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*Full Length Research Paper*

# Molecular identification of bifidobacteria from infant faeces

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***Bifidobacteria*** **have been in the spotlight of scientific research due to their health promoting effects in humans. They play a significant role in controlling pH of the large intestine through liberation of lactic and acetic acid, thereby restricting the growth of many potential pathogens and putrefactive bacteria. This paper discusses the isolation and identification of bifidobacterial species from infant faeces by molecular methods. A study was conducted to isolate and identify bifidobacterial species from the faeces of 46 breast fed infants. Molecular tools like 16S rDNA targeted genus and species specific mPCR primers were used for confirmation. A total of 4 isolates were confirmed namely *Bifidobacterium longum* (Isolate code: $IB_{10}$ and $IB_{12}$) *Bifidobacterium breve* (Isolate code: $IB_{39}$) and *Bifidobacterium bifidum* (Isolate code: $IB_{42}$). Subsequently, the nucleotide sequences of the identified species were submitted to the GenBank for acquisition of accession numbers. The accession numbers assigned to the isolates were JN 656706 and JN 656707 for *B. longum* ($IB_{10}$) and *B. longum* ($IB_{12}$), respectively; JN 656708 for *B. breve* ($IB_{39}$) and JN 656705 for *B. bifidum* ($IB_{42}$).**

**Key words:** Faecal *Bifidobacteria*, multiplex polymerase chain reaction (PCR) for *Bifidobacteria*, 16S rDNA*Bifdobacteria,* isolation of *Bifidobacteria*.

## INTRODUCTION

Bifidobacteria are common members of the infant gut where they form up to 91% of the total micro flora in breast-fed babies (Hadadji et al., 2005). The health and nutritional benefits elaborated by bifidobacteria includes antimicrobial activity, immunocompetence and anticarcinogenic properties. Tissier's discovery of *Bifidobacteria* in breast-fed infants established the concept that specific bacteria take part in maintaining human health. About 29 bifidobacterial species have been identified and among them eleven species have been isolated from infant faeces. The most frequently isolated *Bifidobacterium* species in infant faeces are *Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium infantis* and *Bifidobacterium breve* (Matsuki et al., 2003). These Gram positive, non motile, non spore forming anaerobic pleomorphic rods play a significant role as probiotics. These organisms control the pH of the large intestine through production of lactic and acetic acid thereby restricting the growth of many potential pathogens and putrefactive bacteria (Sullivan and Nord, 2002).

Until recently, identification of isolates of *Bifidobacterium* to the genus level has been done through methods that

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**Table 1.** The sequence of primers.

| Name of the primer | Primer sequence | Product size (bp) | Target bifidobacterial species |
|---|---|---|---|
| PBI245f [b] | GCTTGTTGGTGAGGTAACGGCT | 1180 | *B. bifidum* |
| PBR442f b | AGGGAGCAAGGCACTTTGTGT | 991 | *B. breve* |
| PIN710f b | CTGTTACTGACGCTGAGGAGCT | 723 | *B. infantis* |
| PAD805f b | GTGGGGACCATTCCACGGTC | 628 | *B. adolescentis* |
| PLO965f b | TCCCGACGGTCGTAGAGATAC | 467 | *B. longum* |
| Lm3r-m | CGGGGTGCTGCCCACTTTCATG | | Genus specific primer |

consume time and labour like Gram staining, morphological observations and end product analysis of glucose metabolism as indices. However, these tests do not always provide accurate results. Therefore, there is a need for developing practical techniques that enable rapid and accurate analysis of these anaerobic organisms. Recent research has led to rapid advances in the application of molecular techniques based on 16S rDNA and 23S rDNA gene sequences to study the diversity of *Bifidobacteria* in ecosystem (Scardovi, 1986). The identification methods using 16S rDNA sequence, being nucleic acid sequence based, are less subjective.

In the current study, bifidobacterial species were isolated and identified from infant faecal sample by molecular methods.

