Hierarchical Oligonucleotide Primer Extension as a Time- and Cost-Effective Approach for Quantitative Determination of *Bifidobacterium* spp. in Infant Feces[∇]

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The *Bifidobacterium* spp. present in 10 infant fecal samples (4 from infants with eczema and 6 from healthy infants) were quantified with both hierarchical oligonucleotide primer extension (HOPE) and fluorescence in situ hybridization-flow cytometry. The relative abundances of *Bifidobacterium longum* and *B. catenulatum* with respect to the total bifidobacteria had a poor correlation (ρ , <0.600; P value, >0.208), presumably due to differences in primer specificity and the level of hybridization stringency of both methods. In contrast, the relative abundances of organisms of the genus *Bifidobacterium* against the total amplified 16S rRNA genes and those of *B. adolescentis*, *B. bifidum*, and *B. breve* against the genus *Bifidobacterium* exhibited a good statistical correlation (ρ , >0.783; P value, <0.066). This good comparability supports HOPE as a method to achieve high-throughput quantitative determination of bacterial targets in a time- and cost-effective manner.

The “microflora hygiene” hypothesis states that a lack of exposure to pathogens or certain commensal bacteria in early life may predispose some individuals to allergic disorders (14). However, inconsistent findings on the abundance of health-associated microbes have prevented precise conclusions as to their role in modulating host health. For example, by performing fluorescence in situ hybridization (FISH) on infant feces, *Bifidobacterium* spp. were found in high abundance in healthy infants (11). In contrast, certain species, like *Bifidobacterium pseudocatenulatum*, may be more commonly detected in infants with eczema (3). Therefore, to facilitate our understanding of microbial composition and its correlation to human health, it is essential to use a rapid and high-throughput molecular method to determine the abundances of bacterial targets in a large sample size (16). Although FISH-flow cytometry (FISH-FC) is routinely used to quantify the abundances of bacterial targets in feces (9, 11, 17), it does not suffice as a high-throughput method due to the limited range of spectrally distinct fluorophores that are available in the UV spectrum (10, 13). There is a need to develop a high-throughput technique which can complement the existing molecular methods to rapidly evaluate the relative abundance of bacterial targets.

A molecular method termed hierarchical oligonucleotide primer extension (HOPE) was developed to rapidly determine the relative abundances of bacterial 16S rRNA genes among total PCR-amplified 16S rRNA genes (19). HOPE uses primers of different lengths that were designed to target bacteria at different phylogenetic levels. The primers anneal to complementary regions of the targeted bacteria and extend with a

fluorophore-labeled nucleotide when the bacterial target is present. The extended primers can be differentiated on a genetic analyzer based on primer length and fluorophore color. The relative abundance of the bacterial target against a higher-level primer can then be quantified by calculating the ratio of the peak area of the extended primer with that of a higher-level primer. A subsequent study demonstrated that HOPE can be used for rapid and specific determination of *Bacteroides* spp. present in feces and wastewaters at different taxonomical levels (5). It also has the versatility to be expanded to include other bacterial groups. This can potentially facilitate the identification and quantification of bacterial populations that modulate the health of an individual at different temporal intervals.

This study aimed to demonstrate HOPE as a time- and cost-effective method to quantify the abundances of *Bifidobacterium* spp. in 10 infant fecal samples (4 from infants with eczema and 6 from healthy infants) that were collected at 1, 3, and 12 months of age. The abundances of the *Bifidobacterium* spp. as determined, respectively, by HOPE and FISH-FC were also compared to validate the use of HOPE as a quantitative method.

To obtain the total PCR-amplified 16S rRNA genes, genomic DNA of the fecal microbiota was extracted based on a previously described protocol (12) prior to 20 cycles of PCR amplification (modified 11F forward primer 5'-GTT YGA TYC TGG CTC AG-3' and 1492R reverse primer 5'-GGY TAC CTT GTT ACG ACT T-3') (6, 7). The amplicons were purified, and the concentrations were diluted to 10 ng/μl. For HOPE, a total of 12 primers specifically targeting six *Bifidobacterium* spp. at different taxonomic levels were designed based on a previously described protocol (5). The specificity of the designed primers was verified in silico against entries in RDP II (2), and the sensitivity of the primers was determined as described previously (19). FISH-FC was performed on the same set of fecal samples based on the protocol described by

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TABLE 1. HOPE primers and FISH probes included in this study

