Comparative analysis of fecal microflora of healthy full-term Indian infants born with different methods of delivery (vaginal vs cesarean): *Acinetobacter* sp. prevalence in vaginally born infants

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In this study fecal microflora of human infants born through vaginal delivery (VB) and through cesarean section (CB) were investigated using culture-independent 16S rDNA cloning and sequencing approach. The results obtained clearly revealed that fecal microbiota of VB infants distinctly differ from those in their counterpart CB infants. The intestinal microbiota of infants delivered by cesarean section appears to be more diverse, in terms of bacteria species, than the microbiota of vaginally delivered infants. The most abundant bacterial species present in VB infants were *Acinetobacter* sp., *Bifidobacterium* sp. and *Staphylococcus* sp. However, CB infant's fecal microbiota was dominated with *Citrobacter* sp., *Escherichia coli* and *Clostridium difficile*. The intestinal microbiota of cesarean section delivered infants in this study was also characterized by an absence of *Bifidobacteria* species. An interesting finding of our study was recovery of large number of *Acinetobacter* sp. consisting of *Acinetobacter pittii* (former *Acinetobacter* genomic species 3), *Acinetobacter junii* and *Acinetobacter baumannii* in the VB infants clone library. Among these, *Acinetobacter baumannii* is a known nosocomial pathogen and *Acinetobacter pittii* (genomic species 3) is recently recognized as clinically important taxa within the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex. Although none of the infants had shown any sign of clinical symptoms of disease, this observation warrants a closer look.

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1. Introduction

The human gastrointestinal tract is a host to a large, active and complex community of microbes, known to be composed of a vast number of bacteria, fungi and protozoa (Suau *et al.* 1999; Pandey *et al.* 2012). The total number of these microbes is believed to surpass 10^{14} microbes per gram of feces (Finegold *et al.* 1983). These microbes play significant roles in the host's food digestion, metabolism of endogenous and exogenous compounds, production of vitamins and in the prevention of colonization of gut by a number of pathogens (Suau *et al.* 1999). The gastrointestinal tract of infants at the time of birth use to be sterile and during the birth process and soon after that, microbes from mother and surrounding environment start colonizing this, until a dense, complex community is established (Gronlund *et al.* 1999; Gronlund *et al.* 2000; Benno and Mitsuoka 1986; Palmer *et al.* 2007).There are several factors that are known to have an effect on the initial microbial colonization of the infants gut, i.e. mode of delivery, mode of feeding, etc. Among these, the type of delivery is considered to be the most important one. It is believed that vaginally born infants acquire microbes from their mother,

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Abbreviations used: CB, Cesarean section born breastfed; VB, vaginally born breastfed

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while cesarean born infants get exposed to the environmental microbes, which put them at higher risk of colonization with undesired microbes (Gronlund *et al.* 1999).

Several studies have been performed to monitor the bacterial community structure in infants; however, our understanding of this ecosystem remains limited. Most of the earlier studies have used culture-based approaches, which are time consuming, require extensive hard work and simultaneously do not cover the complete bacterial diversity present in the gastrointestinal tract (Gronlund *et al.* 1999). In recent years with the advancement of molecular methods, out knowledge of gut microflora and their role in various physiological and immunological activities has rapidly increased (Suau *et al.* 1999; Penders *et al*. 2006; Palmer *et al.* 2007). However, most of these works have been carried out in adults leaving aside the infants gut microflora completely unexplored. The first study using molecular methods to explore the establishment and succession of bacterial strains in the gastrointestinal tract of full-term infants was reported by Favier *et al*. In their study they have investigated the development of the gut microflora in two healthy babies, using denaturing gradient gel electrophoresis (Favier *et al.* 2002). In the recent past DGGE and TRFLP techniques are routinely used to deduce the various aspect of the gut microbial diversity in infants including the effect of mode of delivery and other genetic and hygiene factors (Penders *et al*. 2006; Biasucci *et al*. 2008; Biasucci *et al*. 2010). However, owing to the fact that DGGE and TRFLP both have several limitations and there is no information on the infants gut microbiota from the Indian sub-continent, the present study was conducted.

In the present study comparative analysis of bacterial community structure has been carried out at day 7 fecal samples of full-term vaginally born breastfed babies (VB) and full-term cesarean born breastfed babies (CB) by using extensive 16S rDNA cloning and sequencing approach. The purpose of the study was to provide a comprehensive picture of the gut microbiota of infants born with different methods of delivery. The previous work by Gronlund *et al.* had utilized the culture-based method, which fails to provide a broad picture of gut microflora of vaginal and cesarean section born infants (Gronlund *et al.* 1999). The present work is the first of its kind in being from the Indian subcontinent and in utilizing the 16S rDNA cloning and sequencing methodology in comparative study of fecal microbiota of infants born with different method of delivery.

