactobacillus* and *Streptococcus* were observed in all samples except diseased infant. In infant sample, among Enterobacteriaceae family members only *E.coli* and *Proteus* were observed. Genera *Proteus*, *Serratia*, *Yersinia* and *Klebsiella* were present in healthy samples only. *Edwardsiella* and a very high titer of unidentified enterobacteriaceae members was found to be associated only with diarrhea patients. *Shigella* was present in giardiasis patient. Oxidase positive aerobic bacteria were absent in diseased infant, whereas they were present in rest of the samples.

Among gram positive bacteria, *Bacillus*, *Lactobacillus* (low titer in giardiasis patient) and *Streptococcus* were present in all four samples. In healthy female *Candida* represented a high titer, while in other samples it was absent.

Staphylococus titer was higher in diseased infant than giardiasis patient. In D1

a female diarrhea patient, a very high titer for *Sporolactobacillus* was observed whereas it was absent in other samples. The same sample also represented few unidentified gram positive bacteria. *Corynebacterium* and *Micrococcus* were specifically present in giardiasis patient.

Molecular Probes Experiments Results:

The study on diseased samples using PCR based methods indicated absence of metabolically important butyrate-producing genera; *Peptostreptococcus productus*, *Ruminococcus* in D1 and D2 whereas *Clostridium* was absent in D1. In diseased infant D3, only *Bacteroides*, *Lactobacillus*, *E.coli* and *Bifidobacterium* were present. D3 sample was from six days old infant and it is known that in infants gut flora is not rich in diversity. *Campylobacter* known to be pathgenic bacteria was present both dairrhea patients.

The results indicated substantial variation in fecal flora composition in diarrhea condition when compared with healthy controls.

Amebiasis is second most important disease ranking after malaria from the view of morbidity disease. Since amebiasis is caused by the well- known intestinal protozoan *E. histolytica*, it is still not clear of the presence or absence of any specific gut flora members makes a difference in the manifestation of the disease. The experiments were designed to study the impact of disease on the resident gut flora.

For the systematic studies, altogether four groups were made; (1). amebic liver abscess patients (ALA) with diarrhea and high fever (total 36 samples), (2). Moderately infected individuals (Mod) suffering from mild stomach problems (total 25 samples), (3). Individuals found positive for *E. histolytica* but not having any gastric ailment and not taking any antibiotic (total 11 samples) and (4). Healthy individuals not taking any antibiotic treatment (total 19 samples).

Among ALA patients, pus samples were collected from 36 patients. Out of these stool and blood samples were collected from 19 and 17 patients respectively. Blood samples were also collected from 6 healthy individuals. Sera isolated from collected blood samples were stored at -20^0 C for further experiments.

PCR was performed to detect the protozoan *E. histolytica* in DNA isolated from pus and stool samples by using UEE primers (Srivastava *et al* 2005), specific for *E. histolytica*.

Percent incidence values and statistical analysis of presence of amplicons observed for studied categories revealed significant differences in bacterial flora according to the physiological status. Healthy individuals taken in this study belonged to adult age group and were on vegetarian diet and were residents of JNU campus.

Frequency of *Lactobacillus* (P = 0.0011, OR = 31.5 & 95% CI = 2.9776 to 333.235) was significantly lower in case of asymptomatic *E. histolytica* carriers as compared to the healthy individuals, while *Pseudomonas aeruginosa* (P= 0.0024, OR= 0.0462 & 95% CI = 0.0048 to 0.448) was higher in case of asymptomatic individuals as indicated by ODDS ratio (OR) values. Frequency of *Clostridium* (P = 0.0019, OR = 8.866 & 95% CI = 2.2463 to 34.999), *Lactobacillus* (P= 0.0001,OR= 96 & 95% CI= 9.0527 to 1018.0362), *Ruminococcus* (P=0.0037, OR= 10.81 & 95% CI= 2.0478 to 57.1504) and *Peptococcus* (P= 0.0316, OR= 4.408 & 95% CI= 1.2291 to 15.8099) was significantly lower in case of Eh Mod than healthy ones.