## MATERIALS AND METHODS

Forty six fresh faecal samples from clinically healthy newborn infants of both sexes born through normal delivery in and around Madhavaram, North Chennai, Tamil Nadu, India were examined. The infants were solely breast fed and ranged in the age group of 3 to 90 days. About 1 g of freshly voided infant faecal samples collected in sterile sample vials containing Yoshioka broth were plated on Yoshioka agar and incubated at 37°C for 48 h under anaerobic condition using Anaero gas pack (Hi media cat.no. LE002F). Presumptive individual colonies were selected for molecular identification using multiplex PCR specific primers and 16S rDNA primer.

### Molecular identification of bifidobacterial species

Genomic DNA was extracted from the 24 h presumptive bifidobacterial culture using Hi yield genomic DNA mini kit (cat. no. YGB 100/YGB 300) from Real Biotech Corporation, India.

### Multiplex PCR

Multiplex PCR with five primer sets designed by Dong et al. (2000) was used for the detection of bifidobacterial species. The sequence of primers is shown in Table 1.

The reaction mixture consisted of Genei red dye PCR mix (cat.no.2x 105908), nuclease free water (cat. no. 129114), DNA template and the above primers. Multiplex PCR was done with primers for *B. adolescentis, B. bifidum, B. breve, B. longum* and *B. infantis* based on the difference in their PCR product sizes as described by Dong et al. (2000). A 20 µl reaction mixture was prepared in a 0.2 ml thin walled PCR tube. The PCR amplification was carried out in the automated thermal cycler (Eppendorf Mastercycler gradient). Initial denaturation of 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 60 s. Ampification products were detected by electrophoresis on 1% agarose followed by ethidium bromide staining and viewed using UV transilluminator. The PCR products were visualized by electrophoresis on a 1% w/v agarose gel by staining with ethidium bromide and viewed using UV transilluminator, Gel Doc Med C Care Gel Stan 1312 make.

### 16S rDNA

A fragment of the 16S rDNA gene was amplified using Primers fD1–AGAGTTTGATCCTGGCTCAG and rP2-ACGGCTACCTTGTTACGACTT (Weisburg et al., 1991). A 50 µl reaction mixture was prepared in a 0.2 ml thin walled PCR tube. The reaction mixture consisted of Genei red dye PCR mix, forward and reverse primers, DNA template and MilliQ distilled water. Amplification was performed in a thermal cycler (Eppendorf Mastercycler gradient) as per the protocol adopted by Matsuki et al. (2003), which is as follows: 1 cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min and a final cycle of 72°C for 5 min. Amplification products were separated by electrophoresing through 1.5% agarose gel in TBE buffer. Products were purified using a Qiagen Gel purification kit and the amplified products were sequenced. The amplified 16S rDNA sequences were BLAST analyzed using NCBI data base for sequences similarity based identification. The bifido bacterial sequences were deposited in the Gen Bank database using the web-based data submission tool, BankIt(http://www.ncbi.nlm.nih.gov/BankIt).

## RESULTS

### Molecular identification of bifidobacterial species

#### *Multiplex polymerase chain reaction*

Figure 1 illustrates the use of multiplex PCR for detection of species using species specific primer of the required product size.

The present usage of multiplex PCR is based on the work of Dong et al. (2000). The multiplex primers included *B. bifidum, B. breve, B. infantis, B. longum* and *B. adolescentis*. Isolates $IB_{10}$ and $IB_{12}$ presented a product size of 467 bp, $IB_{39}$ and $IB_{42}$ presented a product size of 991