Primer or probe	Target(s) (no. of RDP II hits) ^a	Sequence (5'–3')	Poly(A) tail length (nt)	Type of ddNTP ^b added	HOPE reaction tube no.	Reference
HOPE primers						
Bia183_spe	<i>B. adolescentis</i> (62)	AAG GAC ATG CAT CCA ACT	0	G	2	This study
Biang183_spe	<i>B. angulatum</i> (3)	TTC CCA GAC CAC CAT GCG ATG GAC T	0	G	2	This study
Bibif183_spe	<i>B. bifidum</i> (11)	GAA TCT TTC CCA CAA TCA CAT GCG AT	6	C	2	This study
Bbreve1264_spe	<i>B. breve</i> (11)	CAG GGA TCC GCT CCA GCT CGC A	4	C	2	This study
Bicat181_spe	<i>B. catenulatum</i> , <i>B. pseudocatenulatum</i> (41)	CCA TGC GAG GAG TCG GAG CA	0	T	2	This study
Bil181_spe	<i>B. longum</i> (12)	CAT GCG ATC AAC TGG AA	5	C	1	This study
Bifgp1120_clu	<i>B. breve</i> , some <i>B. longum</i> isolates (19)	ACA ATC CGC TGG CAA CAC G	15	G	1, 2	This study
Bifgp1250_clu	<i>B. dentium</i> , <i>B. bifidum</i> (14)	GTC GCC ATG TCG CAT CCC GC	10	T	1, 2	This study
Bifgp442_clu	<i>B. adolescentis</i> , <i>B. ruminatum</i> (75)	CCG AAG GGC TTG CTC CCA G	15	T	1, 2	This study
Bifgp272_clu	<i>B. angulatum</i> , <i>B. catenulatum</i> , <i>B. pseudocatenulatum</i> (49)	GCC GGC TAC CCG TCG TAG GCT C	8	G	1, 2	This study
Bif660_gen	Most <i>Bifidobacterium</i> spp. (308)	CCA CCG TTA CAC CGG GAA TTC CAG	0	T	1	8 ^c
Eub338Ia_dom	Most <i>Bacteria</i> spp. (221136)	GCT GCC TCC CGT AGG AG	23	T	1	19
FISH probes						
Bado434	<i>B. adolescentis</i> (79)	GCT CCC AGT CAA AAG CG				17
Bang198	<i>B. angulatum</i> (3)	AAT CTT TCC CAG ACC ACC				17
Bbif186	<i>B. bifidum</i> (13)	CCA CAA TCA CAT GCG ATC ATG				17
Bbre198	<i>B. breve</i> (28)	AAA GGC TTT CCC AAC ACA CC				17
Bcat187	<i>B. catenulatum</i> , <i>B. pseudocatenulatum</i> (40)	ACA CCC CAT GCG AGG AGT				17
Blon1004	<i>B. longum</i> (48), some <i>Spirochaeta</i> spp. (16)	AGC CGT ATC TCT ACG ACC GT				17
Bif164	Most <i>Bifidobacterium</i> spp. (293)	CAT CCG GCA TTA CCA CCC				8
Eub338	Most <i>Bacteria</i> spp. (220802)	GCT GCC TCC CGT AGG AGT				1
Non338	Non- <i>Bacteria</i> spp. (0)	ACT CCT ACG GGA GGC AGC				18

^a Denotes that only good-quality 16S rRNA sequences of >1,200 bp were subjected to BLAST analysis to retrieve the number of perfectly matched hits in RDP II.

^b ddNTP, dideoxynucleoside triphosphate.

^c Modified from reference 8.

Lay et al. (9). Table 1 lists the HOPE primers and FISH probes used in this study. A nonparametric Spearson ranked correlation analysis (Minitab) was performed on the abundances of *Bifidobacterium* spp. as quantified by FISH-FC and HOPE, respectively.

The extended HOPE primers were detected in the genetic analyzer when target DNA templates made up more than 0.10% of the total genomic DNA. The lower detection sensitivity of the *Bifidobacterium*-targeting primers than those obtained in previous studies (5, 19) may be due to the high GC content of DNA templates. As *Bifidobacterium* spp. are predominant in infant feces (4, 16), the effect of the low primer sensitivities on subsequent findings could be negligible.

Our findings agree with previous studies and suggested that the genus *Bifidobacterium* was predominant in the infants' fecal microbiota (Fig. 1). Furthermore, it was observed that the abundances varied across individuals and with time (Fig. 1). In most individuals, the relative abundances of the genus *Bifidobacterium* against the total amplified 16S rRNA genes were also highest in the fecal samples that were collected at 1 and 3 months after birth (Fig. 1). On average, *B. adolescentis* was only detected by both HOPE and FISH-FC in the fecal microbiota that was collected 12 months after birth (Fig. 2). In contrast, the *B. catenulatum* group and *B. bifidum* were consistently detected at all sampling times and at relatively high abundances of up to 28.5% and 16.6% of the total bifidobacteria, respectively (Fig. 2). Furthermore, *B. breve* was detected

in the fecal microbiota of infants with eczema at 1 and 3 months after birth and at relative abundances that ranged from 2.8 to 7.9% of the total bifidobacteria (Fig. 2). In contrast, a reverse trend was observed throughout the period in healthy infants (Fig. 2). However, the role of *B. breve* in atopic eczema cannot be conclusively determined from this study as other variables such as the mode of delivery and the dietary regimen were not investigated (15).

