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RESEARCH ARTICLE

Designing and validation of genus-specific primers for human gut flora study

Rani Rekha
School of Life Sciences
Jawaharlal Nehru University
New Delhi 110067
India
Tel: 91 011 26704516
Fax: 91 011 26717580
E-mail: rekhs2004garg@gmail.com

Moshahid Alam Rizvi
Department of Biosciences
Jamia Millia Islamia
New Delhi 110025
India
E-mail: rizvi_ma@yahoo.com

Paul Jaishree*
School of Life Sciences
Jawaharlal Nehru University
New Delhi 110067
India
Tel: 91 011 26704516
Fax: 91 011 26717580
E-mail: jpaul33@hotmail.com

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Abbreviations:
AAD: Antibiotic Associated Diarrhoea
BLAST: Basic Local Alignment Search Tool
IMTECH: Institute of Microbial Technology
MTCC: Microbial Type Culture Collection Center
PCR: polymerase chain reaction
RFLP: Restriction Fragment Length Polymorphism
rRNA: ribosomal ribonucleic acid
U: Units

The aim of this study, was to design and validate 16S rRNA targeted oligonucleotide genus specific primers for amplifying the predominant members of gut flora using polymerase chain reaction. Primers were validated against human faecal samples. Gut flora of a normal individual was compared with that of two diseased individuals. Our observations showed that the genera *Lactobacillus*, *Bacteroides*, *Peptococcus*, *Bifidobacterium*, and *E. coli* were invariably present in all studied subjects however, the absence of butyrate producing bacteria *Ruminococcus* and *Peptostreptococcus* were significant. Presence of the members of the genus, *Campylobacter* in both the diseased samples were also unusual.

The normal gut biota represents a complex microbial ecosystem (the "enterome") that plays a crucial role in homeostasis of the gastrointestinal tract (McCracken and Lorenz, 2001). Human gut microflora is comprised of more than 400 bacterial species. It has been indicated that in normal adults, strictly anaerobic bacteria form 90% of the predominant organisms and 10% of flora are aerobic in nature (Salminen et al. 1995). The predominant anaerobic members of endogenous human gastrointestinal tract belong to the following genera- *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Fusobacterium*,

*Corresponding author

Ruminococcus, *Peptostreptococcus* and *Peptococcus*.

The traditional methods for identifying faecal bacteria include various culture techniques, bacteriological isolations, biochemical tests, morphological examination, and analysis of volatile and non-volatile acid production. These methods have limitations since they are time consuming as well as unable to present the complete status of flora. However application of molecular techniques such as PCR allows the rapid detection of a wide range of bacterial species including both cultivable and non-cultivable microorganisms (Carrino and Lee, 1995; Dutta et al. 2001). The 16S rRNA molecule consists of a mosaic of highly conserved, semi-conserved and highly variable regions, and has become an important tool in molecular phylogeny studies (Woese, 1987). Since the rRNAs differ along their lengths in relative sequence conservation, the targeting of regions of greater or lesser conservation offers exquisite control of probes specificity. Among these regions conserved sequences in all species for a particular genus can be selected as primer sequence; flanking the variable regions. A rapidly accumulating 16S rRNA database facilitated designing of species or group-specific oligonucleotide probes that can be used for the rapid enumeration of anaerobes, independent of cultivation, following shifts in microbial communities to be accurately monitored (Suau et al. 1999).

The human gut microbiota composition may get disturbed under certain circumstances like diarrhoeal condition that may be caused due to infection or antibiotic intake. Antibiotic associated diarrhoea (AAD) may be due to overgrowth of *Clostridium difficile* (Bartlett, 2002). Another possible mechanism is the loss of beneficial metabolic activities of intestinal microbes (Dunne, 2001). Global changes in the composition and quantity of the gut microbiota (even in the absence of overgrowth by pathogenic microorganisms) can result in perturbations of global colonic metabolism that lead to AAD (Högenauer et al. 1998).

In the present study, genus specific primers were designed and validated using faecal samples collected from one healthy and two diarrhoeal patients.

MATERIALS AND METHODS

Designing and validation of primers

Members of predominating gut bacteria were short listed from the literature (Salminen et al. 1995). 16S rRNA sequences of maximum species of a specific genus encountered in the human gut were downloaded from the EMBL database as well as Genbank.

