Clonal Analysis of the Microbiota of Severe Early Childhood Caries

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Abstract

Background/Aims: Severe early childhood caries is a microbial infection that severely compromises the dentition of young children. The aim of this study was to characterize the microbiota of severe early childhood caries. ***Methods:*** Dental plaque samples from 2- to 6-year-old children were analyzed using 16S rRNA gene cloning and sequencing, and by specific PCR amplification for *Streptococcus mutans* and *Bifidobacteriaceae* species. ***Results:*** Children with severe caries (n = 39) had more dental plaque and gingival inflammation than caries-free children (n = 41). Analysis of phylotypes from operational taxonomic unit analysis of 16S rRNA clonal metalibraries from severe caries and caries-free children indicated that while libraries differed significantly (p < 0.0001), there was increased diversity than detected in this clonal analysis. Using the Human Oral Microbiome Database, 139 different taxa were identified. Within the limits of this study, caries-associated taxa included *Granulicatella elegans* (p < 0.01) and *Veillonella* sp. HOT-780 (p < 0.01). The species associated with caries-free children included *Capnocytophaga gingivalis* (p < 0.01), *Abiotrophia defectiva* (p < 0.01), *Lachnospiraceae* sp. HOT-100 (p < 0.05), *Streptococcus sanguinis* (p < 0.05) and *Streptococcus cristatus* (p < 0.05). By specific PCR, *S. mutans* (p < 0.005) and *Bifidobacteriaceae* spp. (p < 0.0001) were significantly associated with severe caries. ***Conclusion:*** Clonal analysis of 80 children identified a diverse microbiota that differed between severe caries and caries-free children, but the association of *S. mutans* with caries was from specific PCR analysis, not from clonal analysis, of samples.

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Aggressive and progressing dental caries in young children is of high clinical significance because affected children can have both extensive caries in primary teeth and a high risk of spread of the caries to the permanent dentition [Li and Wang, 2002]. Early childhood caries (ECC), dental caries in the primary dentition, affects 28% of the US population, particularly disadvantaged ethnic and socioeconomic groups [Beltran-Aguilar et al., 2005]. Severe ECC can affect multiple teeth in children under 6 years of age [Drury et al., 1999], and these children frequently have painful abscesses and reduced ability to sleep or eat, which can lead to poor quality of life [Slade, 2001; Vargas et al., 2005; Clarke et al., 2006]. The exten-

sive restorative therapy needed usually requires general anesthesia [Berkowitz, 2003].

The primary microbial etiology of ECC, determined mainly from selective cultural analysis, is *Streptococcus mutans* [Van Houte et al., 1982]. *Bifidobacterium* species have also been isolated using selective culture from childhood caries [Mantzourani et al., 2009b], and in older adults they were more strongly associated with caries than mutans streptococci [Mantzourani et al., 2009a]. Using 16S rRNA probes, *Bifidobacterium* species were also associated with ECC but were detected at lower levels than *S. mutans* [Aas et al., 2008]. Clonal analyses of environmental samples using broad-range primers have revealed a wide bacterial diversity, including previously uncultivated species [Hugenholtz et al., 1998]. Cloning and sequencing analysis of bacterial plaque samples from the oral cavity detected >700 phylotypes and species [Paster et al., 2001; Relman, 2002; Munson et al., 2004]. Oral species detected by clonal analysis of childhood caries included *S. mutans* and non-mutans *Streptococcus* species, *Lactobacillus, Actinomyces, Bifidobacterium* and *Veillonella* species, and uncultured taxa [Becker et al., 2002; Aas et al., 2008]. Both clonal [Becker et al., 2002; Aas et al., 2008] and cultural analysis of extensive childhood caries [Loesche et al., 1975], however, indicated that *S. mutans* formed only a low proportion of the total caries microbiota. These reports indicated that *S. mutans* was not the dominant species in dental caries, which suggested that other species, including uncultivated ones, could be important in the etiology of this infection.

The aim of this study was to characterize the microbiota associated with severe ECC by comparing the microbiota with that of caries-free children. A 16S rRNA gene broad-range PCR followed by clonal analysis was performed and data were analyzed by phylotypes generated by operational taxonomic unit (OTU) analysis and by comparison with the Human Oral Microbiome Database (HOMD) [Dewhirst et al., 2008]. A specific PCR was used to detect *Streptococcus mutans* and *Bifidobacterium* species. The study hypothesized that the microbiota would be different between severe ECC and caries-free children and that new caries-associated taxa would be candidates as caries pathogens.

Subjects and Methods

Clinical Methods

Eighty preschool children, 39 severe ECC and 41 caries-free, were recruited from 3 dental clinics in the Boston, Massachusetts, area (Boston Medical Center-Boston University, Floating Hospital-Tufts Medical Center, and Cambridge Health Alliance). Inclusion criteria were: the child was 2–6 years of age, was medically healthy, had not used antibiotics within the past 3 months, and the parent or guardian was willing to consent to the child's clinical examination and microbial sampling. The study design, protocol, and informed consent were approved by the institutional review boards of the institutions involved.

Dentists trained in study measurement criteria examined the children. The number of teeth and their caries status were recorded on a 0–4 scale by surface [Drury et al., 1999], dental plaque on a 0–4 scale, gingival inflammation on a 0–3 scale [Loe, 1967] and gingival bleeding as 0–1 (absent or present). The child's age, gender, race and ethnicity were recorded. Plaque samples were taken with sterile wooden toothpicks [Milgrom et al., 2000] from the buccal and interproximal surfaces of molars. In children with severe ECC, plaque from cavities was included. The samples were put in 100 μl of 50 mM Tris-EDTA buffer (pH 7.6) and stored frozen at –70°C [Paster et al., 2001].

Microbiological Methods

DNA was extracted from plaque samples and purified by chemical extraction using Master Pure kits (Epicenter, Madison, Wisc., USA) as previously described [Li et al., 2007]. The purified sample DNA was amplified with universal 16S rDNA primers so that clone libraries could be constructed. Cloning and sequencing were performed as previously described [Paster et al., 2001] with amplification of the 16S rRNA genes using universal 16S rRNA PCR primers (F24, 9–27 forward: 5′-GAG TTT GAT YMT GGC TCA G-3′ and F25, 1,525–1,541 reverse: 5′-AAG GAG GTG WTC CAR CC-3′). The PCR amplification product (1,500 bases) was examined using 1% agarose gel electrophoresis. The PCR product was extracted from the gel and purified using a Qiagen Gel Clean-Up Kit according to the manufacturer's specifications (Qiagen Inc., Valencia, Calif., USA).