2. Materials and methods

2.1 *Subject and sample collection*

The study was carried out in Pune, Maharashtra, India. Twenty-four (12 from each group) infants were included in this study. All infants that were part of this study were healthy full-term delivered and exclusively breastfed. None of these babies had received any antibiotic till the time of sample collection. In the majority of the cases, babies were not in the head-down position near the delivery due date, which lead to cesarean section delivery; however, in the remaining cases, mothers had voluntarily opted for cesarean section delivery. Mothers after the surgery received broad-range antibiotic for the first few days. No rupture of membranes and possible chorioamniontis was observed. Written informed consents had been obtained from the parents of the children's participated in this study. The study was approved by institutional review board of the National Centre for Cell Science, Pune, India. Fresh stool samples were collected in specialized collection tubes at day 7 of the infants' lives. Immediately after collection they were transported to the lab in ice and were stored in −70°C until further processing.

2.2 *DNA extraction, PCR amplification, cloning and sequencing*

The total genomic DNA was extracted from all fecal samples using QIA amp stool DNA Mini Kit in combination with glass bead beating using the manufacturer's instruction. PCR amplification was performed as described earlier using bacterial 16S rRNA universal primers: 530F (5′ GTC CCA GCM GCC GCG G 3′) and 1490R (5′GGT TAC CTT GTT ACG ACT T 3′) (Weisburg *et al.* 1991). Twenty-five cycle PCR reaction was set for each sample in triplicate, to minimize PCR bias and artifacts. The purified PCR products from the same group were pooled together and ligated into pGEM-T Easy vector (Promega, USA). Subsequently, ligated PCR products were transformed into *E. coli* JM109 high-efficiency competent cells (Invitrogen, USA). The transformed cells were plated on Luria–Bertani (LB) agar plates supplemented with Ampicillin (100 µg/ml, Sigma), X-gal (100 µg/mL) and IPTG (0.5 mM; Promega,). The resultant libraries were screened for the positive clones using vector-specific primers. Sequencing of positive clones from both ends was performed on an ABI 3730 sequencer using BigDye chemistry version 3.1 (Applied Biosystems, USA). As a preliminary investigation, before going for the16S rDNA clone library using pooled PCR products, denaturing gradient gel electrophoresis (DGGE) analysis was carried out. However, in DGGE profile of the samples from the same group, we did not observe any significant difference in the band pattern, which led us to prepare the 16S rDNA clone library using pooled PCR products from the same group.

2.3 *Phylogenetic analysis*

All sequences were manually checked and assembled using Chromas-Pro software. Putative chimeras were eliminated

from analysis using MALLARD (Ashelford 2006). The 16S rDNA sequences (mostly around 760 for VB and 850 base pairs for CB infants) were assigned to major phylogenetic groups using DOTUR (Schloss and Handlesman 2005) and search against GenBank and Ez-Taxon database (Kim *et al*. 2012). The sequences from both libraries and public databases with known phylogenetic affiliation were aligned with the Clustal X program (Chenna *et al.* 2003). The alignment was checked and corrected manually using MEGA sequence alignment editor (Kumar *et al*. 2004) and the unaligned portion towards both the ends were trimmed using DAMBE (Xia and Xie 2001).

Phylogenetic analysis was performed with PHYLIP*3.64 package (Felsenstein 1989). Distance and similarity matrices were calculated with the DNADIST program according to the Jukes-Cantor model. Phylogenetic trees were constructed using methods namely Neighbor Joining in MEGA, Maximum Parsimony in Phylip*3.64, Maximum Likelihood using PAUP (Swofford 2001) and Mr Bayes (Huelsenbeck and Ronquist 2001). Bootstrap analysis (1000 replications) was used to estimate the confidence of tree topologies and the CONSENSE program was used to obtain consensus trees. The TREEVIEW program (Page 1996) was used to draw trees. However, the topologies of the tree constructed using all these methods were similar; hence, trees based on the neighbour joining method in MEGA were considered (figures 1 and 2).

2.4 *Statistical analysis*

Rarefaction analysis (Krebs 1998), Shannon index, the reciprocal of Simpson's index (Magurran 1996) and Chao-1 estimator (Chao 1987) were used to characterize the microbial diversity of our samples. Rarefaction compares species richness between unequally sampled sites, treatments or habitats (Hughes *et al.* 2001). The rarefaction curves were generated using Analytic Rarefaction 1.3 (*http://www.uga.edu/~strata/software/index.html*). The Shannon index (H0) is a general diversity index that positively correlates with species richness and evenness, and is considered to be more sensitive to change in abundance of rare species (Magurran 1996; Hughes *et al.* 2001). The Simpson's index (D) is a dominance measure and gives the probability that two clones chosen at random will be from the same species (Hughes *et al.* 2001). The reciprocal of the Simpson index was used to ensure that the value of the index (1/D) increases with increasing diversity (Magurran 1996). Chao-1 was used to estimate the total richness of the microbial community from a sample (Chao 1987). The significance of difference in the composition between the two clone libraries was examined in pairs by using the LIBSHUFF program as described by Singleton *et al.* (2001). The coverage of both clone libraries was calculated using formula $[1-(n/N)]$ as described by Good (1953), where n is the number of phylotypes represented by one clone and N is the total number of clones.

