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J Dent Res 90(11):1298-1305, 2011

ABSTRACT

Severe early childhood caries (ECC) is difficult to treat successfully. This study aimed to characterize the microbiota of severe ECC and evaluate whether baseline or follow-up microbiotas are associated with new lesions post-treatment. Plaque samples from 2- to 6-year-old children were analyzed by a 16S rRNA-based microarray and by PCR for selected taxa. Severe-ECC children were monitored for 12 months post-therapy. By microarray, species associated with severe-ECC ($n = 53$) compared with caries-free ($n = 32$) children included *Slackia exigua* ($p = 0.002$), *Streptococcus parasanguinis* ($p = 0.013$), and *Prevotella* species ($p < 0.02$). By PCR, severe-ECC-associated taxa included *Bifidobacteriaceae* ($p < 0.001$), *Scardovia wiggsiae* ($p = 0.003$), *Streptococcus mutans* with bifidobacteria ($p < 0.001$), and *S. mutans* with *S. wiggsiae* ($p = 0.001$). In follow-up, children without new lesions ($n = 36$) showed lower detection of taxa including *S. mutans*, changes not observed in children with follow-up lesions ($n = 17$). Partial least-squares modeling separated the children into caries-free and two severe-ECC groups with either a stronger bacterial or a stronger dietary component. We conclude that several species, including *S. wiggsiae* and *S. exigua*, are associated with the ecology of advanced caries, that successful treatment is accompanied by a change in the microbiota, and that severe ECC is diverse, with influences from selected bacteria or from diet.

KEY WORDS: pediatric dentistry, clinical outcomes, microbial ecology, *Streptococcus mutans*, *Scardovia wiggsiae*.

DOI: 10.1177/0022034511421201

Received May 19, 2011; Last revision July 29, 2011; Accepted August 1, 2011

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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Microbiota of Severe Early Childhood Caries before and after Therapy

INTRODUCTION

Severe early childhood caries can devastate the primary dentition (Hughes *et al.*, 2011), is a risk factor for caries in permanent teeth (Li and Wang, 2002), and is difficult to treat successfully (Almeida *et al.*, 2000; Graves *et al.*, 2004). In the US, while 28% of children have ECC (Beltran-Aguilar *et al.*, 2005), only a subset of children have advanced disease, mainly concentrated in populations of low socio-economic status (Beighton *et al.*, 2004; Edelstein and Chinn, 2009). Treatment is difficult because of the young age of children and is frequently performed while the children are under general anesthesia. Further treatment may be of limited benefit, because up to 50% of children have new caries lesions after therapy (Almeida *et al.*, 2000; Graves *et al.*, 2004).

The etiology of severe ECC includes infection with selected bacteria, and dietary and host factors, which are influenced by environment and socio-economic status (Beighton *et al.*, 2004). Differences in the plaque microbiota were observed in children from different socio-economic backgrounds, suggesting diversity within ECC (Beighton *et al.*, 2004). We recently confirmed that *Streptococcus mutans* was strongly associated with severe ECC, although at a low proportion of the carious plaque microbiota, using molecular (Kanasi *et al.*, 2010a) and culture-based (Hughes *et al.*, 2011) assays. Other species significantly associated with severe ECC included *Scardovia wiggsiae*, by culture (Tanner *et al.*, 2011), and *Bifidobacteriaceae*, by PCR (Palmer *et al.*, 2010).

To investigate the reasons for new lesions post-treatment, this study aimed to compare the microbiota of caries-free with that of severe-ECC children, and to compare pre-treatment and follow-up microbiotas in children with and without new caries lesions. We modeled microbial, clinical, and dietary characteristics to evaluate whether subgroups existed within severe ECC, to improve our understanding of this complex infectious disease.