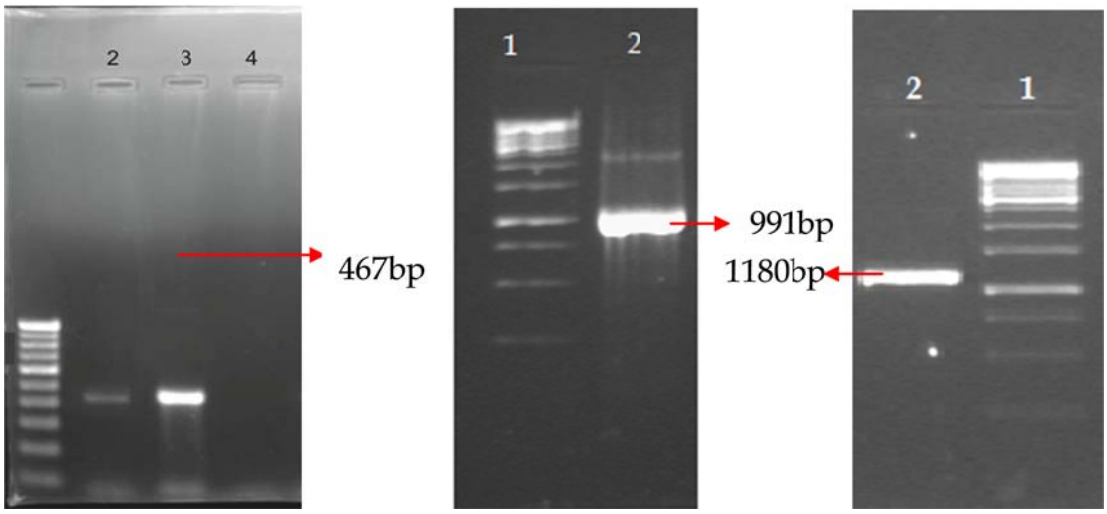


**Figure 1.** Multiplex polymerase chain reaction. Lane 1: Marker; Lane 1: Marker; Lane 1: Marker; Lane 2: *B. longum* (467 bp); Lane 2: *B. breve* (991 bp); Lane 2: *B. bifidum* (1180 bp).

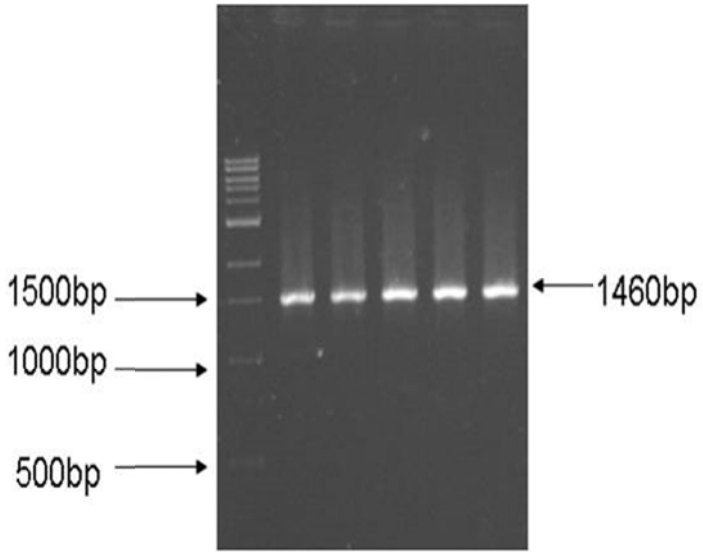


**Figure 2.** 16S rDNA sequencing.

and 1180 bp, respectively. The findings in the present study corroborate with the product size of the reference strains. The results thus showed isolates $IB_{10}$ and $IB_{12}$ belong to *B. longum*, $IB_{39}$ and $IB_{42}$ belong to *B. breve* and *B. bifidum* respectively

## Identification by sequencing 16S rDNA gene

Figure 2 illustrates the product size of 1460 bp of the genus *Bifidobacteria* using universal 16S rDNA based primer on PCR amplification.

The sequences were BLAST analyzed using the NCBI blast site and the isolates were identified as *B. longum, B. breve* and *B. bifidum*. All the 16S rDNA gene sequences were deposited in Genbank for the accession numbers.