Cloning of the PCR-amplified DNA was performed with a commercial kit (TOPO TA Cloning Kit, Invitrogen, San Diego, Calif., USA), according to the manufacturer's instructions. Transformation was performed in competent One Shot TOP 10 *E. coli* cells (Invitrogen, Calif., USA). The transformed cells for each sample were grown overnight at 37°C on Luria-Bertani agar plates supplemented with kanamycin (50 μg/ml). Ninety-six (first 16 subjects, 8 severe ECC, 8 caries-free) or 48 (rest of samples) transformed colonies were picked and suspended in 40 μl of 10 mM Tris buffer. One microliter of cell suspension was used as templates in a PCR with vector primers M13F and M13R (TOPO TA Cloning Kit, Invitrogen, Calif., USA). The amplicons were checked for correct size (approximately 1,500 bases) by 1% agarose gel electrophoresis. Clone amplicons were cleaned using exonuclease (Amersham Pharmacia Biotech Inc., Piscataway, N.J., USA) and shrimp alkaline phosphatase (Amersham). For 16S rRNA sequencing, clone amplicons were sequenced using an ABI prism cycle-sequencing kit on an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, Calif., USA). Sequencing used primer F15 (519–533 reverse: 5′-TTA CCG CGG CTG CTG-3′) as previously described [Paster et al., 2001].

Clone sequences were examined by BLAST analysis against a 16S rRNA reference sequence set at the HOMD [Dewhirst et al., 2008]. The HOMD (version 10) contains 619 species and phylotypes based on 98.5% similarity cutoffs of full 1,540 base 16S rRNA sequences. Each oral species or phylotype in the database

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has a Human Oral Taxon Number (HOT). The threshold for BLAST identification of 500 base partial sequences was 98% match (approximately 8 bases mismatch for partial sequences). Clones that did not match known oral taxa were examined with the Chimera Check program at the Ribosomal Database Project [Cole et al., 2009] and Mallard [Ashelford et al., 2006]. Non-chimera clones which did not match the HOMD references were clustered into provisional additional novel taxa. A description of the analysis of 35,000 oral 16S rRNA clone sequences to extend the HOMD reference set is in preparation. The phylogenetic trees for the species found in this study were created from the full-length reference sequences in HOMD using the neighbor-joining method [Saitou and Nei, 1987].

Specific 16S rRNA gene PCR assay was used to detect *S. mutans* and *Bifidobacterium* species. For *S. mutans*, *S. mutans*-specific reverse primer 5′-ACT CCA GAC TTT CCT GAC CG-3′ (position 587–608) and forward universal primer 5′-GAG TTT GAT YMT GGC TCA G-3′ (position 9–27) [Kanasi et al., 2010] was used. The samples were preheated (94°C, 5 min) followed by amplification under the following conditions: denaturation (94°C, 45 s), annealing (60°C, 45 s), elongation (72°C, 90 s plus an additional second per cycle for 32 cycles with a final extension (72°C, 15 min). *Bifidobacterium* species PCR was performed with 16S rRNA gene genus specific primers as previously described [Matsuki et al., 2004]. Amplicons were verified on 1% agarose gels. The *Bifidobacterium* primers, designed to detect intestinal bifidobacteria, produced appropriate size amplicons around 550 bp for *Bifidobacterium subtile*, *Bifidobacterium dentium*, *Bifidobacterium longum*, *Scardovia inopinata* and *Parascardovia denticolens*. The primers did not amplify strains of *Rothia*, *Propionibacterium*, *Corynebacterium*, *Actinomyces*, *Streptococcus* or *Lactobacillus* species. The reactions indicated that the *Bifidobacterium* primers detected species in the family *Bifidobacteriaceae* (*Bifidobacterium*, *Scardovia*, *Parascardovia*), but not other oral species, including in the phylum *Actinobacteria*.

Statistical Analyses

The subjects were divided into severe ECC and caries-free disease categories. Mean differences between severe ECC and caries-free children with respect to age, plaque, gingival and bleeding indices were evaluated by t test. Associations between disease category and gender and race-ethnicity were compared by χ^2 test.

To compare phylotypes from OTU analysis of 16S rRNA gene libraries, metalibraries were created by combining all sequences of severe ECC children, with a second metalibrary for sequences of caries-free children. These libraries were aligned to the 16S ribosomal SILVA reference database [Pruesse et al., 2007] and analyses performed using MOTHUR software [Schloss et al., 2009]. A distance matrix was created, and sequences were assigned to phylotypes based on OTU calculated from the genetic distance between the sequences. Estimates of phylotype richness were calculated using the abundance-based coverage estimator and the bias-corrected Chao1 [Chao, 1984; Schloss et al., 2009]. The number of observed and estimated phylotypes as a function of the number of clones sequenced was compared at evolutionary distances: 0.02, 0.05 and 0.10 [Hughes et al., 2001].

Good's coverage estimation [Good, 1953; Singleton et al., 2001] was calculated with MOTHUR [Schloss et al., 2009]. The

∫-LIBSHUFF algorithm was used to test whether the structures of 2 metalibraries were the same using the Cramér-von Mises test statistic with Bonferroni correction for multiple comparisons [Schloss et al., 2009] and a Monte Carlo test procedure. The numbers of sequences that were unique to each metalibrary and the probability that the observed differences between the metalibraries were due to chance were calculated. Coverage (percent of estimated diversity) was examined as a function of evolutionary distance. The coverage of severe ECC and caries-free libraries was compared.

To compare phylotypes with HOMD database, HOMD taxa as a percent of total clones identified by subject were averaged over subjects and compared between severe ECC and caries-free children by the McNemar test. Taxa detection frequencies from clonal analysis were calculated by child, then averaged across disease categories and compared by χ^2 test. Detections of *S. mutans* and *Bifidobacteriaceae* in severe ECC and caries-free categories were compared by χ^2 test. A significance threshold of ≤0.05 was used. The results were also adjusted for multiple comparisons using the false discovery rate (α = 0.05) [Benjamini and Hochberg, 1995]. Statistical analyses were performed with SPSS® software (SPSS Inc., Chicago, Ill., USA).

Results

The severe ECC and caries-free (control) groups were balanced by age (4.1 and 4.3 years, respectively) and gender (table 1). The racial classification of the children was: White (34%), Black (29%), Asian (25%) and other/mixed races (11%). Thirty percent of the children were Hispanic. Children with severe ECC exhibited mostly caries into the dentin (12% surfaces), followed by pulpal exposure (9% surfaces), white-spot lesions (7% surfaces) and enamel cavities (5% surfaces). Plaque, gingival and bleeding indexes were all significantly higher in severe ECC than caries-free children (p < 0.001). The sociodemographics did not differ between severe ECC and caries-free children [Palmer et al., 2010].