MATERIALS & METHODS

Clinical Methods

Children aged 2 to 6 yrs were recruited as previously described (Palmer *et al.*, 2010). Children were medically healthy, had not used antibiotics within the

Table. Demographic and Clinical Characteristics of Study Population

	Caries-free n = 32	Severe ECC n = 53	p value
Mean age (yrs) $\pm$ SEM ^a	3.66 $\pm$ 0.17	3.97 $\pm$ 0.13	0.160 ^b
Gender: Male	15 (47%)	30 (56%)	0.44 ^c
Race:			
White	13 (32%)	16 (30%)	0.874 ^c
Black	9 (28%)	20 (37%)	0.398 ^c
Asian	12 (36%)	11 (32%)	0.568 ^c
Other/mixed	1 (3%)	1 (2%)	0.608 ^c
Ethnicity: Hispanic	6 (17%)	13 (24%)	0.565 ^c
Clinical Characteristics			
Mean percent carious surfaces	0	32.4 $\pm$ 2.67	
Mean Plaque Index $\pm$ SEM	0.58 $\pm$ 0.08	4.79 $\pm$ 0.13	< 0.0001 ^b
Mean Gingival Index $\pm$ SEM	0.16 $\pm$ 0.07	1.54 $\pm$ 0.13	< 0.0001 ^b
Mean percent sites bleeding $\pm$ SEM	2.0 $\pm$ 1.2	52.0 $\pm$ 4.9	< 0.0001 ^b
Survey Characteristics			
Parents with cavities	66 %	86%	0.027 ^c
Smoker in household	44%	84%	0.026 ^c
Number of juice drinks/wk $\pm$ SEM	6.5 $\pm$ 0.7	9.7 $\pm$ 0.9	0.011 ^b
Drinks juice between meals	34%	65%	0.016 ^c
Liquid cariogenic foods ^d $\pm$ SEM	2.31 $\pm$ 0.33	3.69 $\pm$ 0.31	0.004 ^b
Juice and juice drinks, ice cream, sweetened yogurt, diet and regular soda			
Solid cariogenic foods ^d $\pm$ SEM	2.47 $\pm$ 0.31	4.88 $\pm$ 0.46	< 0.001 ^b
Bread, potato chips, crackers, jam/jelly, sweetened cereal, bananas, cookies			
Eating or drinking frequency/day	5.29 $\pm$ 0.24	6.16 $\pm$ 0.15	0.0016 ^b

^aSEM = Standard Error of the Mean.^bStudent's *t* test.^cChi-square test.^dCariogenicity coding (from Palmer *et al.*, 2010).

preceding 3 mos, and the parent or guardian provided informed consent for the child's participation. Severe-ECC children (n = 53) had extensive lesions in the primary dentition (Table), and 32 children were caries-free (Hughes *et al.*, 2011). The study design, protocol, and informed consent were approved by the Institutional Review Boards of participating institutions. The children's parents or caregivers completed a demographic and dietary survey (Palmer *et al.*, 2010).

Clinical measurements and plaque sampling were performed on children at baseline, and at 6 and at 12 mos after comprehensive restorative and extraction therapy under general anesthesia and caries-preventive measures for severe-ECC children (Hughes *et al.*, 2011). Plaque samples were taken from the mesial interproximal sites of primary molars, placed in 100 μ L TE buffer, and frozen at -70°C until microbial analysis (Palmer *et al.*, 2010). For children with new caries lesions, the visit during which caries was detected (6 or 12 mos) was the last visit for that child.

Microbiological Methods

DNA from plaque samples was purified with the use of MasterPure kits (Epicenter, Madison, WI, USA) as previously described (Palmer *et al.*, 2010).

Microarray analysis

Samples were analyzed by microarray to over 300 bacterial taxa by the Human Oral Microbe Identification Microarray (HOMIM) assay as previously described (Preza *et al.*, 2009). The lower limit of detection for the HOMIM array is approximately 10⁴ bacterial cells. Only probes that were reactive to at least one sample were evaluated.

