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Genetic Diversity and Evidence for Transmission of *Streptococcus mutans* by DiversiLab rep-PCR

Stephanie S. Momeni^a, Jennifer Whiddon^b, Kyounga Cheon^a, Tariq Ghazal^a, Stephen A. Moser^b, and Noel K. Childers^{a,*}

^aDepartment of Pediatric Dentistry, The University of Alabama at Birmingham, The School of Dentistry Building Room 304, 1720 2nd Avenue South, Birmingham, AL 35294-0007, USA

^bDepartment of Pathology, The University of Alabama at Birmingham, West Pavilion Room, 1720 2nd Avenue South, Birmingham, AL 35294-0007, USA

Abstract

This two-part study investigated the genetic diversity and transmission of *Streptococcus mutans* using the DiversiLab repetitive extragenic palindromic PCR (rep-PCR) approach. For children with *S. mutans* and participating household members, analysis for evidence of unrelated child-to-child as well as intra-familial transmission was evaluated based on commonality of genotypes.

A total of 169 index children and 425 household family members from Uniontown, Alabama were evaluated for genetic diversity using rep-PCR. Thirty-four unique rep-PCR genotypes were observed for 13,906 *S. mutans* isolates. For transmission, 117 child and household isolates were evaluated for shared genotype (by child and by genotype cases, multiple matches possible for each child). Overall, children had 1–9 genotypes and those with multiple genotypes were 2.3 times more likely to have caries experience (decayed, missing and filled teeth/surfaces > 0). Only 28% of children shared all genotypes within the household, while 72% had at least 1 genotype not shared with anyone in the household. Children had genotype(s) not shared with any household members in 155 cases. In 158 cases children and household members shared a genotype in which 55% (87/158 cases) were shared with more than one family member. Children most frequently shared genotypes with their mothers (54%; 85/158), siblings (46%; 72/158) and cousins (23%; 37/158).

A reference library for *S. mutans* for epidemiological surveillance using the DiversiLab rep-PCR approach is detailed. The genetic diversity of *S. mutans* in this population demonstrated frequent

***Corresponding Author:** Dr. Noel K. Childers, Department of Pediatric Dentistry, University of Alabama at Birmingham, School of Dentistry Room 304, 1720 2nd Avenue South, Birmingham, AL 35294-0007, USA, Tel: (+1) 205-934-3230; Fax: (+1) 205-934-7013; nkc@uab.edu.

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The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Table S1. Summary of genotypes observed for children and all participating household family members.

Table S2. Commonality of cases of genotypes that occur only in children (no household family members with matching genotype) indicating child-to-child transmission.

Table S3. Commonality of cases of genotypes that occur in children and their household family members indicating probably mother-to-child and/or intra-familial transmission.

commonality of genotypes. Evidence for both child-to-child and intra-familial transmission of *S. mutans* was observed by rep-PCR.

Keywords

genetic diversity; genotyping; multilocus sequence typing; rep-PCR; *Streptococcus mutans*; transmission