2.4.1 *Nucleotide sequence accession number:* Unique sequences from both libraries were deposited to NCBI GenBank with accession numbers from EU723273 to EU723399 for VB infants library and from EU723400 to EU723483 for CB infants.

3. Results

The bacterial diversity was measured using diversity indices, comparing the phylotype composition and analysing phylogenetic distribution of 16S rDNA clones in both the libraries.

3.1 *Diversity measures*

In order to calculate the diversity measures, the 16S rDNA clone sequences with more than 97% sequence similarity were grouped into the same phylotype, as defined by Suau *et al*. (1999). A total of 211 clones from the both the samples were analysed, 127 for VB and 84 for CB infants. In total, 24 phylotypes were identified with 11 and 13 detected from VB and CB infants respectively (table 1). Rarefaction curves were obtained by plotting the number of phylotypes observed against the number of clones sequenced (figure 3). The decrease in the rate of phylotype detection shown on the curves indicated that the major part of the diversity in the libraries had been detected. Using the formula of Good (1953), the coverage for the both the libraries were calculated to be 94% and 91% respectively. The rarefaction curves also suggested that the sequence population was the least diverse in VB infants and the more diverse in CB infants. This conclusion was further supported by calculating the Shannon indices and the reciprocal of Simpson's indices (table 1). However, the Chao-1 estimator suggested a trend of increasing species richness from CB to VB infants gut (table 1).

3.2 *Comparison of the microbial compositions between the clone libraries*

Bacterial compositions in the clone libraries were compared by calculating the similarity indices and using the LIBSHUFF analysis available freely on RDP II. The *p*-value for the VB vs CB library was calculated to be 0.001, while *p*-value for CB vs VB library was again 0.001. This confirms that both the libraries were significantly different from each other.