In order to avoid any non-specific amplification of non-targeted genera, the sequences of all the genera fetched from the database were subjected to CLUSTALX (Jeanmougin et al. 1998) programme. The blocks of hyper variable regions showing variations among genera were picked up. These sequences were then subjected to second round of alignment where the maximum number of species belonging a genus was aligned and the regions showing conservations were selected as genus specific primers. Genbank program BLAST (Altschul et al. 1990) was used to further ensure that the proposed primers were complementary with the target species and not with the non-targeted one.

Six different primer sets were designed to differentiate following major anaerobic genera (normally encountered in human gut)- *Bifidobacterium* (Bif), *Ruminococcus* (Rum), *Lactobacillus* (Lacb), *Campylobacter* (Camp), *Peptococcus* (Pep), and *Clostridium* (Clos). Primers for *Bacteroides* (Bacto) and *Peptostreptococcus productus* (PSP) were obtained from the published work (Menaja et al. 1996; Wang et al. 1996). For *Staphylococcus aureus*, primers (*mec*A-1 and *mec*A-2) based on methicillin resistant gene were used (Louie et al. 2002). Primer set used for the detection of *E. coli* was based on the *mal*B promoter gene (Wang et al. 1996).

Table 1 lists the sequences of the primers designed for each genus, their nucleotide position with respect to *E. coli* 16S rRNA, size of the PCR product and the annealing temperature value for each primer set, used in this study. The nomenclature for the targeted primers was selected following the OPD database (Alm et al. 1996). All the primers were commercially synthesized from Microsynth (GMBH, Switzerland).

Standard cultures used to ensure primers specificity

Lyophilized cultures of following bacterial strains were procured from the Microbial Type Culture Collection Center (MTCC) located at the Institute of Microbial Technology (IMTECH) Chandigarh, India. They were, *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 740), *Lactobacillus cassie* (MTCC 1423), *Enterococcus faecalis* (MTCC 439), *Escherichia coli* (MTCC 1302), *Salmonella infantis* (MTCC 1107), *Klebsiella pneumoniae* (MTCC 432) and *Pseudomonas aeruginosa* (MTCC 741). All reference strains were cultivated as recommended by MTTC. Genomic DNA was isolated from the lyophilized cultures of bacterial strains using Sarkosyl- Proteinase K method (Hancock, 2002). Genomic DNA of *Bacteroides fragilis* (ATCC 25285D) was provided by the Microbiology Laboratory, All India Institute of Medical Sciences, New Delhi, India.

Clinical status of subjects

Samples were collected from one healthy and two diarrhoeal subjects belonging to adult age group. Sample was collected from healthy individual (an adult female; NF) who didn't consume any antibiotic for a period of six months, prior to sample collection and was not having any

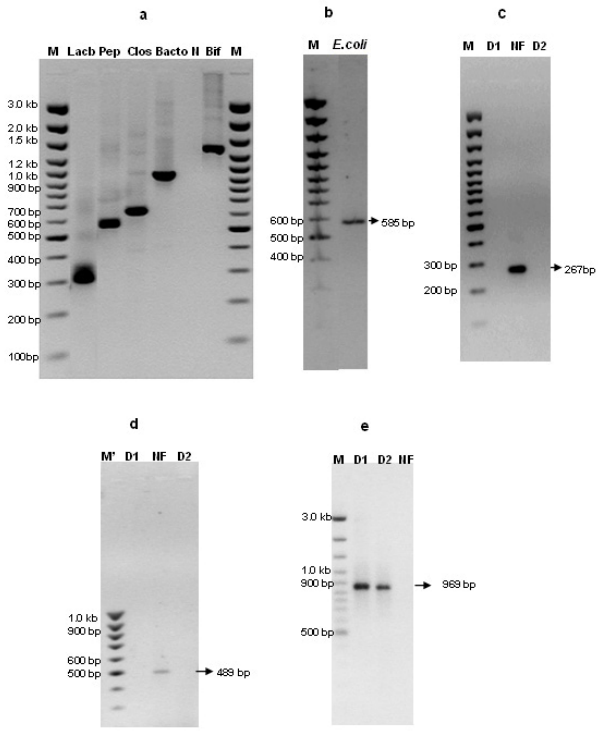

Figure 1. Validation of primers used.
(a) The PCR products amplified with genus-specific primers for genera Lacb, Pep, Clos, Bacto and Bif using healthy individual's template DNA.
(b) PCR product with *E. coli* -specific primers using healthy individual's template DNA.
(c) PCR products with PSP specific primers for all three subjects.
(d) PCR products with Rum specific primers for all three subjects.
(e) PCR products with Camp specific primers for all three subjects. Details of primers from 16S rDNA were designed for various bacterial species (abbreviated on the top of each lane) are represented in Table 1. Details of the subjects NF, D1 and D2 are mentioned in Materials and methods. M and M' represents molecular markers of 100 bp plus and 100 bp (MBI fermentas) N= No DNA template.