1. INTRODUCTION

Streptococcus mutans are bacteria commonly linked with the initiation and progression dental caries (Gross et al., 2012, Loesche, 1986, Tanner et al., 2002). The genetic diversity of *S. mutans* strains has been studied using continually evolving gel based typing methods including randomly amplified polymorphic DNA (RAPD) fingerprinting (Redmo Emanuelsson et al., 2003), arbitrarily primed PCR (AP-PCR) (Baca et al., 2012, Cogulu et al., 2006, Domejean et al., 2010, Gamboa et al., 2010, Gilbert et al., 2014, Hu et al., 2014, Lembo et al., 2007, Li and Caufield, 1998, Liu et al., 2007, Lynch et al., 2015, Napimoga et al., 2004, Palmer et al., 2012, Pieralisi et al., 2010, Teanpaisan et al., 2012, Zhao et al., 2014, Zhou et al., 2011), pulse field gel electrophoresis (PFGE) (Mineyama et al., 2004, Mineyama et al., 2007, Roberts et al., 2002) and repetitive extragenic palindromic PCR (rep-PCR) (Cheon et al., 2011, Moser et al., 2010). Molecular based typing methods used to evaluate genetic diversity include multilocus enzyme electrophoresis (MLEE) (Napimoga, Kamiya, Rosa, Rosa, Hofling, Mattos-Graner and Goncalves, 2004) and more recently, multilocus sequence typing (MLST) (Do et al., 2010, Lapirattanakul et al., 2008, Momeni et al., 2013, Nakano et al., 2007). The overall consensus of these studies has indicated that measure of accuracy for *S. mutans* genotypic diversity is variable depending on the methods used and sample populations evaluated. In general, the number of genotypes (amplitypes, sequence types, etc.) observed indicate limited genetic variation except in the case of MLST, which can be more discriminative than traditional gel based typing methods since a single nucleotide changes can result in a new sequence type (ST). The two most frequently used methods in the literature are AP-PCR and rep-PCR, both of which are currently being used in large-scale epidemiological studies of *S. mutans* in high-risk communities (Cheon et al., 2013, Lynch, Villhauer, Warren, Marshall, Dawson, Blanchette, Phipps, Starr and Drake, 2015). Genotyping is a valuable tool for epidemiological surveillance of clinical microorganisms, allowing for the efficient investigation of microbial diversity, micro-evolutionary shifts, and transmission tracking.

Many studies have addressed the question of horizontal and vertical transmission of *S. mutans*. Vertical transmission has been defined as the sharing of microbes from caregiver to child (typically mother-to-child). Horizontal transmission has been defined as the sharing of microbes between members of a similar age group (e.g., child-to-child, sibling-to-sibling) although horizontal transmission may also occur between other family members (e.g. spouse-to-spouse) (Berkowitz, 2006). For clarification purposes, this paper uses the terms mother-to-child, intra-familial (i.e., sharing of genotypes within a family, regardless of age, beyond mother-to-child), and child-to-child (i.e., sharing of genotypes among the unrelated

originally recruited children) when discussing transmission. Index child or when used broadly children will be used to refer to the originally recruited child in study and not other children within the home (i.e., siblings or cousins).

Mother-to-child transmission is widely accepted as the primary route of infection with *S. mutans*, although other sources of shared genotypes have been reported (i.e., father, nursery and school classmates) (Alves et al., 2009, Binks and Duane, 2015, Domejean, Zhan, DenBesten Stamper, Boyce and Featherstone, 2010, Kozai et al., 1999, Liu, Zou, Shang and Zhou, 2007, Redmo Emanuelsson and Wang, 1998, Tedjosasongko and Kozai, 2002). There are several missing pieces in the current literature. Studies looking at data for transmission by all three routes together in the same study are lacking. Publications considering other household family members (e.g. extended family members within the residence) are rare. Research investigating intra-familial transmission typically evaluates transmission only within families and not between. Furthermore, in many cases, the children recruited for such studies have no known interactions, thus limiting the potential to observe child-to-child transmission. Studies that focus only on one relationship (i.e., mother-to-child or father-to-child) may assume the member compared is the source when other individuals can be possible sources. The dynamic nature of transmission makes it important to evaluate all possible routes (mother-to-child, intra-familial, and child-to-child) of infection when evaluating transmission since children are typically exposed to all of these source types.

Over the last 8 years, this laboratory has utilized the DiversiLab system, an automated rep-PCR method (Healy et al, 2005), to investigate *S. mutans* strain diversity in two cohorts of children and their household family members in Uniontown, AL, USA (Cheon, Moser, Whiddon, Osgood, Momeni, Ruby, Cutter, Allison and Childers, 2011, Cheon, Moser, Wiener, Whiddon, Momeni, Ruby, Cutter and Childers, 2013, Moser, Mitchell, Ruby, Momeni, Osgood, Whiddon and Childers, 2010). The advantages of this approach are that the method is kit based and standardized, has been shown to be reproducible over time and between laboratories, and generates data that is easily shared between laboratories through web-based software (Moser, Mitchell, Ruby, Momeni, Osgood, Whiddon and Childers, 2010, Voets et al., 2013). DiversiLab offers a number of bacterial reference strain libraries for clinical researchers but there currently is no reference library available for *S. mutans*. Therefore, the development of a *S. mutans* library would be beneficial for global epidemiological study of *S. mutans* associated with dental caries and other diseases (e.g. infective endocarditis).