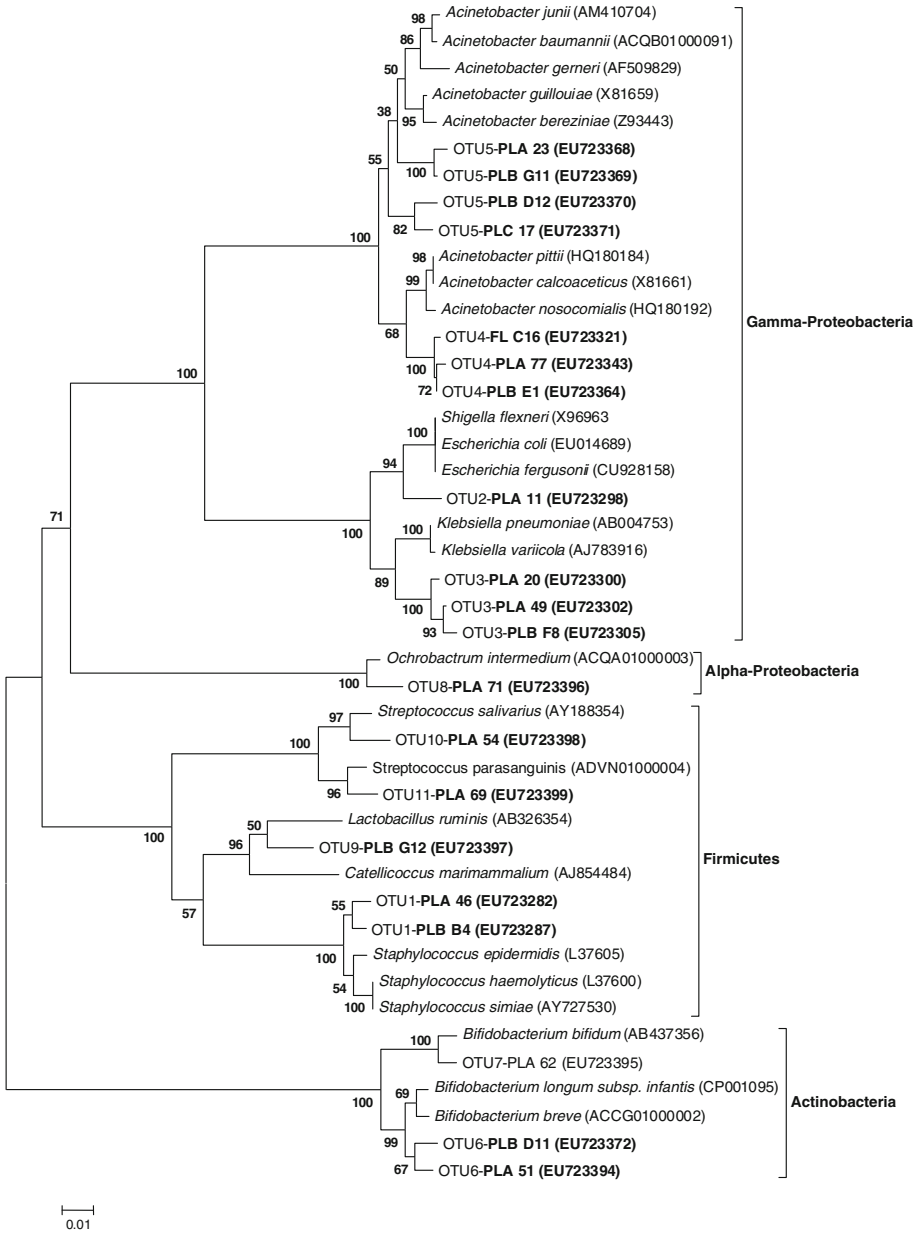

Figure 1. Phylogenetic tree showing relationship among bacterial 16S rRNA gene sequences from full-term VB infants stool sample along with reference sequences retrieved from NCBI database. Bootstrap values (1000 replicates) are shown at the nodes. The scale bar represents genetic distance (1 substitution per 100 nucleotides. The analysis involved 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 777 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Clones obtained from this study appear in bold letters followed by the NCBI accession number. OTU stands for Operational Taxonomic unit.

3.3 *Phylogenetic affiliation of 16S rDNA sequences*

All 16S rDNA sequences obtained in the study were subjected to BLAST search against NCBI GenBank. Majority of the 211 clone sequences showed more than 97% similarity to their nearest database entries. Based on the BLAST results and RDP II classifier, all sequences were assigned into four major phylogenetic group of the domain Bacteria: Firmicutes, Clostridia, Proteobacteria and Actinobacteria (figure 4). The relative abundance of different bacterial species present in each library is summarized in a table using BLAST of representative clone sequences against Ez-Taxon database (table 2).