The complete 16S rDNA gene sequences of the four isolates namely *B. longum* ($IB_{10}$, $IB_{12}$), *B. breve* ($IB_{39}$) and *B. bifidum* ($IB_{42}$) were aligned with the *Bifidobacteria* sequences obtained from the NCBI database by using the ClustalW software. Phylogenetic tree was constructed using DNA Star software. The similarity matrix was constructed.

Figures 3, 4 and 5 shows the phylogenetic tree based on 16S rDNA gene sequence analysis showing the phylogenetic relationship among the global isolates of *Bifidobacteria*.

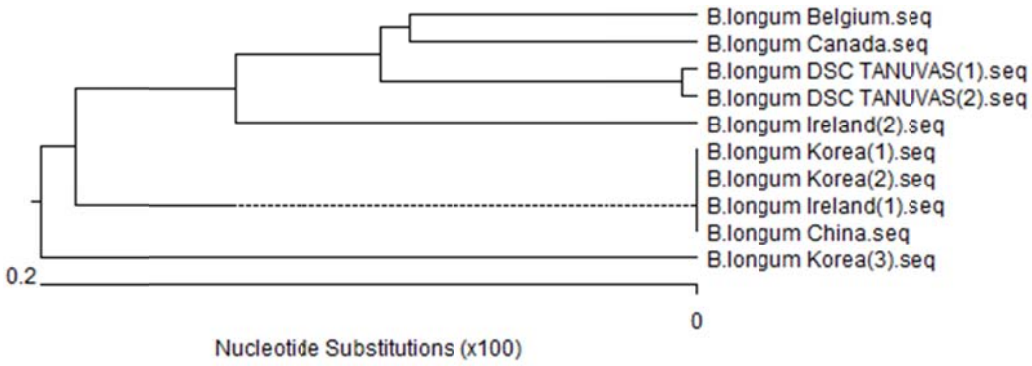


**Figure 3.** Phylogenetic tree based on 16S rDNA sequence showing the phylogenetic relationship of isolated *B. longum* with global isolates.

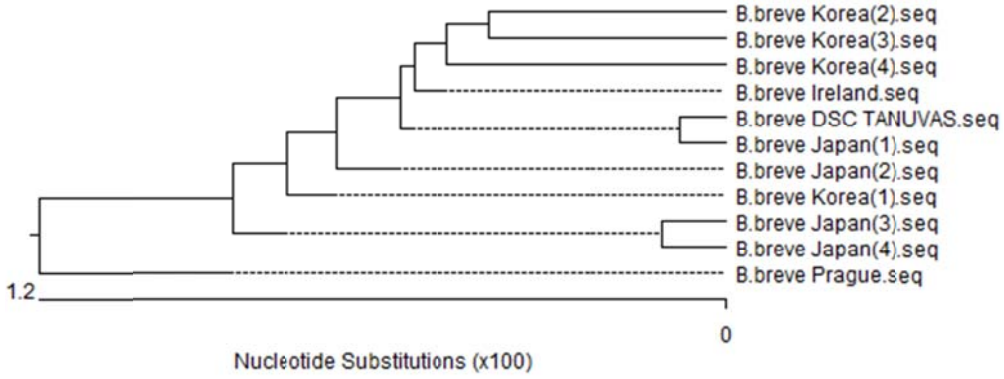


**Figure 4.** Phylogenetic tree based on 16S rDNA sequence showing the phylogenetic relationship of isolated *B. breve* with global isolates.