To determine the comparability of HOPE and FISH-FC, the relative abundances of *Bifidobacterium* spp. quantified by both methods were statistically analyzed by nonparametric Spearson correlation analysis. The relative abundances of *B. longum* and the *B. catenulatum* group against the genus *Bifidobacterium* did not show a significant correlation (*P* values = 0.208 and 0.623, respectively) (Table 2), and the discrepancy may be due to the different specificities of the HOPE primers and FISH-FC probes. Table 1 showed that FISH-FC probe Blon1004 was designed to target *B. longum* at a fourfold higher coverage than the HOPE primer (Bil181) and would understandably result in a significant difference between the abundances detected. Although the HOPE primer and the FISH-FC probe that target the *B. catenulatum* group have similar specificities, the discrepancy in the abundances detected may be due to the difference between the hybridization stringencies of the two methods. In this study, fluorescently labeled probes for FISH-FC were hybridized to their complementary 16S rRNA genes at 35°C (9). The low hybridization temperature may have resulted in cross-

FIG. 1. Relative abundances of bifidobacteria in fecal samples obtained from 10 infants at 1, 3, and 12 months after birth. Abundances were quantified by HOPE (A) and FISH-FC (B).

TABLE 2. Nonparametric correlation analysis of the relative abundances of *Bifidobacterium* spp. determined by HOPE and FISH-FC

Bacteria	Spearson correlation (ρ)	P value	Correlation of abundances quantified by HOPE and FISH-FC
All <i>Bifidobacterium</i> spp. ^a	0.829	0.042	Good at 90% confidence level
<i>B. adolescentis</i> ^b	0.920	0.009	
<i>B. breve</i> ^b	0.857	0.029	
<i>B. bifidum</i> ^b	0.783	0.066	Fairly good at 85% confidence level
<i>B. catenulatum</i> group ^b	0.600	0.208	Not significant
<i>B. longum</i> ^b	0.257	0.623	

^a The relative abundances of *Bifidobacterium* spp. against the total *Bacteria* spp. amplified were compared in this correlation analysis.
^b The relative abundances of the individual *Bifidobacterium* sp. shown against the total amplified *Bifidobacterium* spp. were compared in this correlation analysis.

hybridization with nontargets and therefore comparably higher abundances of the *B. catenulatum* group than those quantified by HOPE (Fig. 1).
Despite the poor correlation of the relative abundances of *B. longum* and the *B. catenulatum* group, a fairly good correlation, ranging from 0.783 to 0.920, was obtained for the relative abundances of the bifidobacteria with respect to the total bacteria and also for the relative abundances of individual species like *B. adolescentis*, *B. bifidum*, and *B. breve* against the genus *Bifidobacterium* (average *P* value = 0.04) (Table 2).
FISH-FC and quantitative PCR are two molecular methods used to examine the *Bifidobacterium* spp. present in the fecal microbiota. Compared to FISH-FC, the entire HOPE procedure for the identification and quantification of *Bifidobacte-*

FIG. 2. Relative abundances of *Bifidobacterium* spp. and total bifidobacteria. Abundances in samples from the infants in the respective health groups and time points were averaged and quantified by HOPE (○) and FISH-FC (△).

rium spp. after primary DNA extraction and PCR amplification took less than 120 min (5, 19), which was significantly shorter than that required for FISH-FC. Furthermore, we demonstrate the use of inexpensive unlabeled oligonucleotide primers to achieve up to nine-plexing per reaction. Compared to quantitative PCR, which uses fluorescently labeled PCR assays like the TaqMan, HOPE would allow a relatively more cost-effective examination of up to 864 targets in a 96-well plate format.

Furthermore, HOPE is highly adaptable and allows the total number of detectable bacterial targets to be easily increased simply by adding HOPE reactions or by adding a primer(s) to individual reactions. For example, although the HOPE primer targeting *B. longum* is highly specific, it did not achieve satisfactory coverage of the entire *B. longum* group. Primers that target *B. dentium* and *B. infantis* were also not included in this study. These species may constitute the large unidentified fraction of *Bifidobacterium* spp. that was not accounted for. Besides profiling for these known *Bifidobacterium* spp., the yet-to-be-cultured *Bifidobacterium* spp. can also be identified by the construction of 16S rRNA gene libraries and designed with new HOPE primers that target the unrepresented *Bifidobacterium* spp. The current list of primer assays can be easily expanded to include these new primers so as to provide more comprehensive coverage of the bifidobacterial population that is present in infant feces.

In summary, this study has demonstrated the potential of HOPE as a time- and cost-effective detection method that can examine the relative abundances of bacterial targets at various taxonomic levels. It can be used to capture possible changes in the abundances of *Bifidobacterium* spp. and/or other bacterial targets present in infant feces. The abundances can then be correlated with clinical disorders such as allergic diseases, and the findings will eventually assist in the elucidation of the roles played by microorganisms in the mediation of immune responses.

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