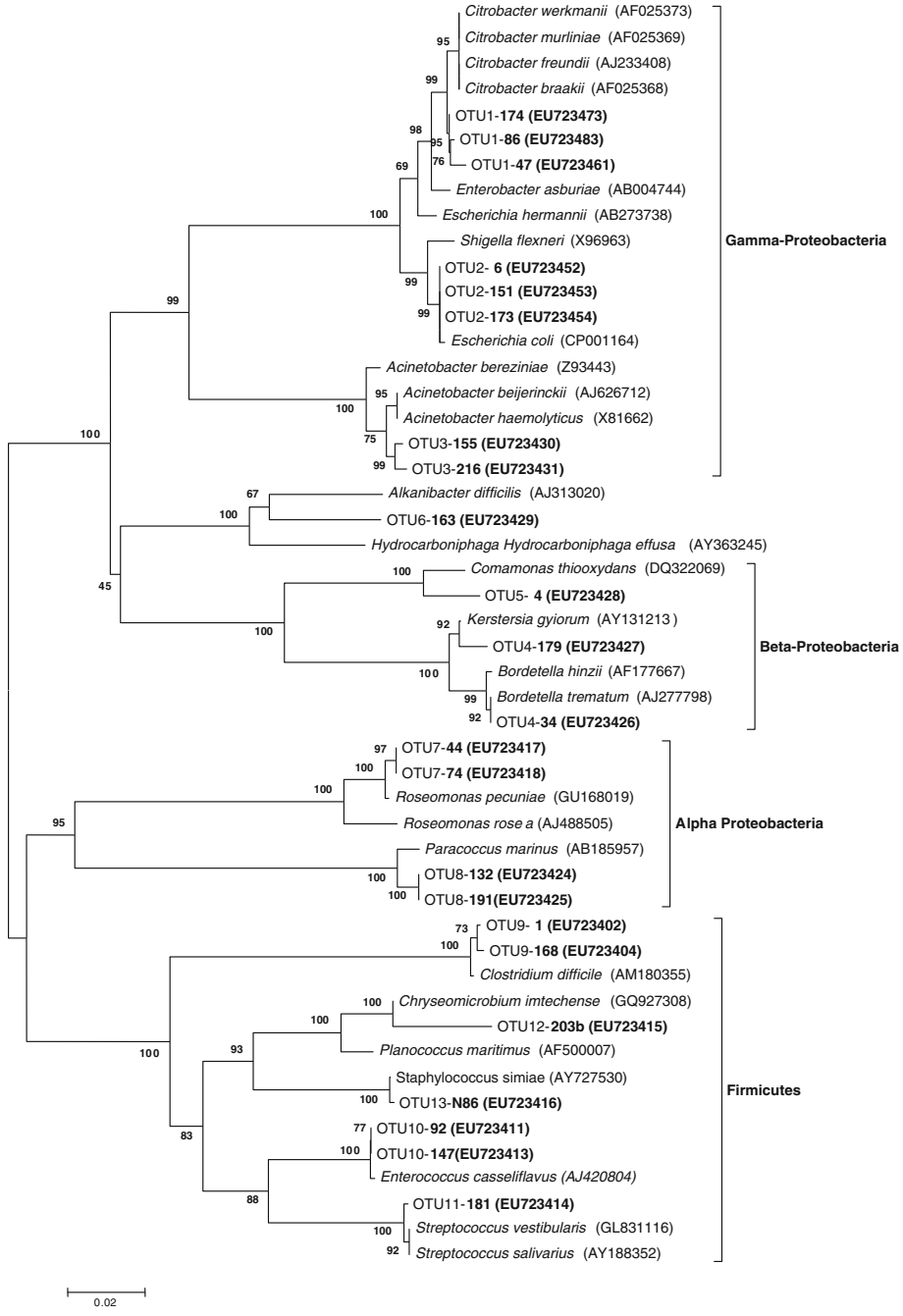

Figure 2. Phylogenetic tree showing the relationship among bacterial 16S rRNA gene sequences from full term CB infants stool sample along with reference sequences retrieved from NCBI database. Bootstrap values (1000 replicates) are shown at the nodes. The scale bar represents genetic distance (2 substitutions per 100 nucleotides). The analysis involved 50 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 807 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Clones obtained from this study appear in bold letters followed by the NCBI accession number. OTU stands for Operational Taxonomic unit.

3.3.1 *Firmicutes:* Twenty-seven out of the 127 clones were affiliated to the firmicutes family in the VB library, constituting 21% of total clone sequences. In CB infants 15 out of the 84 were from firmicutes, which is 17.85% of the total derived sequences. The most abundant firmicute member present in the VB library was *Staphylococcus haemolyticus*,

Table 1. Diversity indices for 16S rDNA libraries obtained for the VB and CB infants day 7 libraries

Community	No. of sequences	No. of phylotypes observed	Diversity measure		
			H0*	1/D**	Chao-1†
VB Day 7	127	11	1.61204	3.7857	21 (13, 63)
CB Day 7	84	13	1.81661	4.4962	15.5 (13, 30)

* Shannon index.

** Reciprocal of Simpson index.

† The values in parentheses are the 95% confidence intervals.

represented by the 24 out of the 127 clone sequences. *Lactobacillus ruminis Streptococcus salivarius* and *Streptococcus parasanguinis* were also found to be represented by one clone sequence each. CB infants library firmicute family was represented by the *Roseomonas pecuniae*, *Paracoccus* sp., *Enterococcus* sp., *Streptococcus vestibularis*, *Chryseomicrobium imtechense* and *Staphylococcus* sp. Among these, *Muricoccus roseus* was the most abundant. Seven clone sequences were affiliated to this species, while others were represented by 2, 3, 1, 1 and 1 clones respectively.

3.3.2 *Clostridium:* In the CB infants *Clostridium difficile* was recovered in relatively large numbers; however, they could not be detected in the VB infants. *Clostridium difficile* is a known noso-comial pathogen and has been implicated in several gut-related disorders (Park *et al.* 2005). The presence of this species in the CB infants may be an indication of the aberrant gut microbial composition in these infants, although further work is required to affirm the same. The percentage of the *C. difficile* in the CB infants was 11%; i.e. 9 out of total 84 clone sequences belong to this species. This was relatively high but no disease symptoms were observed in these babies, which is in agreement with earlier studies (Gewolb *et al.* 1999; Park *et al.* 2005).

3.3.3 *Proteobacteria:* Out of 211 clones sequences, 147(69%) sequences were associated with proteobacteria group, 77 and 70, respectively, for VB and CB infants libraries. In the VB library, a member of beta proteobacteria sub-group could not be observed; however, CB infants library was represented by all three sub-groups, alpha, beta and gamma proteobacteria. Among the sub-groups, gamma proteobacteria were predominant in comparison to the other sub-groups. In VB infants 76 sequences belonged to gamma proteobacteria, while alpha proteobacteria was represented by *Ochrobactrum intermedium*. The number of sequences affiliated to this group was found to be just 1. In CB infants 9 sequences were from alpha proteobacteria, 3 were from beta proteobacteria and the remaining 58 belonged to the gamma proteobacteria sub-group. An interesting finding of the present work was the recovery of the *Acinetobacter* species in the large number from the normal VB infants' stool samples. The *Acinetobacter* sp,. which belongs to gamma proteobacteria sub-group, was the most abundant bacterial group. The *Acinetobacter* species recovered in the clone library were *Acinetobacter pittii* (former Acinetobacter genomic species 3), *Acinetobacter junii* and *Acinetobacter baumannii* constituting approximately 44% of the total clones in this library. Among these, *Acinetobacter pittii*, which was represented by 56 clones, has been recently recognized as a clinically important taxa within the *Acinetobacter calcoaceticus-Acinetobacter*