In the clonal analysis, out of 4,516 clones sequenced, 714 (15%) were not used because they were short, of poor quality or were chimeras. Of the remaining 3,802 clones, 1,846 were from severe ECC and 1,956 were from caries-free children. The observed and estimated diversity of phylotypes from OTU was similar for both metalibraries at 0.10 evolutionary distances (90% similarity). At 0.05 and 0.02 evolutionary distances (95 and 98% similarity, respectively) there were consistently higher values for the estimated than observed phylotypes. For the severe ECC library at 0.05 evolutionary distances there were 116 observed OTU and 147 estimated OTU; at 0.02 evolutionary distances 247 OTU were observed and 366 were estimated. For the caries-free library at 0.05 evolu-

tionary distances there were 113 observed OTU and 133 estimated OTU; at 0.02 evolutionary distances 240 OTU were observed and 341 were estimated. Less than 50% of the observed and estimated phylotypes were shared between the severe ECC and caries-free libraries. The number of observed phylotypes at 98% sequence similarity was 92 for severe ECC, 84 for caries-free with 157 shared by severe ECC and caries-free, whereas estimated phylotypes were 134 for severe ECC, 117 for caries-free with 224 shared by severe ECC and caries-free libraries. Good's coverage estimation at 0.02 evolutional distances, which approximate species level, for severe

ECC and caries-free libraries was 96.5 and 96.7%, respectively. At 0.05 evolutional distances, which approximate genus at a narrow cutoff, severe ECC and caries-free libraries had 98.6% coverage, and at 0.10 evolutional distances, which approximate genus at a broad cutoff, severe ECC and caries-free libraries had 99.2 and 99.4% coverage. There were significant differences by ∫-LIBSHUFF between severe ECC and caries-free metalibraries ($p < 0.0001$) with dCXY score: severe ECC – caries-free = 0.00017562; and caries-free – severe ECC = 0.00013673.

Table 1. Demographic and clinical characteristics of the study population

	Caries-free (n = 41)	Caries (n = 39)	Caries-positive, %	p value
Mean age ± SEM, years	4.3 ± 0.2	4.1 ± 0.2		0.536[a]
Gender				
Male	26	23	47	
Female	15	16	52	0.684[b]
Race				
White	18	9	33	
Black	11	13	54	
Asian	10	10	50	
Other/mixed	2	7	78	0.117[b]
Ethnicity				
Hispanic	13	11	46	
Non-Hispanic	28	28	50	0.733[b]
Caries, percent surfaces affected				
Intact surface	100	66		
White or opaque patches	0	7		
Enamel cavity	0	5		
Cavity into dentine	0	12		
Pulpal exposure	0	9		
Mean plaque index ± SEM	0.65 ± 0.01	1.58 ± 0.01		<0.001[a]
Mean gingival index ± SEM	0.26 ± 0.01	1.96 ± 0.02		<0.001[a]
Mean bleeding index ± SEM	0.00 ± 0.00	0.57 ± 0.01		<0.001[a]

SEM = Standard error of the mean. [a] Student's t test. [b] χ^2 test.

Fig. 1. Phylogenetic trees of species and phylotypes identified from clones compared with the HOMD. The trees were created from full 16S rRNA reference sequences although it is recognized that use of 500-bp data from study clones could have created a different, but less accurate, treeing topology. Taxa in trees include identifications at >0.1 mean percent positive taxa by subject. A total of 139 taxa were identified. Numbers of children with taxa detected are in the table to the right of each tree. Species or phylotypes that differed in detection frequencies are noted at * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, by χ^2 analysis, or Fishers exact test. **a** Phylogenetic tree of 51 taxa in *Proteobacteria, Bacteroidetes* and *Actinobacteria* detected in severe caries and caries-free children, with positions of *Clostridia* and *Bacillus* as phylogenetic references. The tree includes 28 taxa of *Proteobacteria*, 15 taxa of *Bacteroidetes*, 1 *Treponema* species, 5 *Actinobacteria* and 2 *Leptotrichia* taxa.