episode of clinical disorder. Among diseased individuals, sample D1 was collected from an old age female suffering from bronchitis infection and was prescribed augmentin for 8 days (875 mg / 12 hrs). Patient had no history of chronic gastrointestinal disease and did not consume any antibiotics at least for a year. On the third day of antibiotic intake, patient noted the onset of bulky, loose stools. The patient denied of any abdominal pain or fever. The second diarrhoea patient; an adult male (D2) was suffering from acute giardiasis. Giardiasis patient was on flagyl treatment for three days, when the sample was collected. All three individuals were consuming regular vegetarian diet.

Sample collection

Faecal samples collected in sterile vials were transported to laboratory within 2 hrs after collection and immediately frozen at -20ºC for DNA isolation purpose.

Morphological and microscopic observations

To identify ova and cysts of the intestinal parasite in giardiasis patient's faecal sample, wet mount preparations were prepared in normal saline and stained with Lugol's iodine for cysts.

DNA isolation

DNA isolation from frozen faecal samples was done using Qiagen stool DNA mini kit; Qiagen, according to the instructions of the manufacturer. 180 mg of sample was used and concentration of DNA was determined by eye estimation on 0.6% agarose gel electrophoresis.

Testing of primers for PCR amplification

PCR was performed in Tech gene, thermal cycler (Nugen Scientific, USA). PCR was performed in 20 µl (final volume) containing 1X of 10X PCR buffer (containing 750 mM Tris-HCl (pH 8.8 at 25ºC), 200 mM $(NH_4)_2$ SO_4, 0.1% Tween-20), 2 mM of each dNTP, 2 mM $MgCl_2$ (except for *Lactobacillus*, where 1.5 mM $MgCl_2$ was used), 20 pmol of each primer and 2 U of Taq DNA polymerase (MBI Fermentas, USA) and 2.5 µl of template DNA (Faecal sample DNA). The amplification conditions were; one cycle of 94ºC for 5 min followed by 30 cycles of 94ºC for 30 sec, annealing (annealing temperatures mentioned in Table 1) for 1 min, 72ºC for 1 min, final extension at 72ºC for 8 min and finally cool down to 4ºC. PCR products were checked for expected sizes on 1.5% agarose gel by loading 20 µl of the PCR reaction along with molecular marker of 100 bp ladder plus (MBI Fermentas, USA). Gel run was carried out for 3-4 hrs. Sizes of various amplicons generated by genus specific primers were checked as per Table 1. DNA isolated from the above mentioned pure strains were used as controls (~80 ng genomic DNA) for the subsequent PCR experiments.

Validation of primers specificity

PCR products were amplified using faecal DNA from healthy individual using genus specific primers. Later these products were purified using Qiagen gel extraction kit and cloned into pGEMT®- easy vector (Promega co., Madison, USA). Similarly PCR product for *Campylobacter* amplified from diseased individual's sample DNA was purified and cloned into pGEMT-easy vector. The cloned products were sequenced and analyzed using NCBI BLASTN program. Sequences were submitted to EMBL nucleotide database (WEBIN). These cloned products were used as control DNA when PCR reactions were carried out with diseased individuals. Each PCR reaction was repeated thrice for all samples to confirm the reproducibility of the technique.

RESULTS

Analysis of PCR products generated using the genus specific primers

In order to test specificity of the primers (Table 1) target and non- target DNA were used as positive and negative controls with each PCR reactions. In order to further authenticate primers, amplicons generated using genus specific primers for healthy individual and *Campylobacter* for diseased one (Figure 1) were cloned and sequenced. Primers used for amplification of targeted genera (genomic DNA of pure strains) worked well whereas, no amplification was observed with non- targeted genera and non-targeted clones DNA.

The accession numbers were: AM042696 (*Bacteroides vulgatus* partial 16S rRNA gene), AM042697 (Uncultured *Clostridium* sp. partial 16S rRNA gene), AM042698 (Uncultured *Bifidobacterium* sp. partial 16S rRNA gene), AM042699 (*Campylobacter coli* partial 16S rRNA gene), AM042700 (*Peptococcus* sp. RR-2005 species partial 16S rRNA gene), AM042701 (*Lactobacillus acidophilus* partial 16S rRNA gene), AM117587 (*Peptostreptococcus productus* partial 16S rRNA gene) and AM117597 (*Ruminococcus* partial 16S rRNA gene). The species names, in given accession numbers have been mentioned on the basis of BLAST results showing highest hits with particular species.