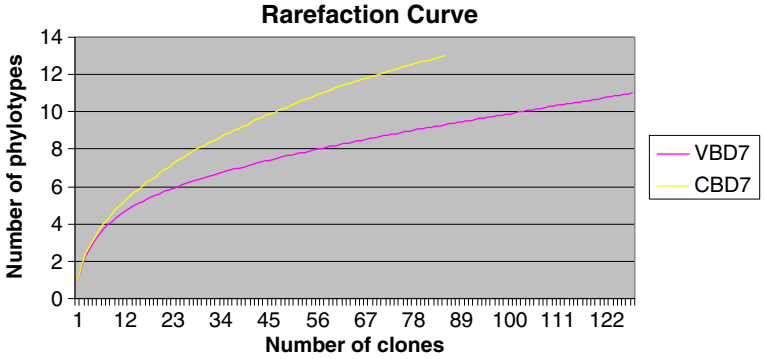

Figure 3. Rarefaction curves for VB (pink line) and CB (yellow line) day 7 Libraries.

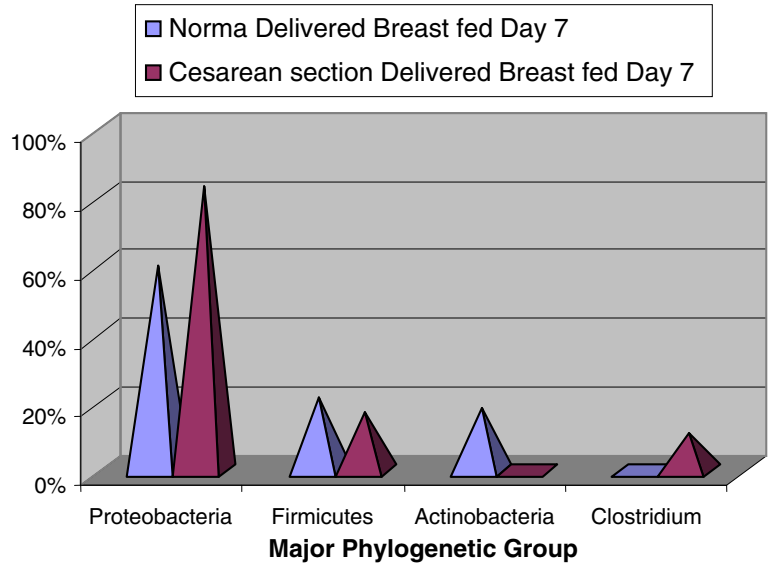

Figure 4. Distribution of clone library sequences in major phylogenetic groups. VB and CB clone libraries were represented by blue and brown bars respectively.

baumannii (ACB) complex. The detection of this species along with the *Acinetobacter baumannii*, a well-known opportunistic pathogen, *in the stool samples of the VB infants requires close attention* (Nemec *et al.* 2011). *However,* the most abundant species from the gamma proteobacteria subgroup in CB infants were *Citrobacter* sp. and *Escherichia coli;* both are the members of Enterobacteriaceae family. Among these, *Citrobacter* sp. was represented by the 34% of total clones, while *Escherichia coli* constituted about 31% of total clones, and together they constituted 65% of total clones in this library.

3.3.4 *Actinobacteria:* The members of actinobacteria, represented mainly by *Bifidobacteria*, are known primary colonizers of neonatal gut. There are several reports that highlight the importance of the *Bifidobacteria* in the health and wellbeing of their hosts (Saarela *et al.* 2002). In the present study, the number of sequences affiliated to the actinobacteria group was found to be 24. However, all of these clone sequences were obtained form the VB infants library. The *Bifidobacterium* species recovered in the VB infants library were *Bifidobacterium longum* sub sp. *infantis*, *Bifidobacterium bifidum* and *Bifidobacterium breve*. This group together constituted 18% of the total clones of the VB library. It was surprising not to get a single clone sequences belonging to the actinobacteria group in CB infants. This confirms the previous reports regarding the disturbed gut microflora of infants born by cesarean section (Gronlund *et al.* 1999).