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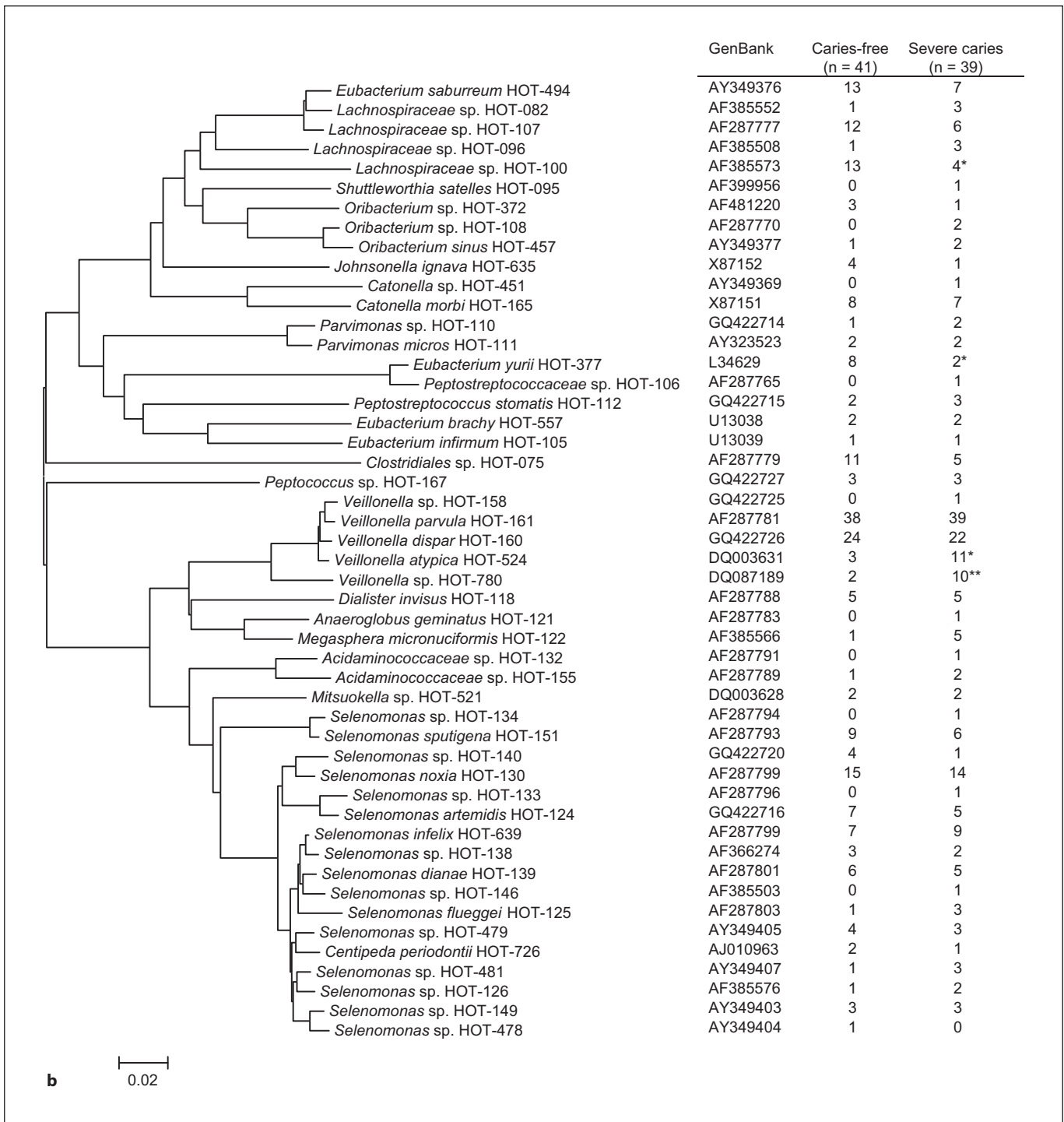|	GenBank	Caries-free (n = 41)	Severe caries (n = 39)
Aggregatibacter segnis HOT-762	M75043	1	2
Aggregatibacter sp. HOT-513	DQ003635	1	0
Aggregatibacter sp. HOT-458	AY349380	2	1
Aggregatibacter paraphrophilus HOT-720	M75042	2	0
Aggregatibacter aphrophilus HOT-545	M75041	1	1
Haemophilus sp. HOT-036	AY005033	0	2
Haemophilus sp. HOT-035	AY005034	1	1
Aggregatibacter actinomycetemcomitans HOT-531	M75036	0	1
Haemophilus parainfluenzae HOT-718	EU083530	0	1
Terrahaemophilus aromaticivorans HOT-826	AB098612	6	4
Cardiobacterium hominis HOT-633	M35014	2	0
Lautropia mirabilis HOT-022	AY005030	0	1
Neisseria sp. HOT-499	AY349388	3	0
Kingella oralis HOT-706	L06164	2	2
Kingella sp. HOT-012	AF366267	2	0
Kingella denitrificans HOT-582	L06166	4	0
Eikenella corrodens HOT-577	AF320620	2	3
Neisseria elongata HOT-598	L06171	1	2
Neisseria sp. HOT-016	AY005024	2	0
Neisseria flavescens HOT-610	AJ239280	1	7*
Neisseria meningitidis HOT-669	AF382273	2	4
Neisseria subflava HOT-476	AJ239291	5	2
Neisseria mucosa HOT-682	AJ239282	6	3
Neisseria sicca HOT-764	AJ239292	6	7
Neisseria flava HOT-609	AJ239301	5	0*
Campylobacter concisus HOT-575	L06977	5	2
Campylobacter showae HOT-763	L06974	0	4*
Campylobacter gracilis HOT-623	L04320	8	4
Capnocytophaga granulosa HOT-325	AY005074	26	19
Capnocytophaga gingivalis HOT-337	AY004073	27	9 ***
Capnocytophaga sp. HOT-338	U41352	6	2
Capnocytophaga sp. HOT-332	AF366270	0	1
Capnocytophaga sputigena HOT-775	X67609	0	1
Porphyromonas sp. HOT-284	AY008313	1	0
Porphyromonas catoniae HOT-283	AF366271	1	2
Porphyromonas sp. HOT-279	AY008310	0	1
Tannerella sp. HOT-286	AY008308	1	1
Prevotella sp. HOT-473	AY349399	0	1
Prevotella sp. HOT-317	AY005061	2	0
Prevotella oris HOT-311	AY005056	1	2
Prevotella nigrescens HOT-693	X73963	1	0
Prevotella melaninogenica HOT-469	AY349395	0	1
Prevotella sp. HOT-306	AF385511	0	1
Treponema socranskii HOT-769	AF033306	3	2
Actinomyces sp. HOT-414	AY278610	1	0
Actinomyces sp. HOT-525	DQ003632	1	0
Actinomyces israelii HOT-645	X82450	1	0
Actinomyces sp. HOT-171	AF385553	1	2
Corynebacterium matrucHOT-ii HOT-666	X82065	1	2
Leptotrichia sp. HOT-212	AY008309	1	0
Leptotrichia sp. HOT-498	AY349387	0	1
Clostridia			
Bacilli			

a   0.05

Taxon	GenBank	Caries-free (n = 41)	Severe caries (n = 39)
Eubacterium saburreum HOT-494	AY349376	13	7
Lachnospiraceae sp. HOT-082	AF385552	1	3
Lachnospiraceae sp. HOT-107	AF287777	12	6
Lachnospiraceae sp. HOT-096	AF385508	1	3
Lachnospiraceae sp. HOT-100	AF385573	13	4*
Shuttleworthia satelles HOT-095	AF399956	0	1
Oribacterium sp. HOT-372	AF481220	3	1
Oribacterium sp. HOT-108	AF287770	0	2
Oribacterium sinus HOT-457	AY349377	1	2
Johnsonella ignava HOT-635	X87152	4	1
Catonella sp. HOT-451	AY349369	0	1
Catonella morbi HOT-165	X87151	8	7
Parvimonas sp. HOT-110	GQ422714	1	2
Parvimonas micros HOT-111	AY323523	2	2
Eubacterium yurii HOT-377	L34629	8	2*
Peptostreptococcaceae sp. HOT-106	AF287765	0	1
Peptostreptococcus stomatis HOT-112	GQ422715	2	3
Eubacterium brachy HOT-557	U13038	2	2
Eubacterium infirmum HOT-105	U13039	1	1
Clostridiales sp. HOT-075	AF287779	11	5
Peptococcus sp. HOT-167	GQ422727	3	3
Veillonella sp. HOT-158	GQ422725	0	1
Veillonella parvula HOT-161	AF287781	38	39
Veillonella dispar HOT-160	GQ422726	24	22
Veillonella atypica HOT-524	DQ003631	3	11*
Veillonella sp. HOT-780	DQ087189	2	10**
Dialister invisus HOT-118	AF287788	5	5
Anaeroglobus geminatus HOT-121	AF287783	0	1
Megasphera micronuciformis HOT-122	AF385566	1	5
Acidaminococcaceae sp. HOT-132	AF287791	0	1
Acidaminococcaceae sp. HOT-155	AF287789	1	2
Mitsuokella sp. HOT-521	DQ003628	2	2
Selenomonas sp. HOT-134	AF287794	0	1
Selenomonas sputigena HOT-151	AF287793	9	6
Selenomonas sp. HOT-140	GQ422720	4	1
Selenomonas noxia HOT-130	AF287799	15	14
Selenomonas sp. HOT-133	AF287796	0	1
Selenomonas artemidis HOT-124	GQ422716	7	5
Selenomonas infelix HOT-639	AF287799	7	9
Selenomonas sp. HOT-138	AF366274	3	2
Selenomonas dianae HOT-139	AF287801	6	5
Selenomonas sp. HOT-146	AF385503	0	1
Selenomonas flueggei HOT-125	AF287803	1	3
Selenomonas sp. HOT-479	AY349405	4	3
Centipeda periodontii HOT-726	AJ010963	2	1
Selenomonas sp. HOT-481	AY349407	1	3
Selenomonas sp. HOT-126	AF385576	1	2
Selenomonas sp. HOT-149	AY349403	3	3
Selenomonas sp. HOT-478	AY349404	1	0