In addition to rep-PCR analysis, this laboratory also utilizes MLST to validate new representative library rep-PCR genotypes. This approach is consistent with the recommendation that genotypes should be evaluated by an alternate method to validate lineage assignments (Foley et al., 2006, van Belkum et al., 2007). Previously, 22 representative rep-PCR library strains were reported for *S. mutans* using MLST (Momeni, Whiddon, Moser, Cheon, Ruby and Childers, 2013). Additional rep-PCR genotypes have been discovered and are evaluated here with MLST to validate strain uniqueness.

The present study has two aims. The first is to determine the overall genetic diversity of *S. mutans* isolates obtained from an 8-year longitudinal study of children and their household

family members using rep-PCR. The second aim is to provide an overview of evidence for mother-to-child, intra-familial, and child-to-child transmission of *S. mutans* using commonality of rep-PCR genotypes for children and their household members.

2. MATERIALS AND METHODS

2.1. Sample collection

The University of Alabama at Birmingham Institutional Review Board approved this study. Adult household family members provided informed consent and waiver of assent for participation of children. Two cohorts of children and their household family members were enrolled over a two-year period as part of a longitudinal epidemiological study of *S. mutans* in rural Uniontown, Alabama, USA. This minority (all index children were African-American) sample population was determined to be high risk for early childhood caries due to low socioeconomic status and high caries prevalence (68.5% of children have caries by 4 years of age) (Ghazal et al., 2014). Cohort 1 (CH1) consisted of 91 schoolchildren (age 5–6 years at enrollment) and their household family members recruited from an elementary school. Cohort 2 (CH2) consisted of 90 infants (age 6–18 months initially) and their household family members recruited at a local community center. Inclusion criteria were that household members must reside in the same residence as the child and that all index children were unrelated. Generally, most family members elected to participate; however, enrollment was not required. Subjects were provided compensation for their participation along with oral health education and dental hygiene supplies (toothbrushes, toothpaste). In total 593 individuals were evaluated. Household members included mother, father and siblings as well as extended family members (i.e., grandparents, aunts, uncles, cousins). The decayed, missing and filled teeth/surfaces (i.e., dmfs/DMFS) were recorded according to the World Health Organization (WHO) criteria (WHO, 1997) for evaluation of caries activity (i.e., unrestored cavitation) and caries experience.

Periodic (i.e., 6–12 month) samples were collected as previously described (Moser, Mitchell, Ruby, Momeni, Osgood, Whiddon and Childers, 2010). In this study plaque, saliva and tongue scraped samples were evaluated and the preferred sample type was plaque samples if *S. mutans* isolates were available. Plaque samples from children were generally collected with a sterile toothpick from all surfaces of specific permanent or primary molar teeth for cohort 1 and 2, respectively. Household family members had plaque collected from multiple molar teeth with sterile toothpick for a pooled sample. Caries status of specific teeth were recorded but not used as the criteria for specific teeth collected. Samples were inoculated on mitis salivarius media supplemented with bacitracin and sucrose (MSB, Gold's Media) and incubated anaerobically for 48 hours after which up to 10 presumptive *S. mutans* isolated colonies were selected based on morphological appearance (Gold et al., 1973). Sub-cultured isolates were stored in 80% glycerol (20% culture) at –80°C until processed with rep-PCR. In total, 30,822 putative *S. mutans* isolates were collected over 8 years of which 13,906 samples were submitted for DNA extraction and were of sufficient quality to be processed with rep-PCR. Confirmation of *S. mutans* was performed using SYBR Green PCR with primers specific to the *gtfB* gene to screen for *S. mutans* (Moser, Mitchell, Ruby, Momeni, Osgood, Whiddon and Childers, 2010, Yoshida et al., 2003)

