A COMPARATIVE STUDY ON COLONIC AND MILK BACTERIA BY DOUBLE-DIGESTED 16S rRNA GENE AMPLICONS

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ABSTRACT
16S rRNA gene was amplified from faecal or milk total genomic DNA of eight women by polymerase chain reactions (PCR) using a pair of bacteria specific universal oligonucleotide primers. Amplification products were digested with both HaeIII and TaqI restriction enzymes. Sampling period covered the second and third trimester of pregnancy and further 6 months after birth for the collection of breast milk. Digestion products were resolved in an 8% polyacrylamide gel and restriction fragment length polymorphism (RFLP) profiles were visualized by silver staining. A control group was also prepared from eight other women who were single and without children. In the RFLP profiles, clearly discernible 66 digestion products (bands) were selected and subjected to unweighted pair-group method with arithmetic mean analysis (UPGMA). Results indicated that almost 58% of the clustering comprised common banding patterns, shared by faecal, milk and control samples. Nine of the clusters (approximately 13%) were exclusive to faecal and milk samples. Prominent bands present in the milk RFLP patterns indicated that four of the mothers who adopted traditional food sources could have almost identical prominent bacteria in their milk.


Keywords: breast, breast milk, commensal bacteria, RFLP, 16S rRNA gene, molecular typing

Introduction
Gastrointestinal tract constitutes a complex symbiotic ecosystem formed by hundreds of bacterial species and their eukaryotic host. The largest bacterial community resides in the large intestine having up to $10^{14}$ cells in 1g of faecal sample (3). Thus, it has been estimated that the number of colon bacteria is greater than that of cells found within the body of an average human adult (3, 9). The bacterial element of the ecosystem appears to have formed by complicated means of interactions within itself and with the epithelial cells of mucosal surfaces during the course of evolution. Many attempts have been made to understand the interrelationships by identifying the types of bacteria in the tract and by studying the molecular communication between them. In the human adults approximately 500 species have been known to exist. It appears however that 99% of the microbiota are only formed by 30 or 40 of the species within which anaerobic bacteria predominate (3). In a recent culture-independent study it has been reported that large numbers of genes are shared by the identifiable individual organisms and these have been considered as a “core microbiome” at gene or functional level (14). These findings suggest that a healthy adult is expected to have an almost fixed microflora content and that these bacteria might be held responsible for the establishment of permanent (autochthonous) flora and for the long term interactions with the host (14). Extensive research performed on the functional aspects of the microbiota has demonstrated that it can modulate the expression of genes whose functions include nutrient absorption, carbohydrate and xenobiotic metabolisms, intestine motility, mucosal barrier function, angiogenesis, morphogenesis and postnatal maturation of intestinal cell lineages, and maturation of the gut associated lymphoid tissue (GALT) (9). Aseptically collected human breast milk has been shown to possess an endogenous bacterial flora of less than $10^3$ cfu/ml. It has been speculated that this flora might originate from the maternal luminal microbiota via an internal route to mammary glands involving mononuclear phagocytes (10) or dendritic cells (6). Infants are born without having a sizeable microbial community and colonization of a healthy infant’s gut microbiota is initiated by the milk flora through breast feeding (6). In the lights of the data summarized above, in the present study, it was first intended to obtain a qualitative picture of the faecal flora during pregnancy and then to compare it with the corresponding milk flora collected by 15 d/sample periodic sampling for 6 months.

Materials and Methods
Collection of faecal and milk samples
Pregnant women included in the study had not experienced any viral diseases and were not on medication. Faecal samples were collected from 8 women administered at Cumhuriyet University Medical Hospital, and two district clinics (Kadiburhanettin and Cayyurt) in Sivas immediately after the official confirmation of pregnancy. Sampling were started towards the beginning of second trimester because the individuals usually visited the institutions when they were
already pregnant for more than 6 weeks and because obtaining the consent of each of the subjects required persuasion and time. Sampling was started immediately after obtaining the consents, continued by taking one sample per month, and ended by the date of delivery. Thus, the numbers of samples obtained from each of the subjects varied: from A- 6 samples; C- 4 samples; D- three samples; E- three samples; F- 6 samples; and N- O-, and P- 5 samples. At each collection, 10 or 20 g of faeces were taken into sterile plastic containers. The samples were suspended in 30 ml TNE buffer (50 mM Tris [pH 8], 5 mM EDTA [pH 8], 50 mM NaCl) in 50 ml sterile Falcon tubes and were then centrifuged for 15 min at 1000 rpm at 4°C to remove particulate material. Cell suspensions were transferred into new tubes and centrifuged for 15 min at 5000 rpm. Pellets were washed three times in 30 ml TNE buffer, resuspended in sterile peptone water containing 20% glycerol, and four aliquots were prepared in 2 ml cryotubes and stored at -80°C. Milk sampling was performed for 6 months by taking 2 samples per month. At each sampling, 50 to 100 ml of milk was aseptically obtained by using a power-operated milking machine (Morton TBM 2100 Breast Pump, Turkey). Milk samples were transferred into 50 ml sterile Falcon tubes and centrifuged for 10 min at 5000 rpm. Pellets were suspended in 20% glycerol and stored at -80°C.