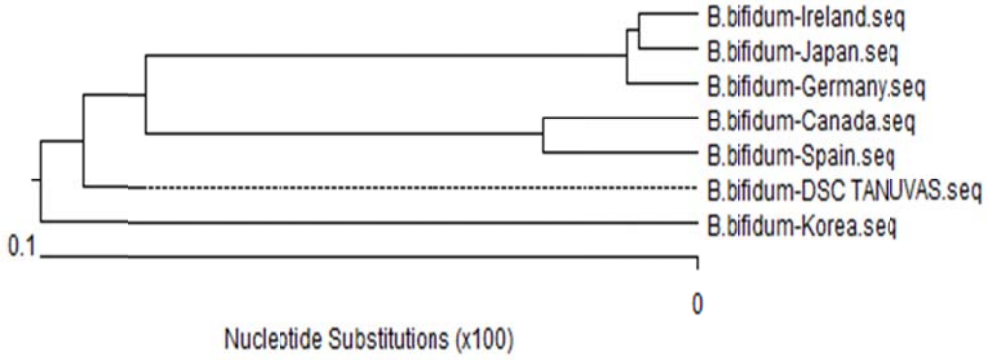


**Figure 5.** Phylogenetic tree based on 16S rDNA sequence showing the phylogenetic relationship of isolated *B.mbifidum* with global isolates.

### Accession number of *Bifidobacterium* species based on molecular tools using multiplex PCR and 16S rDNA

Table 2 gives the accession number of the isolates obtained from the Genbank.

## DISCUSSION

### Identification of bifidobacterial species in infant faeces

The incidence and isolation of *Bifidobacteria* from breast

**Table 2.** Accession number of *Bifidobacterium* species based on molecular tools using multiplex PCR and 16S rDNA.

| Isolate identity code no. | Species | Accession number obtained from GenBank |
|---|---|---|
| $IB_{10}$ | *Bifidobacterium longum* | JN 656706 |
| $IB_{12}$ | *Bifidobacterium longum* | JN 656707 |
| $IB_{39}$ | *Bifidobacterium breve* | JN 656708 |
| $IB_{42}$ | *Bifidobacterium bifidum* | JN 656705 |

fed infant faeces in the present study corroborates with Roberts et al. (1985), Tamime et al. (1995), Silvi et al. (1996) and Martin et al. (2009) who reported that human milk is favourable for the growth and sustenance of *Bifidobacteria* in the large intestine of infants.

Wasilewska and Bielecka (2003) also isolated and identified fourteen bifidobacterial strains from faeces harbouring the gut of 3-month old breast fed infant. Vlkova et al. (2005) reported the presence of *Bifidobacteria* from twenty nine infant faeces out of the ninety five faecal samples collected. Hence the breast fed infant faecal sample seemed to be an ideal source of *Bifidobacteria*.

## Multiplex polymerase chain reaction

Matsuki et al. (2003) suggested that for the isolation of *Bifidobacterium* from infant faeces it is efficient to apply the primers for *B. breve, B. bifidum, B. longum* and *B. infantis* as he observed the presence of these species most frequently in infant faeces. Accordingly in this study, the use of these species specific primers, designed using the reference primers of Dong et al. (2000) identified four of the faecal isolates as *B. longum* ($IB_{10}$ and $IB_{12}$), *B. breve* ($IB_{39}$) and *B. bifidum (*$IB_{42}$*)*. Isolates $IB_{10}$ and $IB_{12}$ presented a product size of 467 bp, $IB_{39}$ and $IB_{42}$ presented a product size of 991 and 1180 bp, respectively. The reference species specific primers designed by Dong et al. (2000) showed *B. longum* to have a product size of 467 bp. *B. breve* and *B. bifidum* had a product size of 991 and 1180 bp, respectively. The findings in the present study thus corroborates with the product size of the reference strains designed by Dong et al. (2000).

Cleusixa et al. (2010) also used molecular tools like multiplex qPCR assay which enabled the enumeration of *Bifidobacterium* spp. by targeting the bifidobacterial xylulose-5-7 phosphate/fructose-6-phosphate phosphoketolase gene (xfp).

## Identification by sequencing of the 16S rDNA gene

Figure 2 illustrated the product size of approximately 1460 bp of the genus *Bifidobacteria* using universal 16S rDNA based primer. Similarly Ruben et al. (1996) also amplified a fragment of 1468 bp of 16S rDNA of *Bifidobacterium* sp. strain LW 420 through PCR using primers.