4. Discussion

The human gut is a complex ecosystem composed of the numerous genera, species and strains of bacteria. The role of these micro-organisms in various useful metabolic activities and in the maintenance of healthy gut environment is well acknowledged (Finegold *et al.* 1983; Benno and Mitsuoka 1986; Suau *et al.* 1999; Gronlund *et al.* 1999; Gronlund *et al.* 2000; Palmer *et al.* 2007; Favier *et al.* 2002). Microbial colonization of the neonatal gut is a very important event in their lives. The initial colonization and acquisition of the gut microbiota can profoundly influence the status of cellular and humoral elements of the gut mucosal immune system during the lives of neonates (Park *et al.* 2005). The composition of enteric microbiota in the early days of life appears to be an important factor for achieving and maintaining good health in the years to come. The type of delivery, gestational period and feeding habitat has been known to greatly influence initial acquisition and colonization of the infant's gut (Saarela *et al.* 2002). Several studies have effectively demonstrated this, but surprisingly there is no report related to the similarities and differences in the gut microbial community composition of infants born by different modes of delivery (vaginal vs cesarean) at their very early stage of life using modern molecular techniques. Our basic understanding of this ecosystem is exclusively based on culture techniques, which are laborious, time consuming and underestimate the bacterial diversity of these ecosystems. Recent studies on the bacterial composition of infant gut

Table 2. Summary of VB and CB Day 7 clone libraries sequences

Clone name (Assigned NCBI accession no.)	Closest neighbour present in public database (Ez-Taxon)	Accession no.	Sequence similarity (%)	No. of clones	
				VB	CB
PLA_46 (EU723282)	*Staphylococcus haemolyticus*	L37600	99.736	24	ND
PLA_11 (EU723298)	*Shigella flexneri*	X96963	99.873	3	ND
PLA_20 (EU723300)	*Klebsiella pneumoniae*	AB004753	99.350	12	ND
FL_C16 (EU723321)	*Acinetobacter pittii*	HQ180184	99.318	56	ND
PLB_G11(EU723369)/ PLA_23(EU723368)	*Acinetobacter junii*/*Acinetobacter baumannii*	AM410704/ACQB01000091	98.800/99.113	4	ND
PLA_62 (EU723395)	*Bifidobacterium bifidum*	AB437356	99.631	1	ND
PLB_D11(EU723372)/ PLA_51 (EU723394)	*Bifidobacterium longum subsp. Infantis/Bifidobacterium breve*	CP001095/ACCG01000002	99.255/99.504	23	ND
PLA_71 (EU723396)	*Ochrobactrum intermedium*	ACQA01000003	99.239	1	ND
PLB_G12 (EU723397)	*Lactobacillus ruminis*	AB326354	97.647	1	ND
PLA_54 (EU723398)	*Streptococcus salivarius*	AY188354	98.965	1	ND
PLA_69 (EU723399)	*Streptococcus parasanguinis*	ADVN01000004	98.726	1	ND
174 (EU723473)/86 (EU723483)	*Citrobacter murliniae*/*Citrobacter werkmanii*	AF025369/AF025373	99.492/98.092	ND	26
151 (EU723453)	*Escherichia coli*	CP001164	99.746	ND	28
155 (EU723430)/216 (EU723431)	*Acinetobacter beijerinckii*	AJ626712	99.007/ 98.968	ND	2
34 (EU723426)/179 (EU723427)	*Bordetella trematum*/*Kerstersia gyiorum*	AJ277798/ AY131213	100.000/ 99.198	ND	2
4 (EU723428)	*Comamonas thiooxydans*	DQ322069	97.722	ND	1
163 (EU723429)	*Alkanibacter difficilis*	AJ313020	94.770	ND	1
44 (EU723417)	*Roseomonas pecuniae*	GU168019	99.381	ND	7
132 (EU723424)	*Paracoccus marinus*	AB185957	98.452	ND	2
168 (EU723404)	*Clostridium difficile*	AM180355	98.970	ND	9
92 (EU723411)	*Enterococcus casseliflavus*	AJ420804	100.000	ND	3
181 (EU723414)	*Streptococcus vestibularis*	GL831116	99.610	ND	1
203b (EU723415)	*Chryseomicrobium imtechense*	GQ927308	97.956	ND	1
N86 (EU723416)	*Staphylococcus simiae*	AY727530	99.736	ND	1
Total number of clones screened				127	84

ND, not detected.

were restricted to the use of a few selective media suited for the isolation of a narrow spectrum of gram-positive bacteria, mainly S*taphylococci*, *Streptococci*, *Bifidobacteria*, and lactic acid bacteria (Gronlund *et al.* 1999; Hughes *et al.* 2001; Kumar *et al*. 2004). The results of such studies cannot be considered illustrative of the total bacterial diversity present in the infant gut. In this context, the aim of present work was to apply 16S rDNA cloning and sequencing approach to carry out a comparative study to describe bacterial diversity present in the infants born by different modes of delivery. There are other 16S-rRNA-based molecular techniques, such as DGGE, TGGE and FISH, which have been widely used and have proved to be valuable tools in characterizing bacterial diversity in the human intestinal tract (Songjinda *et al.* 2005; Schwiertz *et al.* 2003; Leser *et al.* 2002; Mackie *et al.* 1999). An advantage of cloning and sequencing approach is that data generated through cloning and sequencing can be used to design group-specific probes and primers for further studies. The data resulting from present study is important since little or no 16S rDNA sequence information has been previously reported from the gut of these two groups from the Indian subcontinent.