Bacterial-specific PCR analysis

PCR was performed for the detection of *S. mutans*, *S. sobrinus*, and *Bifidobacteriaceae* (Palmer *et al.*, 2010). *Scardovia wiggisiae* PCR was performed with species-specific primers designed with AlleleID[®] (PREMIER Biosoft International, Palo Alto, CA, USA) in 16S rRNA. The forward primer was (Scar448F) 5'-GTG GAC TTT ATG AAT AAG C -3' and reverse primer (Scar619R) 5'-CTA CCG TTA AGC AGT AAG -3'. The samples were amplified by pre-heating (94°C, 2 min), denaturation (94°C, 20 sec), annealing (51°C, 20 sec), and elongation (72°C, 20 sec) (40 cycles), with a final extension (72°C, 5 min). Amplicons were verified on 1.5% low-melt agarose gels (NuSieve[®] GTG[®], Lonza, Rockland, ME, USA). The specificity of the primers was evaluated by testing against *Scardovia inopinata*, *Parascardovia denticolens*, *Alloscardovia omnicolens*,

A. Species detected by HOMIM microarray

B. Species detected by taxon-specific PCR

Figure 1. (A) Microbiota of severe-ECC and caries-free children by microarray. Percentage of children with reactivity to probes in HOMIM microarray. Taxa were selected as showing some difference between disease categories, with taxa detected more frequently in severe ECC to the left and in caries-free to the right. For data from all probes, see Appendix Table. Taxa showing associations with severe ECC included *Slackia exigua*, *S. parasanguinis* I & II, *Prevotella* species, and *Neisseria flavescens*. *S. mutans* was detected in 25% severe-ECC children in the microarray. (B) Microbiota of severe-ECC and caries-free children by taxon-specific PCR. Taxa showing greatest association with severe ECC were *Bifidobacteriaceae*, *S. wiggsiae*, and *S. mutans* in combination with these taxa. *S. mutans* was detected in 56% severe-ECC children by species-specific PCR.

Bifidobacterium dentium, *Bifidobacterium subtilis*, and selected *Actinomyces*, *Corynebacterium*, *Propionibacterium*, *Mobiluncus*, and *Rothia* species. Amplicons from samples from the *S. wiggsiae* primers were sequenced and compared against the Human Oral Microbiome Database (HOMD) (Dewhirst *et al.*, 2010).

Statistical Analyses

Mean differences between caries-free and severe-ECC children in age, plaque, gingival, and bleeding indices and dietary characteristics were evaluated by *t* test. Data from microarray and PCR were analyzed as detected or not-detected by child.

Differences in proportions between disease categories and demographic characteristics, probe reactions from the microarray, and PCR were tested by Chi-square. Severe-ECC children, divided into children with and without new lesions post-therapy, and baseline and follow-up microbial data were compared by McNemar Chi-square. A significance threshold of $p \leq 0.05$ was used. In addition, adjustment for multiple comparisons was by the false-discovery rate (Benjamini and Hochberg, 1995). Statistical analyses were performed with SPSS® software.

Partial least-squares analysis (OPLS procedure) with SIMCA P+ (version 12.0, Umetrics AB, Umeå, Sweden) was used for multivariate analysis of microarray, PCR, dietary, clinical, and demographic information (Table) as previously described (Kanasi *et al.*, 2010b; Lif Holgersson *et al.*, 2011). PLS is a multivariate linear regression model method suitable for the detection of correlations between matrices of descriptor and response variables in datasets where the descriptor variables co-vary and/or the numbers exceed the numbers of observations. Variable auto-scaling to unit variance and cross-validation (Q^2) was done by the software.

RESULTS

There were no differences in child age, gender, race, or ethnicity between the 53 severe-ECC and 32 caries-free children (Table). Severe-ECC children had higher plaque and gingival indices and bleeding scores than caries-free children. Putative cariogenic foods and eating and drinking frequencies were more frequent in severe-ECC than caries-free children (Table).

Taxa detected more frequently by microarray at baseline in severe-ECC than caries-free children included *Slackia exigua* ($p = 0.002$), *Streptococcus parasanguinis* I and II ($p = 0.013$), *Prevotella melaninogenica* ($p = 0.013$), and *Prevotella* Cluster IV ($p = 0.018$) (Fig. 1A, Appendix Table). Species detected more frequently in caries-free children included *Cardiobacterium hominis* ($p = 0.003$), *Leptotrichia hofstadii* ($p = 0.006$), and *Corynebacterium matruchotii* ($p = 0.017$). Species detected more frequently at baseline in severe-ECC children who subsequently had new caries lesions than in ECC children without new lesions included *Prevotella* Cluster IV ($p = 0.001$), *Prevotella nigrescens* ($p = 0.01$), and several *Capnocytophaga* taxa. None of the differences in microarray data was significant after adjustment for multiple comparisons.