In modern bacterial taxonomy, ribosomal gene sequences are considered to have the potential to provide a powerful approach to the investigation of phylogenetic relationship. Amplification of 16S rDNA genes was carried out in this study as that of Michael et al. (2000) who reported that the highly conserved portions of 16S rDNA genes are ideal for designing primers that will amplify small subunit of rDNA genes. Giovanna and Dellaglio (2007) also studied the phylogenetic tree based on 16S rDNA gene sequence analysis showing the phylogenetic relationships among the species of *Bifidobacterium*. Similarly, in the present study, the information gained from the 16S rDNA gene sequence gave a comparison to deduce detailed phylogenetic relationship with other global isolates. In the present study, the isolated species of the genus *Bifidobacterium* formed a coherent phylogenetic unit and generally showed over 98% similarity of 16S rDNA sequences with other members of the global isolates.

The phylogenetic tree of the isolates $IB_{10}$ and $IB_{12}$ (*B. longum*), $IB_{39}$ (*B. breve*) and $IB_{42}$ (*B. bifidum*) are shown in Figure 3, 4 and 5. The phylogenetic tree showed that the isolates $IB_{10}$ and $IB_{12}$ *(B. longum)* were closely related to and in the same clade as the isolates from Belgium, Canada and Ireland. The multiple alignment identity matrix showed that the isolates $IB_{10}$ and $IB_{12}$ had a homology of over 99% with all selected global *B. longum* species. Both isolates had a divergence of 0.1 with the isolates of Belgium, Canada and Ireland.

The phylogenetic tree showed that the isolate $IB_{39}$ (*B.breve*) was closely related to the isolate from Japan (1) and was in the same clade as the isolates from Korea (2), Korea (3), Korea (4) and Ireland. The multiple alignment identity matrix showed that the isolate $IB_{39}$ had a homology of over 98% with selected global *B.breve* species. $IB_{39}$ had a divergence of 0.2 with the isolates of Japan (1) and Ireland. The isolate had a divergence of 0.8, 1.0 and 0.5 with the isolates of Korea (2), Korea (3) and Korea (4).

The phylogenetic tree showed that the isolate $IB_{42}$ (*B. bifidum*) was closely related to the isolates from Japan, Germany and Ireland. The multiple alignment identity matrix showed that the isolate $IB_{42}$ had a homology of over 99% with selected global *B. bifidum* species.

The rDNA- or rRNA targeted studies sheds new light on the bacterial colonization of the intestines and offers possibilities to accurately monitor the development of the population of this organism in the intestinal flora of new-born infants. Furthermore, PCR with the strain-specific primers, together with phenotypic tests, can well serve as important markers for identification of species, as pro-biotic strains as reported by Giovanna and Dellaglio (2007).

The isolation of *B. breve, B. bifidum* and *B. longum* in the present study coincides with the findings of Mullie et al. (2006) who reported in their findings the occurrence *of B. breve, B. bifidum* and *B. longum* as the frequently isolated species of *Bifidobacteria* in the infant faeces.

The findings in Table 2 are in accordance with Tamime et al*.* (1995) and Vlkova et al. (2005) who reported the dominating species of *Bifidobacteria* in intestinal flora of breast fed infants as *B. longum*, *B. breve and B. bifidum.*

Loquasto et al. (2013) sequenced the genome of *B. animalis subsp. lactis* ATCC 27673 and compared it with other strains using molecular tools for probiotic activity.

Baffoni et al. (2013) identified *Bifidobacterium* species by adopting hsp60 as the universal primers in a simple PCR procedure. They reported this molecular tool as simple and cheap for identification of bifidobacteria in a mixed population. Hence, the use of molecular tools like mPCR can aid in detecting probiotic bifidobacteria from mixed population

## Conflict of interest

The author(s) have not declared any conflict of interests.

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