Comparison between healthy versus ALA patients fecal sample flora shown that there is a marked reduction in *Bifidobacterium* (P =0.0002, OR= 18.6667, 95% CI= 3.5494 to 98.169),*Bacteroides* (P = 0.0069, OR = 8.5714, 95% CI = 1.8175 to 40.4239) and *Lactobacillus* (P= 0.0001, OR = 90, 95% CI = 8.4582 to 957.64).

Asymptomatic carriers versus ALA fecal samples shown frequency of *Bifidobacterium* (P =0.0086, OR = 9.33, 95% CI= 1.6534 to 52.686), *Clostridium* (P =0.0086, OR = 9.33, 95% CI= 1.6534 to 52.686) and *Bacteroides* (P = 0.001, OR = 22.5, 95% CI = 3.1351 to 161.4788) was significantly lower in amebic liver abscess patient's samples.

On the other hand Eh Mod versus ASS comparison indicated significantly low titer of *Bacteroides* in ASS (P= 0.0001, OR= 21.33 & 95% CI= 4.4156 to 103.069). Statistical comparison between asymptomatic versus Eh Mod indicated, low titer of *Clostridium* in Eh Mod individuals (P= 0.0097, OR=8.444, CI= 1.6821 to 42.3913).

The most significant information revealed by PCR based detection was presence of *Bacteroides* (present in 5 samples) and *Peptostreptococcus productus* (present in 25 samples out of 36) in pus samples of ALA patients.

Metronidazole Resistance Genes In Stool and Pus Samples:

Nitroimidazole resistant gene (*nim* E) was identified (using PCR approach) in stool samples of Eh Mod (88% incidence) and ALA patients (68% incidence). Few of the pus samples also shown presence of *nim* E gene (38% incidence).

Nim E gene is known to be plasmid- associated gene (Rysset *et al* 1992). Higher incidence of *nim* gene in stool sample DNA of Eh Mod individuals than ALA patients may be related to presence or absence of plasmid containing *nim* E gene in these samples. However, clinical details about metronidazole (dosage period) intake for Eh Mod individuals were not available. For ALA patients mean duration of metronidazole intake was 6 days (400mg to 800 mg either BD or TID).

Change in Cytokines Profile In ALA patients:

Since *E. histolytica* is known to provoke cell-mediated immunity (Kasper and Buzoni-Gatel 2001) during severe infection, cytokines profile for few of pro-inflammatory cytokines was compared in blood samples of ALA patients with control group. Higher titers were observed for IFN- g and IFN-a in ALA patients indicating induction of cellular immunity in amebic liver abscess condition.

Conclusions:

Major achievement of this study was development of genus-specific primers for important gut resident genera.

Following important conclusions can be drawn from this study;(1). There is a variation in fecal flora in diarrhea condition, (2). There is a negative effect of presence of pathogenic organism *E. histolytica* on predominating intestinal flora as seen in asymptomatic and moderately infected individuals and (3). A significant loss of intestinal flora in ALA patients indicated the effect of severe invasiveness of the disease. In ALA patients there is a stimulation of cellular immunity as revealed by high titers of interferons in blood samples. Besides it, this is first report showing presence of anaerobic bacteria in pus samples. It is difficult to comment on the viability of the reported bacteria. In addition, it is also difficult to assess if these bacteria translocated to liver due to leaky gut condition or if they have a

tendency to remain associated with migrating population of specific strain *of E. histolytica*.

Future perspectives of this study are; quantitative PCR techniques are required to establish the quantum of fluctuations observed during disease conditions. Animal model and culture-based studies are required to study (1) the interaction between *E. histolytica* and gut bacteria especially during amebic liver abscess condition, (2) the effect of metronidazole on *E. histolytica* strains isolated from pus samples, and (3) the translocation of bacteria to extraintestinal tissues like liver due to breaching of intestinal mucosa barrier by the protozoa; (4)experiments are required to explain the role of *nim* gene with the presence of bacteria in pus samples since there are indications that nim resistant genes are quite prevalent in some bacteria.