PCR results

DNA isolation using Qiagen kit yielded DNA of high

Table 1. Positions of genus specific primer sequences with respect to *E. coli* 16S rRNA.

Genus	Primer Sequence	Primer Position vs E. coli 16S rRNA†	PCR Product	Annealing Temp‡
Bacteroides	GGGGTTCTGAGAGGAAG ACCCCCCATTGTACCAC	S-G- Bacto- 0297- S- 17 S-G- Bacto-1245- A- 17	950 bp	5 2.0
Bifidobacterium	CGCTGGCGGCGTGCTTAACACAT CGCGATTACTAGCGACTCCGCCTTCA	S-G- Bif- 0008- S- 23 S-G- Bif- 1306- b- A- 26	1300 bp	60.0
Lactobacillus	TGCCTAATACATGCAAGTCGA GTTTGGGCCGTGTCTCAGT	S-G-Lacb-0018-S-21 S-G-Lacb-336-A-19	318 bp	52.0
Ruminococcus	CCTCTGACCGCTCTTTAATCGGAGCTTTCCTTC CCAGTTATCGGTCCCACCTTCGGCAGCT	S-G-Rum-0998-S-33 S-G-Rum-1447-A-28	482 bp	61.0
Peptostreptococcus productus	AACTCCGGTGGTATCAGATG GGGGCTTCTGAGTCAGGTA	S-G-PSP-0212-S-20 S-G-PSP-0503-A-19	270 bp	51.0
Peptococcus	GGTGCCGCAGTAAACACAATAAGT AAGGCCCGGGAACGTATTCA	S-G-Pep-0830-S-24 S-G-Pep-1369-A-20	539 bp	53.0
Clostridium	CTCAACTTGGGTGCTGCATTT ATTGTAGTACGTGTGTAGCCC	S-G-Clos-0586-S-21 S-G-Clos-1205-A-20	619 bp	53.0
Campylobacter	AGGGAATATTGCGCAATGGGGGAAA TTACTAGCGATTCCGGCTTCATGC	S-G-Camp-0466-S-25 S-G-Camp-1435-A-24	969 bp	55.0
E. coli	ECO-1 GACCTCGGTTTAGTTCACAGA ECO-2 CACACGCTGACGCTGACCA	Mal B promoter gene	585 bp	53.0
Staphylococcus aureus	*mec*A-1 AAAATCGATGGTAAAGGTTGGC *mec*A-2 AGTTCTGCAGTACCGGATTTGC	Methicillin resistance gene	533 bp	51.0

† Primer positions are represented according to the OPD nomenclature (Alm et al. 1996). As per nomenclature; 'S': Small subunit rRNA, A hyphen (S-). Each character or group of characters in the primer name is set off with a hyphen to facilitate computer –aided manipulations. G: Genus; A: antisense template strand; S; DNA sense strand; 4 digit number in the middle represents the position of the primer with respect to *E. coli* and the last two digit number depicts the number of nucleotides present in the primer; *e.g.* (S-G-Bif-1306-A-26). Bacto: *Bacteroides;* Bif: *Bifidobacterium*; Rum: *Ruminococcus*; Lacb: *Lactobacillus*; Pep: *Peptoccoccus*; PSP: *Peptostreptococcus productus*; Clos: *Clostridium*. Primer sets for Bacto (Menaja et al. 1996); PSP (Wang et al. 1996); *E. coli* (Wang et al. 1996); and *S. aureus* (Louie et al. 2002) used in the present study are cited in the literature.

‡: Annealing temperatures are based on standardized conditions.

purity grade suitable for PCR reaction and concentration of DNA was approximately 50 ng / µl. Volume of template DNA used (2.5 µl; ~62 ng) worked fine for PCR amplification. Comparison of PCR results for all three samples showed that the genera *Lactobacillus*, *Bacteroides*, *Peptococcus*, *Bifidobacterium*, and *E. coli* were invariably present in all subjects. However, the two genera, *Ruminococcus* and *Peptostreptococcus* were absent in the diseased subjects (Figure 1c and Figure 1d). *Clostridium* was absent in the antibiotic associated diarrhoea patient (D1). Interestingly, *Campylobacter* was present in both the diseased individuals while absent in healthy subject (Figure 1e). No amplicon was observed in any samples for methicillin resistant gene specific primers (*mec*A-1 and *mec*A-2) used for detecting methicillin resistant *Staphylococcus aureus* strains.