2.2. Rep-PCR

Rep-PCR analysis was performed as previously described (Moser, Mitchell, Ruby, Momeni, Osgood, Whiddon and Childers, 2010). In addition to the clinical isolates, various prototype (control) strains of *S. mutans* (Ingbritt C, UA96, OMZ-175, GS-5, kpsp2, LM7, OR22P1, 10449, YT1, NG8, UA159, UA130 & FT1) were also evaluated. Briefly, DNA was isolated using the UltraClean microbial DNA isolation kit (bioMérieux, Inc., Durham, NC). Quality and quantity of DNA was measured to determine acceptable purity using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Rep-PCR was performed using the Diversilab system with the Streptococcus DNA fingerprinting kit. PCR was performed on a GeneAmp 9700 with gold heating block (Applied Biosystems). Amplicons were then analyzed with microfluidics LabChip (bioMérieux, Inc.) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The DiversiLab web-based software was used to generate reports including similarity matrix, dendrograms, and electropherograms. Genotypes were determined using a three minor or 1 major band difference guideline. Percent similarity was not used for determining genotypes as percent similarity becomes increasing unreliable when larger numbers of isolates are analyzed (Momeni et al., 2015). Major and minor bands were differentiated using a cut-off of 100 fluorescence units.

2.3. Genetic Diversity

The genetic diversity of *S. mutans* was evaluated using rep-PCR genotype data for the 13,906 isolates from children and the household family members. For each child, the number of genotypes, sex, age group (i.e., cohorts) and caries experience were included in the analysis. For household members only the number of genotypes was evaluated. Analysis per child was performed to determine if sharing of genotypes with mother, father or any household members was associated with children's sex, age group, or caries experience.

2.4. Evaluation of Commonality for Possible Transmission

To investigate potential transmission, data was evaluated two ways: by child and by rep-PCR genotype. Only children with 1 or more household members with *S. mutans* isolates were considered in the analysis (n = 118). One child had no *S. mutans* detected and was therefore excluded from analysis since genotypes were required to be included. Seven mothers had no *S. mutans* detected but were included in statistical analysis because their child did have genotype information. Thus a total of 117 children and their household family members were available for analysis. For each rep-PCR genotype, each child was evaluated as having either 1) no match with any household member or 2) matching with one or more household member. Children with no match to any household member were counted as likely child-to-child transmission since the source is probably not within the household. Children in this group were further evaluated by dividing into three subgroups based on the number of isolates with the genotype (groups: children with only 1 isolate termed "minor genotypes", children with 2–9 isolates termed "borderline genotypes", and children with 10 isolates "established genotypes"). Since the focus of this analysis was based on the genotype perspective, in this paper the term "cases" is used since some children had more than one genotype. For instance, a child may have 3 genotypes that did not match any household member. These would be counted at 3 cases for only 1 child.

Children with a genotype matching a household member were counted as evidence for interfamilial transmission. This group was further evaluated as having either 1) only one household member with the genotype or 2) more than 1 household member with the genotype. Children with more than one household member with the same genotype were considered stronger candidates for interfamilial transmission.

2.5. MLST

In addition to previously reported ST, MLST was performed on 13 newly identified rep-PCR genotype representative Library strains (Momeni, Whiddon, Moser, Cheon, Ruby and Childers, 2013). Briefly, independent PCR reactions were performed using primer sets specific to the eight housekeeping gene fragments of the MLST Scheme for *Streptococcus mutans* as published by Nakano et al. (Nakano, Lapirattanakul, Nomura, Nemoto, Alaluusua Gronroos, Vaara, Hamada, Ooshima and Nakagawa, 2007). Resulting amplicons were purified using the UltraClean PCR Clean-up kit (MoBio Laboratories, Inc., Carlsbad, CA) and sequenced by Sanger sequencing using the UAB Heflin Genomic Core Laboratory. Sequence data analysis was performed using the CLC DNA workbench with MLST module version 5.7.1 (CLC bio USA, Cambridge, MA). Sequences were aligned with a reference strain *Streptococcus mutans* UA159. Alleles and ST were compared with the PubMLST database (www.pubmlst.org) for numerical assignment. New alleles were repeated from PCR through sequencing to confirm new sequences prior to submitting to PubMLST. All new alleles and ST were submitted to the PubMLST database and added to GenBank.