The PCR primers for *S. wiggsiae* detected *S. wiggsiae* but did not react with species in *Bifidobacteriaceae* or the Phylum/Class *Actinobacteria*. All amplicons from PCR of plaque samples for *S. wiggsiae* showed homology to *S. wiggsiae* sequences in HOMD. By specific PCR, *S. mutans*, *S. sobrinus*, *Bifidobacteriaceae*, and *Scardovia wiggsiae* individually, and the combinations *S. mutans* with *S. sobrinus* ($p < 0.02$), *S. mutans* with *Bifidobacteriaceae* ($p < 0.0001$), and *S. mutans* with *Scardovia wiggsiae* ($p < 0.005$) were associated with severe ECC (Fig. 1B).

Children with subsequent lesions ($n = 17$) compared with none ($n = 36$) had no differences in baseline gingival index, bleeding, number of carious surfaces, restorations placed, or teeth extracted (data not shown). For 16 out of 17 children, the new lesions on follow-up were on surfaces without a previous restoration.

By microarray, severe-ECC children without new lesions (Fig. 2A) showed post-therapy reductions in *S. mutans* (not detected in follow-up), *Streptococcus mitis* biovar 2 ($p < 0.035$), *Campylobacter gracilis* ($p < 0.02$), *Campylobacter concisus/rectus* ($p = 0.004$), *Capnocytophaga* species Human Oral Taxon from HOMD (HOT) 335 ($p = 0.004$), and *Selenomonas* taxa. Species that were detected more frequently in these children post-therapy were *Propionibacterium propionicum* ($p < 0.002$), *Lautropia mirabilis* ($p < 0.026$), and 3 *Capnocytophaga* species. Children without new lesions also had significantly lower plaque index ($p < 0.001$), gingival index ($p < 0.0001$), and bleeding gingival sites ($p < 0.0001$) in follow-up compared with pre-treatment. By PCR, only *S. mutans* was detected less frequently in follow-up ($p = 0.035$).

There were no significant differences in baseline and follow-up microbiota of severe-ECC children with new caries lesions (Fig. 2B), although there were reductions at follow-up in plaque index ($p < 0.02$), gingival index ($p < 0.0001$), and bleeding gingival sites ($p < 0.04$).

The multivariate model of baseline microbial, clinical, and food cariogenicity data yielded a strong model with 2 significant components ($R^2 = 0.646$, $Q^2 = 0.462$). This model clustered the children into caries-free children ($n = 32$) and two subgroups [C1 ($n = 34$) and C2 ($n = 19$)] of severe-ECC children (Fig. 3). The factors associated with being caries-free were consistent with the univariate observations (Fig. 3, Appendix Figure).

Restricting the multivariate model to severe-ECC children (model $R^2 = 0.784$, $Q^2 = 0.622$) confirmed separation into two caries subgroups (C1 and C2). Scores for plaque, gingivitis and bleeding; PCR detection of *S. mutans*, *S. wiggsiae*, *S. mutans* combined with *S. wiggsiae*, *S. mutans* combined with *S. sobrinus*; and microarray detection of *Slackia exigua* did not differ significantly between the two caries subgroups, though values tended to be higher in group C2. Children in subgroup C1, however, were specifically characterized by higher intake of solid cariogenic foods, being White and presence of *Haemophilus parainfluenzae* (Fig. 3, Appendix Fig.). Children in caries subgroup C2 were specifically characterized by higher detection frequencies of multiple bacterial species, including *Prevotella* by microarray, and PCR detection of *S. sobrinus* and *Bifidobacteriaceae* alone or in combination with *S. mutans* (Fig. 3, Appendix Fig.). The differences between cluster groups were also significant by univariate analyses (data not shown).

Clinically, although not significant, children in subgroup C1 had more carious surfaces (44%) than those in subgroup C2 (32%) before treatment ($p = 0.07$), and on follow-up, more children in subgroup C2 (47%) experienced new caries lesions than those in subgroup C1 (15%) ($p = 0.06$).