b 0.02

Fig. 1. b Phylogenetic tree of 49 taxa in the *Clostridia* branch of *Firmicutes,* which includes the Gram-negative *Veillonella* and *Selenomonas* genera, detected in severe ECC and caries-free children. This group comprises several named and unnamed taxa, including the *Centipeda* species.
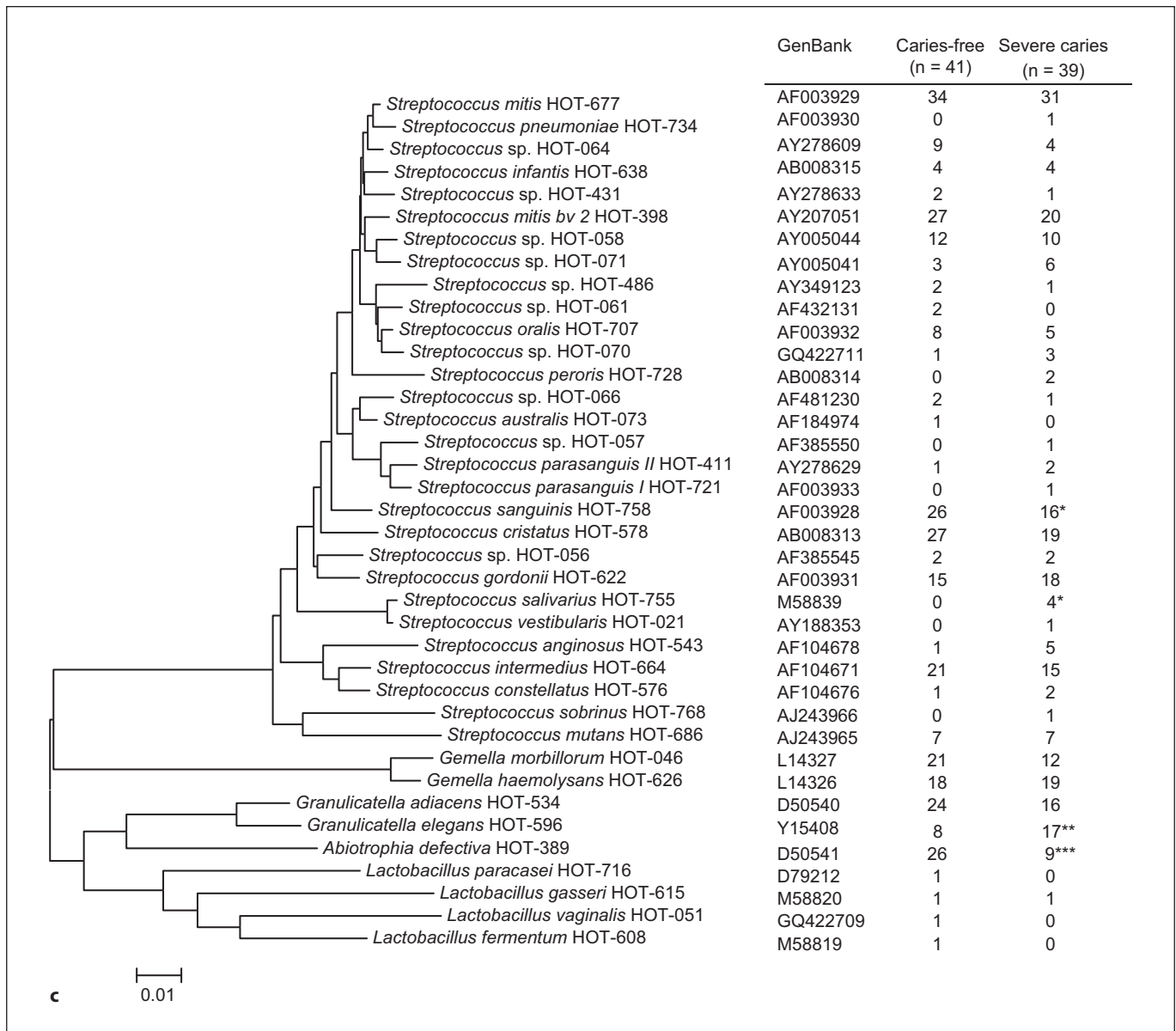
	GenBank	Caries-free (n = 41)	Severe caries (n = 39)
Streptococcus mitis HOT-677	AF003929	34	31
Streptococcus pneumoniae HOT-734	AF003930	0	1
Streptococcus sp. HOT-064	AY278609	9	4
Streptococcus infantis HOT-638	AB008315	4	4
Streptococcus sp. HOT-431	AY278633	2	1
Streptococcus mitis bv 2 HOT-398	AY207051	27	20
Streptococcus sp. HOT-058	AY005044	12	10
Streptococcus sp. HOT-071	AY005041	3	6
Streptococcus sp. HOT-486	AY349123	2	1
Streptococcus sp. HOT-061	AF432131	2	0
Streptococcus oralis HOT-707	AF003932	8	5
Streptococcus sp. HOT-070	GQ422711	1	3
Streptococcus peroris HOT-728	AB008314	0	2
Streptococcus sp. HOT-066	AF481230	2	1
Streptococcus australis HOT-073	AF184974	1	0
Streptococcus sp. HOT-057	AF385550	0	1
Streptococcus parasanguis II HOT-411	AY278629	1	2
Streptococcus parasanguis I HOT-721	AF003933	0	1
Streptococcus sanguinis HOT-758	AF003928	26	16*
Streptococcus cristatus HOT-578	AB008313	27	19
Streptococcus sp. HOT-056	AF385545	2	2
Streptococcus gordonii HOT-622	AF003931	15	18
Streptococcus salivarius HOT-755	M58839	0	4*
Streptococcus vestibularis HOT-021	AY188353	0	1
Streptococcus anginosus HOT-543	AF104678	1	5
Streptococcus intermedius HOT-664	AF104671	21	15
Streptococcus constellatus HOT-576	AF104676	1	2
Streptococcus sobrinus HOT-768	AJ243966	0	1
Streptococcus mutans HOT-686	AJ243965	7	7
Gemella morbillorum HOT-046	L14327	21	12
Gemella haemolysans HOT-626	L14326	18	19
Granulicatella adiacens HOT-534	D50540	24	16
Granulicatella elegans HOT-596	Y15408	8	17**
Abiotrophia defectiva HOT-389	D50541	26	9***
Lactobacillus paracasei HOT-716	D79212	1	0
Lactobacillus gasseri HOT-615	M58820	1	1
Lactobacillus vaginalis HOT-051	GQ422709	1	0
Lactobacillus fermentum HOT-608	M58819	1	0

c 0.01

Fig. 1. c Phylogenetic tree of 38 taxa in the *Bacillus* branch of *Firmicutes* detected in severe caries and caries-free children. The tree includes 29 *Streptococcus* species, comprising 10 unnamed species, 2 *Gemella*, 2 *Granulicatella* and 4 *Lactobacillus* species.