Genomic DNA extraction
One faecal aliquot was resuspended in 15 ml TNE buffer and particulate matter was removed by centrifugation for 5 min at 1000 rpm. Suspension was transferred into a new tube and centrifuged for 10 min at 5000 rpm to pellet cells. For the milk samples, cell pellets corresponding to 400 ml milk were pooled. Genomic DNA was essentially prepared following the protocol described by Bulut et al. (1), except that TNE buffer with which the milk pellets were washed, included 125 mM EDTA.

Amplification of 16S rRNA gene by PCR
Amplification of 16S rRNA gene region was performed by using the following primers: Forward, 5′-AGAGTTTGATCCTGCTCAG-3′ and reverse, 5′-CTACGGCTACCTTGTTACGA-3′ (15). Each of the polymerase chain reactions (PCR) was performed in a 50 ml reaction volume containing 200 ng genomic DNA as the template, 0.2 mM-dNTPs, 1.5 mM-MgCl₂, 10 pmol each of the DNA primers in 1x PCR buffer (10 mM-Tris–HCl, 50 mM-KCl, 8 g Nonident P40/l, pH 8; MBI Fermentas, Lithuania), and 1.25 U Taq DNA polymerase (MBI Fermentas). Amplification conditions were as follows: an initial denaturing step of 5 min at 94°C; 40 amplification cycles, each consisting of steps of: 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min elongation at 72°C. Reactions were terminated with a final extension step for 10 min at 72°C. PCR amplifications were performed in a Thermo Cycler System (Thermo Electron Corp., USA).

Restriction digestion of the amplification products, electrophoresis and staining procedures
PCR products, on average 1500 bp in length, were chloroform extracted and 2 µg of the amplified DNA were digested with two restriction endonucleases, HaeIII and TaqI (MBI Fermentas), as described by Bulut et al. (1). Digestion products were separated in an 8% polyacrylamide gel with 35x45x0.4 cm dimensions (12) for 12 h at 150 mA. After the completion of electrophoresis, the gel was stained as described by Qu et al. (11).

UPGMA of the RFLP profiles
RFLP patterns were analysed by UPGMA with the PAUP software (4.0b10, Altivec, Massachusetts, USA). Bands were numbered starting from 600 bp. Each of the lanes was inspected and the presence or the absence of the corresponding bands was designated as “1” or “0”, respectively. The two digit matrix thus obtained was used in the analysis.

Results and Discussion
RFLP of 16S rRNA gene
The technique is essentially based on the amplification of 16S rRNA gene using a pair of universal oligonucleotide primers specific to Bacteria and restriction of the amplification products with appropriately chosen restriction endonucleases. Digestion products are usually separated in an agarose gel (8). Main advantage of this technique is that it has the highest capacity in obtaining reproducible results among all of the PCR based fingerprinting methods (8). However, because rRNA gene sequences are very conserved, its resolution power remains relatively low. Having such shortcomings of the technique in mind, genus, species or intraspecies specific restriction enzymes could be selected to increase its discriminative power. Many investigations have been available in the literature

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TABLE 1

Some background information on the pregnant women (donors of faecal samples)
by which the discriminative capacity of certain restriction endonucleases has been assessed on different genera or species. In the present study, the amplicons of rRNA gene were double-digested with HaeIII and TaqI. The former cleaves DNA at guanine and cytosine (GC) rich nucleotides and it has been shown to discriminate bifidobacteria and lactococci at species level (1, 4). The other enzyme, TaqI, hydrolyses DNA between adenine and cytosine nucleotides and has been reported to have more discriminative power on the rRNA genes of Bacteroides, lactobacilli (1), and staphilococci (13). Resolution power was also increased by using nondenaturing polyacrylamide gel electrophoresis and by visualizing the restriction patterns by a sensitive silver-staining method (11). In the gel, 66 distinct restriction banding patterns were counted. On average, a 16S rRNA gene is 1500 bp in length and when it is divided by the number of these distinct bands, 66, theoretically each of the bands represents approximately 23 bp of 16S rDNA. In order to increase the number of bands within the restriction profiles PCR products could also be digested with a third enzyme. The resolution power of a 6 or 8% polyacrylamide gel is however within the range of 600 bp DNA (12), it was thought that further degradation of DNA would make the RFLP patterns much more complicated.

The use of PAUP for RFLP analysis

Similarity between the restriction profiles of faecal, milk and control samples was assessed by using UPGMA in PAUP (phylogenetic analysis using parsimony). The reasons for analysing the RFLP results by UPGMA were threefold: (I) it is a tree-building algorithm, (II) it graphically represents similarity among genotypes, and (III) it can be used without reference to any specific model of evolution (5).