DISCUSSION

In this study, we confirmed differences in the microbiota of caries-free children and S-ECC children, and observed differences in the microbiota of children with and without caries progression after treatment. In the children without new lesions, there was reduction in several species in addition to *S. mutans*. Using modeling based on the microbiota, high cariogenic diet,

A. Severe-ECC children (n=36) without new lesions

B. Severe-ECC children (n=17) with new carious lesions

Figure 2. (A) Microbiota pre-treatment and at follow-up of severe-ECC children without new lesions after 12 mos monitoring. Taxa were selected as showing some difference between pre-treatment and follow-up, with taxa detected more frequently pre-treatment to the left and in follow-up to the right. Taxa detected less frequently 12 mos after treatment included *S. mutans*, *C. concisus*/*C. rectus*, *C. gracilis*, *S. mitis* biovar 2, and unnamed *Capnocytophaga* and *Selenomonas* taxa. Species detected more frequently in follow-up included *P. propionicum*, *L. mirabilis*, and *C. sputigena*. (B) Microbiota pre-treatment and at follow-up of severe-ECC children with new lesions during 12 mos of monitoring. Taxa are the same and in the same order as in Fig. 2A. While there is variation in taxa detection between pre-treatment and follow-up, none of the differences was significantly different. Comparison of Figs. 2A and 2B also illustrates baseline differences between the microbiota of children with and without new lesions in follow-up, including *Prevotella* Cluster IV ($p = 0.001$), *Prevotella nigrescens* ($p = 0.01$), and several *Capnocytophaga* taxa (Appendix Table).

Figure 3. PLS modeling plots. **Inset:** PLS score plot based on demographics, diet, and microbiota of severe-ECC and caries-free children. Children clustered into three groups, caries-free (●) and two ECC subgroups, C1 and C2 (●). The PLS model used caries at baseline as the dependent variable, and the microarray, PCR, dietary, clinical, and demographic information as the independent matrix. **Main Plot:** PLS loading scatter plot illustrating important variables for clustering. Cluster groups are as in the score plot (inset). Taxa with VIP-values > 1.0 are indicated as follows: *Haemophilus parainfluenzae*, *Capnocytophaga* sp. HOT329, *Actinomyces* Cluster, *C. hominis*, *C. matruchotii*, *L. hofstadii*, *Aggregatibacter segnis*, *S. mitis* bv2, *Prevotella melaninogenica*, *Prevotella* cluster IV, *Prevotella* cluster I, *P. denticola*, *P. nigrescens*, *Prevotella* sp. HOT308, *Selenomonas sputigena*, *S. noxia*, *S. artemidis*, *Selenomonas* sp. HOT138/146, *Treponema maltophilum*, *T. socranskii*, *Eubacterium infirmum*, *E. saphenum*, *Tannerella* sp. HOT286, *Gemella sanguinis*, *Bacteroidetes G-2:HOT274 (AU125)*, *Actinomyces gerencseriae*, *Veillonella atypica*.

and clinical features, we observed two groups of severe-ECC children, suggesting diversity within severe ECC.

A new finding was the association of *Slackia exigua* with severe ECC, suggesting that the species may be a risk factor for this infection. *S. exigua* is a fastidious anaerobic Gram-positive rod (Wade *et al.*, 1999) in *Actinobacteria* (Dewhirst *et al.*, 2010) and has been associated with periodontitis (Abiko *et al.*, 2010). *Prevotella* species were also associated with severe ECC. *Prevotella* species were detected in ECC (Corby *et al.*, 2005), and cluster IV species *P. melaninogenica* and *Prevotella nigrescens* were cultured from the current population (Tanner *et al.*, 2011). *Prevotella* species were not strongly aciduric (Tanner *et al.*, 2011) or acidogenic, suggesting that their presence reflects the ecological niche of deep dentinal caries (Nadkarni *et al.*, 2004; Chhour *et al.*, 2005), rather than contributing to caries progression.