DISCUSSION

The microbiota of the gastrointestinal tract of humans needs to be studied extensively because of the role played by gut bacteria both in the maintenance of gut homeostasis during healthy and disease conditions. Analysis of bacteria based on 16S rRNA primers is a sensitive and specific technique to identify gut flora that are difficult to cultivate. The reliability of the results is determined mainly by the specificity of the selected primers and experimental conditions. Among the 16S rRNA sequence based genus specific primers tested for BLASTN analysis, *Bacteroides* specific primers showed partial homology with *Streptomyces*, *Cytophagals* that are not inhabitants of human intestine. No homology was observed with any non-targeted genera for *Bifidobacterium* specific primers. Similarly other genus specific primers showed partial homology with *Marichromatium purpuratum, Roseospirillum parvum* and *Thiomicrospira* species that are also not encountered in a healthy human gut. Identification of closely related members of Enterobacteriaceae, for example, *Shigella* spp. and *E. coli*, is difficult to achieve using molecular probes since specific primers are difficult to design from 16SrRNA region (Christense et al. 1998). We have used *mal* B promoter gene sequence based primer set for *E. coli* detection (Wang et al.1996).

It is advantageous to use genus specific primers since it encompasses maximum number of species of a genus. Using RFLP technique for single PCR product, species differentiation can be accomplished for a genus along with the observation of variations, if any.

Primers used for the PCR reaction showed specificity for targeted genera. The sequences obtained from the cloned PCR products were subjected to BLAST analysis for confirming the specific genus. These clones were further used as appropriate controls.

An interesting observation in this study was the absence of *Ruminococcus* and *Peptostreptococcus* in the diseased samples whereas they were present in healthy individual. Both the organisms are well-known butyrate -producing bacteria (Schwiertz et al. 2002, Wilson et al. 2000), which is a preferred energy source for colonic epithelial cells, and is thought to play an important role in maintaining colonic health in humans (Barcenilla et al. 2000). *Clostridium* was absent in old age female diarrhoea patient. There are reports indicating loss of normal resident flora due to antibiotic intake in diarrhoea and other infectious diseases (Wang et al. 2002; Tanaka et al. 2005). Loss of these bacteria due to antibiotic treatment can lead to increased amounts of carbohydrate in the colonic lumen, leading to an osmotic diarrhea. Augmentin (taken by D1, old age AAD patient) contains amoxicillin which is a broad spectrum drug used against bacterial infection (Bartlett, 2002). Our results for AAD patient also support the study by Young and Schmidt (2004) where loss of Clostridia cluster IV was observed due to intake of Amoxicillin. The drug Flagyl (metronidazole) used for giardiasis patient (D2) treatment is again a broad spectrum drug effective against protozoa and anaerobic bacteria (Muller, 1983). This may be responsible for the absence of the above flora in the giardiasis patient.

Presence of the members of Enterobacteriaceae *e.g. E. coli* as observed by us both in healthy and in diseased conditions has been reported earlier by Young and Schmidt (2004). This indicates that these members are rather stable even in diseased conditions.

In order to check if any of these patients acquired nosocomial infection due to *Staphylococcus aureus*, PCR was performed using *mec*A primer set. No methicillin resistant strains of *Staphylococcus aureus* was observed in any of the sample studied. Presence of *Campylobacter* in diarrhoeal subjects supported the earlier studies (Haque et al. 2003) showing chances of mixed infection in giardiasis patients. Our results indicated that both the patients suffering from diarrhoea were probably more prone to mixed infections. Our results show that *Lactobacillus*, *Bacteroides*, *Peptococcus* and *Bifidobacterium* were more stable flora and did not alter in diarrhoea conditions. However, due to lack of quantitative PCR data, it is difficult to comment on any change in titer of a specific genus due to diseased condition. Loss of predominant beneficial butyrate producing bacteria in diarrhoea conditions along with the presence of pathogenic genus *Campylobacter* was important observation.

Through this study we were able to design and validate genus specific primers and use them as molecular probes for PCR based detection of important gut bacteria in faecal samples. Observations made here, are preliminary ones, therefore whether the variation in flora is due to physiological status cannot be concluded out of the limited number of the samples analyzed. More number of samples, in each category needs to be analyzed for comparing gut flora profile between healthy and diseased individuals.

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