Our results highlight the distinct difference present between the fecal microflora of vaginally born and cesarean section born infants at day 7 of their lives. The vaginally born infants at day 7 of there lives has lower species richness than their counterpart cesarean babies. However, the community composition of the VB infants in our study was similar to that in the earlier works related to the gut microflora of infants, excluding the *Acinetobacter* sp. (Based on

DOTUR, the number of OTUs generated for VB and CB infants were 11 and 13 at 97% cut-off of definition of a species, showing greater species abundance in the latter one.) This finding supports the previous observation of the researches that gut of the CB infants are susceptible to the colonization of many other undesired microbes from the environment, while the VB infants, which acquire their initial flora from their mothers, provides resistance, leading to the less diverse microflora but are relatively stable over the time (Gronlund *et al.* 1999).

The significant difference was observed in case of *Bifidobacterium* sp. prevalence. *Palmer* et al. in their recent paper, using DNA microarrays and gene sequencing to reconstruct the colonization process, had doubted the earlier claims by many researchers that this species constitute the major microflora of the infants gut at very early stages of their lives (Palmer *et al.* 2007). They have reported in their paper that *Bifidobacterium* does not appear to be dominating bacteria in the infant's gut, at least up to several weeks of their life. However, in our study we have found that second most abundant microbe in the gut of the infants born with normal delivery at the day 7 was *Bifidobacterium*, which is in agreement with earlier researchers (Gronlund *et al.* 1999; Park *et al.* 2005). This difference with the findings of the Palmer *et al.* may be attributed to geographical location and different climatic condition of their sample group. On the other hand, cesarean babies from our study were completely devoid of this species, which clearly points towards the role of the mode of delivery in the initial microbial colonization of infants gut.

In the present study, members of Enterobacteriaceae family were represented by the *Shigella flexneri* and *Klebsiella pneumoniae* in VB infants but their number was less in comparison to the other recovered phyla. However, In CB infants *Citrobacter* sp. and *E. coli* species together constituted the 65% of the total bacterial sequences retrieved. This number was very high in comparison to that in an earlier study by Park *et al.* (2005). In recent reports, a high number of aerobic microorganisms has been implicated to be associated with diseases like atopic eczema and asthma in children. Kirjavainen *et al*., in their unpublished data on 69 infants with cow's milk allergy, showed that of those infants who had a detectable concentration of IgE in serum, 74% (36/51) had *E. coli* as part of their most predominant cultivable aerobic flora during breast feeding, while among infants in whom serum IgE was not detected, the respective value was only 39% (7/18). The presence of a large number of *Escherichia coli* in cesarean-section-delivered babies in comparison to their counterpart vaginally delivered babies again points towards the aberrant and disturbed gut microbial community structure in these infants.

Besides all these in our study, surprisingly, we found that *Acinetobacter* sp. were the most abundant bacterial species, constituting a major 44% of the total number of clones in VB infants. These infants were born in hospitals but had spent only few days (1 or 2) in the hospital, whereas cesarean section infants, who spent there initial few days (>5 days) till sample collection in maternity wards, lacked substantial number of *Acinetobacter* sp. The presence of *Acinetobacter* sp. in the feces of VB infants can be explained by the ubiquitous nature of this species. Members of the *Acinetobacter* genus are known to be widely present in soil and water. The infants from our study group might have picked up these from environment during their stay outside the hospital. This speculation is supported by an assumption of Dijkshoorn *et al.* that acinetobacter have some sort of reservoir in the outside patients and these outside patients carry them to the hospitals where they may cause an epidemic in immune suppressed individuals (Dijkshoorn *et al.* 2005).

In conclusion, we successively demonstrated using 16S rDNA library approach that type of delivery exerts direct effect on composition of the gut microflora of infants. Our results confirm that the fecal microflora of VB and CB infants at day 7 of their lives are completely different from each other, which is in agreement with earlier reports (Benno and Mitsuoka 1986). Thus, we hypothesize that further work, to acquire adequate knowledge of the types of microorganism, as well as the events that influence the initial acquisition and colonization of neonatal gut, may provide an important tool to modulate the gut microbiota to enhance the functions beneficial to host physiology and immunity.

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