We developed a PCR assay for *S. wiggsiae* because of its association with severe ECC by culture (Tanner *et al.*, 2011) and by 16S rRNA gene probes as CX101 (Becker *et al.*, 2002), but this species was detected relatively infrequently in the HOMIM assay. In contrast to our culture data, *S. wiggsiae* by PCR showed a stronger association with severe ECC than *S. mutans*, suggesting that the relative importance of these species needs further study. Combinations of *S. mutans* and *S. sobrinus* were

associated with S-ECC, consistent with improved risk assessment over these species individually for ECC (Okada *et al.*, 2005; Choi *et al.*, 2009). The associations of combinations of bifidobacteria and *S. wiggsiae* with *S. mutans* suggest that additional species combinations could be valuable in risk assessment for ECC. None of the species combinations tested, however, was associated with detection of new lesions post-therapy, including *S. mutans* with *S. sobrinus*, suggesting that risk assessment for ECC in the general population is different from predicting new lesions in children who already have advanced disease.

Species at baseline associated with children without new lesions post-therapy included *Cardiobacterium hominis* and *S. mitis* biovar 2. *C. hominis* was also associated with control sites for root caries (Preza *et al.*, 2009), and *S. mitis* was associated with caries-free children by culture as compared with severe-ECC children (Marchant *et al.*, 2001; Tanner *et al.*, 2011), consistent with the current study findings. Several unnamed *Capnocytophaga* species were detected, but their taxonomy needs clarification before their role in disease can be assessed.

We examined pre-treatment and follow-up microbiotas and observed that several species, including *S. mutans*, changed in detection in the children without new lesions, suggesting a shift in the plaque microbial complex. There were no significant changes in children with new caries, suggesting minimal change

in the microbiota, and that persistence of the pre-treatment microbiota was responsible for caries progression. These observations are in accord with the role of the composition of dental plaque as a bacterial community in health and caries (Marsh, 2006; Takahashi and Nyvad, 2011). Studies focusing on *S. mutans* observed reduction in this species post-treatment in children without new caries lesions (Simratvir *et al.*, 2010), as observed in the current report and by selective isolation of *S. mutans* (Hughes *et al.*, 2011).

Multivariate modeling by baseline characteristics grouped children by caries-free or severe-ECC status, with characteristics that were similar to those in univariate analysis. Modeling also revealed diversity within severe ECC. Both severe-ECC groups had poor oral hygiene and a similar level of *S. mutans* infection, but one group ate more cariogenic foods while the other was characterized by a multispecies complex including *Bifidobacteriaceae* and *S. sobrinus*. It seems possible that one group of children had disease associated with an increased infection by cariogenic bacteria compared with the second group. Diversity within ECC is consistent with the observed differences within ECC, based on higher counts of cariogenic bacteria in subsets of children (Beighton *et al.*, 2004), and warrants further investigation.

A strength of this study was the use of the HOMIM microarray that facilitated detection of multiple taxa and revealed ecological changes that occurred after therapy that would not have been possible by assaying only for mutans streptococci and lactobacilli. The major study limitation was in loss to follow-up of children with severe ECC, which limited the post-treatment monitoring time to 12 mos, and reduced statistical power for examining pre- and post-treatment microbiotas in children in the new-lesion group. While a longer follow-up time could have identified additional children with new lesions, in children with 2 years' follow-up, most of the new lesions were recurrent caries (Jamieson and Vargas, 2007). In the current study, children had new lesions on unrestored surfaces, suggesting that we captured a population with aggressive caries. A technical limitation was reduced detection for specific species with the universal primers needed for the microarray assay. We addressed this by adding specific PCR to selected taxa.

This study aimed to understand the influence of the microbiota on treatment relapses after therapy for severe ECC. Our findings suggest that there are differences in the microbiota before treatment, with increased infection in children with new lesions post-treatment, and differences in treatment response. Further, we propose that severe ECC is a complex infection, with the possibility that the microbiota and diet may have different degrees of influence in different children.

ACKNOWLEDGMENTS

This investigation was supported by USPHS grants DE-015847 and DE-007327 from the NIDCR of NIH, the Colgate-Palmolive Company, and the Henning and Johan Throne-Holst's Foundation.

We acknowledge Vanja Klepac-Ceraj for exploratory data analyses. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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