2.6. Statistical Analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The maximum caries score observed for all periods were used for caries experience except for children with less than 7 periods of rep-PCR data or for whom caries exam data was unavailable, in which cases the outcome value was imputed by using last observation carried forward (e.g., if a child in CH2 was last seen at age 2 and was caries free, this was recorded as caries free at age 4). Logistic Regression was used for both bivariate and multivariable analysis. For bivariate analyses, logistic regression modeling was used to assess individually the relationship between each covariate and the outcome. In the multivariable model building, only variables with an overall p-value <0.25 in the bivariate analyses were included. Backward selection was used to eliminate consecutively the variables with p-values >0.1 from the models. The final model contained only variables with p-values <0.1. Changes in Akaike Information Criterion (AIC) were not considered when evaluating the fitted model generated from the preceding step, because AIC is sensitive to changes in sample size associated with adding variables. The outcome variable was defined as the presence of more than one *S. mutans* genotypes (yes/no) across all study visits (e.g., if a child had only one specific genotype at all periods evaluated, this was recorded as a child with a single genotype; or if a child had more than one genotype at any one period, or over different periods, then this was recorded as a child with multiple genotypes). The main explanatory variables were caries at ages 12 and 4 years (for CH1 and CH2, respectively), cohort, sex, matching the genotype with household member and number of isolates group (isolates group were defined as group 1 (0–9 isolates), group 2 (10–29 isolates), group 3 (30–82 isolates), and group 4 (more than 83 isolates)).

3. RESULTS

3.1. Genetic Diversity

Thirty-four unique rep-PCR genotypes were observed for 13,906 *S. mutans* isolates from 593 individuals (Fig. 1). The total number of individuals in Fig. 1 is higher than the total from CH1 and CH2 because individuals can have more than one genotype. *S. mutans* genotypes were reported for 82 children and 191 household family members (mean 3.58, range 0–11 members) in CH1 and 87 children and 234 household family members (mean 3.13, range 0–10 members) in CH2. Of these children, 30 CH1 and 13 CH2 children had no household members evaluated. The most frequently occurring genotypes were G18 (2,110 isolates), G09 (1,686 isolates), and G01a (1,363 isolates). The least reported genotypes were G21 (3), G42 (4) and G38 (5). G09 and G18 were the most prevalent genotypes, being found in 174 and 133 different individual subjects respectively. Four genotypes (G27, G10, G42, and G21) occurred in only one individual each, although the number of isolates with these genotypes found in these individuals ranged from 3–112. For the more common *S. mutans* prototype strains, UA159 demonstrated a rep-PCR genotype similar to G01b, Ingbritt C was most like G15, LM7 was similar to G23, and OMZ-175 and GS-5 matched G18.

A summary of children with single versus multiple genotypes is compared based on sex, age group, and caries experience in Table 1. Although more males were included in the study (91 males versus 77 females), the ratio of children with multiple genotypes, as evaluated by sex, were comparable. A comparable number of children were included in each age group (CH1 and CH2); however, a greater percentage of younger children had multiple genotypes (72% for CH2 versus 58% in CH1). A larger number of children in this population had caries experience (127 children versus 41 children in the caries free group). As would be expected, children in the caries free group had far fewer *S. mutans* isolates available and were less likely to have multiple genotypes (60% presented only 1 genotype).

Table 2 provides the results of the bivariate and multivariable statistical analysis for having multiple genotypes based on sex, age group (cohorts), caries status, and number of isolates analyzed. Younger children were significantly more likely to have multiple genotypes than older children ($p=0.053$) and having multiple genotypes was highly associated with dental caries experience ($p<0.001$). The probability for having multiple genotypes was significantly higher for children having great than 10 isolates evaluated ($p<0.001$). When applying multivariable analysis, the younger age group remained significant, as did having greater than 10 isolates. Accounting for the number of isolates evaluated between the caries groups for the multivariable analysis indicated that the p-value for multiple genotypes was approaching significance with an odds ratio of 2.6. Having multiple genotypes was not significantly different by gender. No significant associations were observed for sharing of genotypes with mother, father or any household members based on sex, age group, or caries experience. Interestingly, having multiple genotypes did not improve the odds ratio of matching any household members (data not shown, $p=0.2070$, OR 0.548).