Comparison of phylotypes with HOMD indicated that 139 HOMD taxa were identified, with an additional 35 provisional taxa. From severe ECC children >98% clones were identified at the >98% sequence similarity level to HOMD groups and 1.78% to additional provisional groups, whereas from caries-free children 97.02% clones were identified to HOMD groups and 2.28% to provisional groups. Of the 139 HOMD taxa identified, 88 (63%) were detected in both severe ECC and caries-free children, 22 (16%) were detected only in severe ECC children, and 29 (21%) were detected only in caries-free children. The numbers of taxa detected in severe ECC (mean ± SEM = 14.8 ± 0.73) and caries-free children (mean ± SEM = 16.6 ± 0.77) (p = 0.09, t test) did not differ.

Unidentified clones in provisional phylotypes were closely related to recognized species, and in descending

order of detection frequencies were identified to the genera or higher taxonomic level: *Capnocytophaga, Veillonella, Actinomyces, Kingella, Lachnospiraceae, Peptococcus, Selenomonas, Streptococcus, Aggregatibacter, Gemella, Granulicatella, Lautropia, Neisseria* and *Synergistetes*. There were no differences in mean percentages or detection frequencies in the provisional phylotypes between severe ECC and caries-free children. These potentially novel taxa are being evaluated for inclusion into HOMD.

Clones identified to HOMD species and phylotype groups are shown in phylogenetic trees (fig. 1), which include the numbers of children and the taxa detected. The most frequently detected taxa (fig. 2), *Veillonella, Streptococcus* and *Capnocytophaga,* were detected in >50% of the children, particularly *Veillonella parvula, Streptococcus mitis* 1 and 2, and *Capnocytophaga granulosa*. Among the *Proteobacteria, Bacteroidetes* and *Actinobacteria* (fig. 1a) most clones were *Neisseria* and *Capnocytophaga. Neisseria flavescens* and *Campylobacter showae* were detected more frequently in severe ECC children, while *Capnocytophaga gingivalis* and *Neisseria flavia* occurred more frequently and in higher proportions in caries-free children (fig. 1a, 2a, b). *Clostridia* species and phylotypes within Firmicutes (fig. 1b) included 6 species of *Selenomonas,* 11 closely related *Selenomonas* phylotypes, 3 species of *Veillonella* and 2 *Veillonella* phylotypes. *Veillonella atypica* and *Veillonella* sp. HOT-780 (fig. 1b) were detected more frequently in severe ECC, whereas *Lachnospiraceae* sp. HOT-100 and *Eubacterium yurii* were found more frequently in caries-free children (fig. 1b, 2a, b). The phylogenetic tree of the *Bacillus* branch of Firmicutes included 19 species and 10 phylotypes in *Streptococcus,* 4 species of *Lactobacillus,* 2 *Granulicatella* and 2 *Gemella* species (fig. 2b). *Granulicatella elegans* and *Streptococcus salivarius* were detected more frequently in severe ECC children, whereas *Abiotrophia defectiva* and *Streptococcus sanguinis* (fig. 1c), also *Streptococcus cristatus* in mean levels (fig. 2b), were associated with caries-free children. Associations for *A. defectiva* and *C. gingivalis* remained significant after adjustment for multiple comparisons [Benjamini and Hochberg, 1995].

S. mutans was detected by clonal analysis in 20% of the severe ECC and caries-free children, at around 1% clones, with no significant differences between severe ECC and caries-free children (fig. 1c, 2b). *Streptococcus sobrinus* was detected in 1 caries child. No *Bifidobacteriaceae (Bifidobacterium, Scardovia* or *Parascardovia)* species were detected in the clonal analysis.

S. mutans *and* Bifidobacteriaceae *Species Detection by Specific PCR*

S. mutans was detected in 73% of the severe ECC children and 38% of the caries-free children (p = 0.002, χ^2 test, fig. 3). *S. mutans* detection was significantly associated with plaque (p = 0.004), gingival inflammation (p = 0.003) and bleeding (p = 0.003) (Kruskal Wallis test) (data not shown). *Bifidobacteriaceae* species were detected in 87% of the severe ECC children and 21% of the caries-free children (p < 0.0001, χ^2 test, fig. 3) *Bifidobacteriaceae* detection was significantly associated with plaque (p < 0.0001), gingival inflammation (p < 0.0001) and bleeding (p < 0.0001) (Kruskal Wallis test) (data not shown).

Discussion

The study aimed to use 16S rRNA gene broad-range PCR followed by cloning and sequencing, a noncultural approach to detect the diversity of cultured and uncultured taxa in an environment, to describe the microbiota of severe ECC. The study examined 80 children to overcome variation between individuals [Diaz et al., 2006; Aas et al., 2008], and to detect significant microbial differences between severe-ECC and caries-free populations. Statistical tools were developed [Schloss et al., 2009] to analyze extensive sequence-based data from metagenomic studies of the Sargasso Sea, which had 2 million sequencing reads [Venter et al., 2004]. We applied these OTU analyses to evaluate observed and estimated population diversity and coverage and also differences between clone libraries from severe ECC and caries-free children. Significant differences were detected between severe ECC caries-free children using the clone sequence data by OTU analysis and phylotype classification using HOMD. Association of selected target caries pathogens with severe ECC, however, required specific 16S rRNA PCR analysis of samples.