Interpretation of the UPGMA result

The similarity tree obtained in PAUP software programme divided the restriction products into two cluster groups: A and B (Fig. 1). Group A clusters consisted solely of the common banding patterns for the faecal (F), control faecal (C), and milk (M) samples and each of these patterns was designated as cFM. Bottom three branches of B group clustering also included many of the cFM clusters. Towards the top of the tree this cluster still existed with a decreasing frequency and it formed the largest clustering type with 58% frequency in the whole tree. This was followed by the FM (faecal and milk samples) type clusters with 13% frequency and these were located within the B2 branches. Third most frequent (7%) cluster type was M (milk samples) and these were scattered in the upper parts of the tree. F and CF clusters had the lowest frequency (4%). These results might indicate the existence of a “core” microbial flora in all three types of the samples (C, F, and M) and these could be specifically represented by the 13 cFM clusters making up group A. Another prominent feature of the tree could be that the lower half of the tree was almost formed by the CFM type of clustering.

Amount of milk used for genomic DNA extraction

Initially 10 ml of the collected milk sample were used for the genomic DNA extraction. Visible DNA wools were obtained. When, however, this DNA preparation was used as the template, the yield of PCR was very low. Milk genomic DNA was then checked along with faecal genomic DNA samples by agarose gel electrophoresis. It was observed that milk genomic DNA had a much higher molecular weight. This observation was accounted for by the presence of a population of eukaryotic cells in the milk samples. Finally, the amount of the milk used for DNA extraction had to be increased up to 400 ml/per sample by pooling half of the milk pellets together, collected during the whole sampling period. Therefore, in the results (Fig. 2) each of the milk donors was represented by only one RFLP profile. The other half of the milk pellets were stored as glycerol stocks for future experiments.

Milk donors and interpretation of the RFLP profiles obtained from milk samples

After the date of delivery each of the faecal sample donors (pregnant women) became milk donors (mothers). Some background information was provided for the mothers (Table 1). Half of the mothers had stated to have adopted traditional food supplies (mothers C, E, F, and N; CEFN group), such as artisanal cheese, yoghurt or organic fruits and vegetables provided from nearby villages, whereas the other half (mothers A, D, O, and P; ADOP group) have long been adapted to urban life and consumed mostly processed food available in the supermarkets. In the former group, one of the mothers (mother E) gave birth by a caesarean section and the remaining mothers
(C, F, and N) had vaginal delivery. In the ADOP group two of the mothers (A and D) had caesarean section while the other two (mothers O and P) gave birth vaginally (Table 1). These features, together with the remaining information in the table, were found to be very helpful for the interpretation of milk RFLP profiles (Fig. 2). The following observations could be made by a visual inspection of these RFLP banding patterns: 1) the RFLP profiles of CEFN mothers consisted of more prominent but fewer bands; 2) most of these bands also appeared to be present, with varying and much lower intensities, in the profiles of ADOP group (small arrows, Fig. 2); 3) one of the prominent band patterns (large arrow) was exclusive to the CEFN group; 4) ADOP profiles consisted of more heterogeneous bands and indicated much less pattern similarity within themselves, except a few bands located between 120 and 150 bp of the size marker bands.

The first two observations could imply that traditional food consumption had a reducing effect on the diversity of milk bacteria, perhaps, by leading to more defined population, because the RFLP patterns of the CEFN group shared most of the prominent bands (Fig. 2). Furthermore, the type of delivery (vaginal or caesarean section) did not seem to modulate or change this overall RFLP pattern similarity. Thus, it could be suggested that one key player dictating the composition of milk bacteria may well be the nature of nutritional environment in the breast. Presence of some of the CEFN prominent bands in the ADOP profiles might also imply the existence of common bacteria in the breast milk. The above observations, however, seemed to argue against the possibilities that the type of delivery or even the number of pregnancy or age of the mother (Table 1) would have a discernable influence on the formation or the composition of endogenous milk bacteria.

In the present study, the results indicated that large and complex ecosystems could initially be studied by adopting relatively simpler methods and by using large number of samples, and that important information would be gained, for example, in the temporal assessment and tracing of local commensal bacteria that posses diagnostic values.

Conclusions

Mother’s breast can be envisaged to be acting as an enrichment and propagation vessel

From the studies on the environmental pollutants possessing estrogen-like activity (xenoestrogens) in the body (2), and on the measurement of transfer factors (TF) for essential and heavy elements from food into mother’s milk (7), it can be envisaged that human breast is acting as a transfer organ of the metabolized food components or stored elements from blood to milk, and because milk serves as a concentrated food source, breast environment must also act as a “concentrator”. At the same time it might also operate as an open fermenter with a feeding mechanism at its upstream (blood vessels) to maintain growth conditions for the endogenous bacteria, while feeding them to the baby. These features might be expected to be common in healthy mothers as the underlying genetic makeup should almost be the same. One of the key variables here could be the food source which is solely determined by the mother’s diet.

Acknowledgements

This work was supported by CUBAP (The Scientific Research Projects Council of Cumhuriyet University) with the project number F-168.

REFERENCES