DNA sequences from the clonal libraries were grouped into OTU phylotypes at different levels of evolutionary distance to determine the numbers of observed phylotypes. The estimated phylotypes, which indicate the total diversity of the population [Hughes et al., 2001; Schloss and Handelsman, 2005] were greater than observed at each threshold, suggesting that there is increased diversity in the populations than reported from this clonal analysis. Coverage of the populations, however, was quite high at 0.02 evolutionary distances, suggesting that clinically relevant comparisons between health and disease
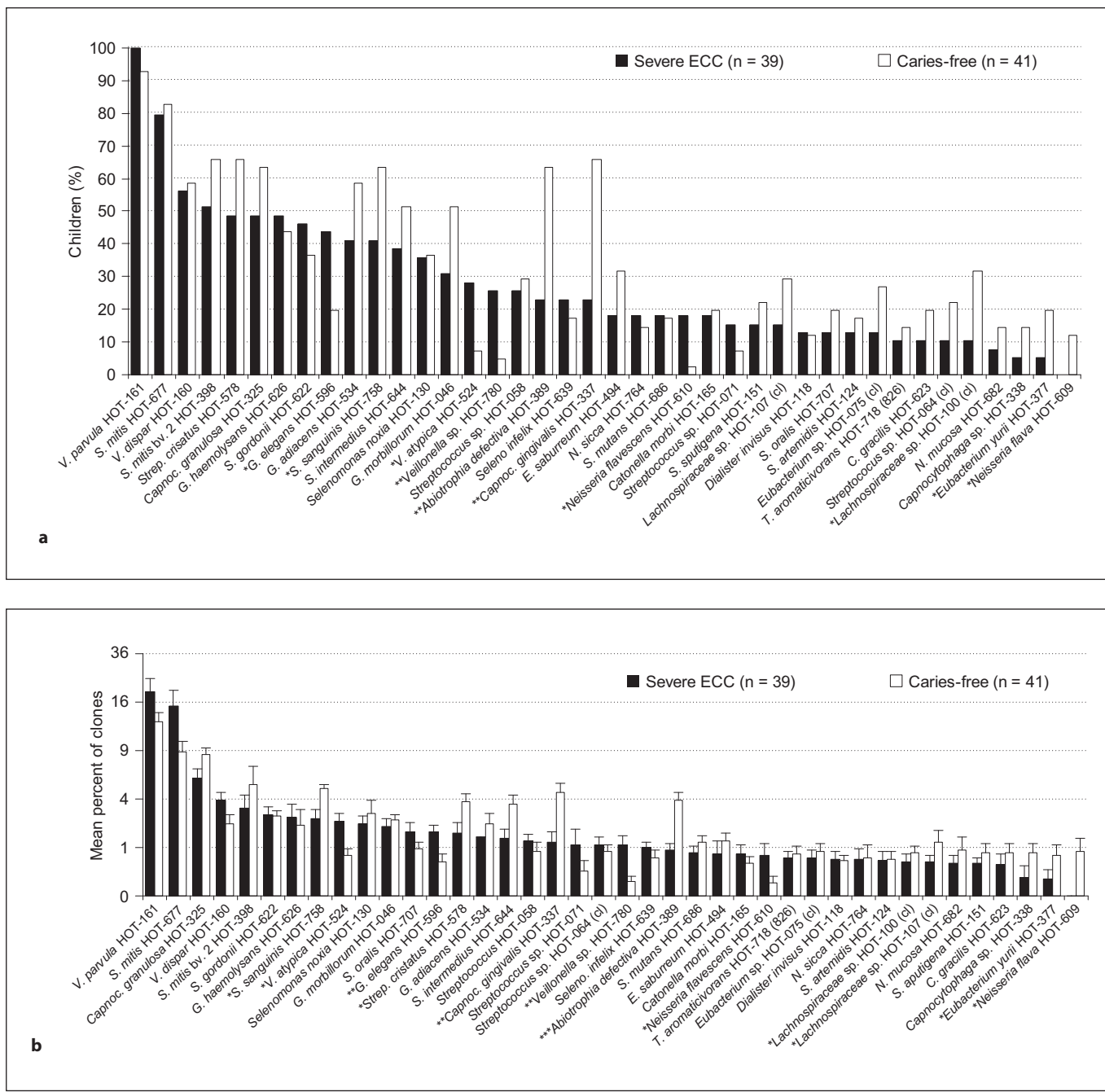
Kanasi/Dewhirst/Chalmers/Kent/
Moore/Hughes/Pradhan/Loo/Tanner

Fig. 2. The most frequently detected species from severe caries and caries-free children. The scale represents the square root of data to enhance the visualization of taxa less frequently detected or in lower proportions. **a** Detection frequencies of taxa by child in severe caries and caries-free children. Taxa are ordered in decreasing order of detection in severe ECC children. Taxa that differed in detection frequencies are noted at * $p \leq 0.05$, ** $p \leq 0.01$ by χ^2 analysis. **b** Mean proportions of taxa detected in severe caries and caries-free children. Taxa are given in decreasing order of mean clone levels in severe ECC children. Taxa that differed in detection frequencies are noted at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by Mann Whitney U test. Error bars = standard error of the mean.
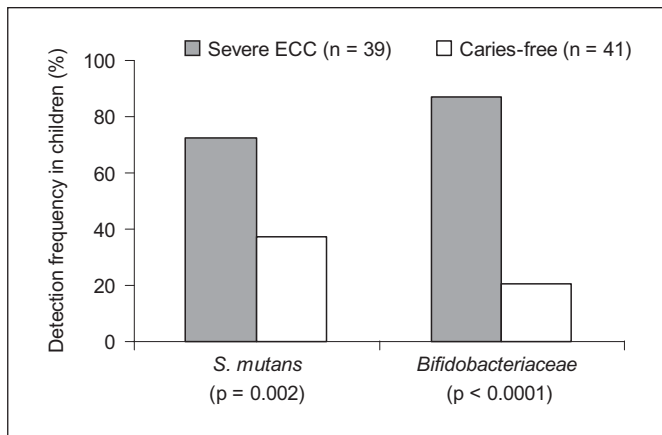
Fig. 3. Detection frequencies by child of *S. mutans* and *Bifidobacteriaceae* species assayed by species-specific PCR. *Streptococcus mutans* (p < 0.002) and *Bifidobacteriaceae* species (p < 0.0001) were strongly associated with severe early childhood caries.

could be made. The differences in childhood caries and caries-free populations previously reported from clonal analyses [Becker et al., 2002; Aas et al., 2008] was confirmed in the OTU phylotypes with over half of the phylotypes detected only in either severe ECC or caries-free populations, at differences of evolutionary distance <0.02 or 98% similarity.

To identify the clones, sequences were identified by BLAST analysis against the HOMD reference set of 619 species and phylotypes. A wide diversity of bacterial taxa (species and phylotypes) was detected including more species/phylotypes of *Streptococcus, Capnocytophaga, Selenomonas* and *Neisseria* than previously reported in children [Becker et al., 2002; Aas et al., 2008], reflecting, in part, the large number of clones sequenced and identification to HOMD taxa.

The microbiota of children with severe ECC was associated with *V. atypica* and *Veillonella* sp. HOT-780 compared with caries-free children. In other studies *Veillonella* species were related to ECC by culture [Marchant et al., 2001] and DNA probes [Becker et al., 2002]. *Veillonella* species also dominated in early and established dental cavities in primary and secondary teeth of children by clonal and 16S rRNA probe analyses [Aas et al., 2008]. Physiologically *Veillonella* species use lactate as a carbon energy source, and their detection in dental caries reflects the lactic acid rich environment derived from bacterial metabolic end products, particularly *S. mutans*. In biofilm experiments, *Veillonella* enhanced growth of *S. mutans,* suggesting a close symbiotic relationship be-

tween these species [Egland et al., 2004; Chalmers et al., 2008]. *Granulicatella elegans* was detected in higher mean proportions from severe-ECC than caries-free children. This species, previously recognized as a nutritionally variant *Streptococcus,* has been detected in children with caries [Aas et al., 2008], but clinical significance of this species has not been reported. *Neisseria flavescens* was not reported from other clonal analyses of plaque samples of young children but was detected in refractory periodontitis [Paster et al., 2001]. The non-mutans streptococci, *S. mitis* and *Streptococcus* sp. HOT-071 in the *S. mitis/S. oralis* group, were detected more frequently from severe-ECC than caries-free children while not significantly, and have also been associated with childhood caries by 16S rRNA probes [Becker et al., 2002]. Associations of certain species with severe-ECC may also reflect the increased levels of gingival inflammation in these children with abundant dental plaque and frequently jagged teeth or root stumps irritating the gingival margin.

The microbiota of caries-free children was associated with higher proportions of *C. gingivalis, Abiotrophia defectiva, S. cristatus, S. sanguinis* and *Lachnospiraceae* sp. HOT-100 clones than severe ECC children. *C. gingivalis* is frequently detected in subgingival plaque and was found in young children but not as frequently as *C. granulosa* [Corby et al., 2005], as observed in the current study. *Abiotrophia defectiva,* another nutritionally variant *Streptococcus,* has been detected in endocarditis and bacteremia and generally requires 16S rRNA sequence data for identification [Senn et al., 2006]. *A. defectiva* was previously detected at higher levels in caries-free than caries children [Becker et al., 2002; Corby et al., 2005]. *S. cristatus* and *S. sanguinis* are health-related species. *S. cristatus* was associated with caries-free children in primary and secondary teeth [Aas et al., 2008]. *S. sanguinis* was previously associated with caries-free children [Becker et al., 2002; Corby et al., 2005] and had an inverse relationship with *S. mutans* with respect to childhood caries; children colonized with *S. sanguinis* before *S. mutans* showed delayed *S. mutans* infection and detection of dental caries [Caufield et al., 2000]. *Lachnospiraceae* sp. HOT-100 is an uncultivated phylotype which appears from our data associated with caries-free children. The role of these species in dental health should be examined further.

Despite an extensive literature relating *S. mutans* to ECC, lack of *S. mutans* association by clonal analysis was previously reported in childhood caries, advanced carious lesions and root caries [Chhour et al., 2005; Aas et al.,

2008; Preza et al., 2008]. While bias in the clonal analyses could have resulted from PCR and primer bias [Ishii and Fukui, 2001; Munson et al., 2004] low proportions of *S. mutans* in caries were also reported in a cultural analysis of children with extensive caries [Loesche et al., 1975]. Our findings are thus consistent with literature studies. *Streptococcus sobrinus* was detected in only 1 child with severe ECC in the current study, consistent with lower *S. sobrinus* detection levels than *S. mutans* and lack of association with childhood caries by 16S rRNA probes [Becker et al., 2002].

Plaque samples were also analyzed by specific PCR to detect *S. mutans* and *Bifidobacteriaceae*. *S. mutans* detection by PCR was significantly associated with severe ECC. Recent studies linking *S. mutans* with severe ECC have been performed by culture [Marchant et al., 2001], PCR [Seki et al., 2006] and by 16S rRNA probes [Becker et al., 2002; Corby et al., 2005; Aas et al., 2008]. The discrepancy between clonal counts and selective detection (PCR, selective isolation, DNA probes) is likely related to these different approaches used for species detection, with selective detection having the ability to identify species in low proportions of the microbiota. These data are thus consistent with the observation that carious sites may harbor widely diverse bacteria, and *S. mutans,* while important in disease, may be present only in low proportions of carious sites.

The strong association of species in *Bifidobacteriaceae* represents new findings by this PCR detection method, although bifidobacteria have been detected and previously associated with childhood caries [Becker et al., 2002; Aas et al., 2008] and initial caries [Van Houte et al., 1996; Svensater et al., 2003]. These studies, however, did not report associations at the detection frequency and significance of the current study. Future clonal analyses need to be expanded to detect species in *Bifidobacteriaceae.*

Discrepancies in the current study data compared with literature reports included lack of detection using clonal analysis of *Bifidobacteria* and several other species in the *Actinobacteria* phylum *(Actinomyces, Corynebacterium, Rothia)* and *Lactobacillus*, especially since selected *Lactobacillus* species were recently associated with ECC [Kanasi et al., 2010]. Possible reasons for not detecting certain species include DNA extraction and bacterial lysis methods, and PCR and primer bias [Ishii and Fukui, 2001; Munson et al., 2004]. While differences in populations detected varied with different primers in dental caries [Munson et al., 2004], a recent report clarified improved primers to detect *Actinobacteria* [Frank et al.,

2008]. Only 1 spirochete species, *Treponema denticola,* was detected, thus several primer sets would be needed for the recovery of all major taxa in the microbiota of dental caries.

In summary, the present study compared the microbiota of severe ECC with caries-free children by clonal analysis. Phylotypes based on OTU analyses indicated that there was more diversity than detected in this clonal analysis and that severe ECC and caries-free populations differed. Significant differences were also detected in HOMD taxa between severe ECC and caries-free children. *V. atypica, Veillonella* sp. HOT-780, *N. flavescens* and *G. elegans* were associated with severe ECC, and *Streptococcus* sp. HOT-071 was detected more frequently from and in higher proportions with severe ECC but not significantly as in other studies. By clonal analysis *S. mutans* was detected infrequently and detection did not differ between severe ECC and caries-free populations, but *S. mutans* and *Bifidobacteriaceae* species were significantly associated with severe ECC using specific PCR assays. We conclude that candidates as new caries pathogens from this study were *G. elegans* and species within *Bifidobacteriaceae.* Other caries-associated species including *Neisseria* and *Veillonella* are not acidogenic and thus not caries pathogens but could play a key role in supporting the biofilm of this infection.

Disclosure Statement

There are no conflicts of interest.

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