3.2. Commonality for Possible Transmission

Evidence for transmission is presented first as an overall basic summary for the children and then as individual genotype cases (since children may have multiple genotypes).

Overall, 117 children and their household members were evaluated in this part of the study representing 13,124 *S. mutans* isolates. Index children with more than one household member were evaluated for 76% (89/117, mean 3.24, range 1–11) of families. This analysis included 50 children from CH1 and 67 from CH2 (Table S1). Of these, 27 children (23%, 27/117) shared no genotypes with any household members. Eighty-four children (71%, 84/117) had at least one genotype not shared with any household members (genotypes range 1–5 per child); however this data does not consider if genotypes are minor or established. Analysis also found 33 children (28%, 33/117) that shared all genotypes (range 1–4 genotypes) within the household. The number of genotypes per child ranged from 1–9 genotypes with an average of 2.72 genotypes per child. There were 75 children (63%, 75/105; total number adjusted for children with mothers) that shared at least 1 genotype with their mothers.

Table 3 (Table S2) lists 157 cases among 21 genotypes where a genotype occurred in a child, but did not occur in any of the corresponding household members. The number of cases is higher than the number of families evaluated (n=117) because each genotype was considered independently and thus a child may have multiple genotypes that did not match any household members. Thirteen genotypes were excluded from the table because 1 genotype (G19) did not match the profile for child-to-child transmission (intra-familial only), 4 genotypes (G10, G20, G24, G45) had only 1 child with no matching household members and did not match other children, and 8 genotypes did not have any *S. mutans* isolates from children (household member only; G03, G17, G21, G25, G27, G30, G38, G42). Three genotypes (G01d, G02, G11) had only one child in Table S2 but these matched other children that were excluded from this study due to the lack of household members for comparison but were included in the counts for child-to-child transmission.

For the children that did not share a genotype with a household family member, further analysis was done to evaluate minor, undetermined, and established genotypes (Fig. 2). Fifty-three cases (34%, 53/157) were considered minor or transient genotypes since only one isolate of these genotypes were found in one or more children. Cases where a child had 2–9 isolates of a genotype were considered borderline, thus, 57 cases (36%, 57/157) were treated as having undetermined significance. However, there were 47 cases (30%, 47/157) in which the child presented with 10 isolates of the genotype (established genotypes); 37 (24% 37/157) of these cases had more than one household family member evaluated.

Table 3 (and Table S3) lists the 158 cases among 21 genotypes where children and one or more household members shared a genotype. In 55% (87/158) of these cases more than one household member shared the genotype with the child. Thirteen genotypes were excluded from the table. Of these 4 genotypes, (G20, G24, G45, and G49) had profiles that did not match possible intra-familial transmission (child-to-child only); 6 genotypes (G03, G17, G21, G25, G30, and G38) had no children to compare with household members and 3 genotypes (G10, G27, G42) were excluded because these genotypes had no household

members to compare with the child. Of these 87 cases, 44 were cases where the child shared the genotype with 2 household members, 21 with 3 household members, 12 with 4 household members, and 10 with 5 household members.

The distribution of the shared genotypes among family members can be seen in Fig. 3 (and Table S3). It is possible that a child shared a genotype with multiple siblings, grandmothers, aunts, uncles, and/or cousins. Children sharing a genotype with any respective household member were counted only once. For instance, if a child had genotype G01 in common with 5 siblings and 3 cousins, they were counted as only 1 sibling case and 1 cousin case. Note that totals for the individual household members will not add to the total number of cases since a child may share the genotype with multiple family members. However, totals for each household member type are listed to indicate the number of cases in which each household member type was shared.

Fig. 4 compares the number of children with evidence for child-to-child and intra-familial transmission by rep-PCR. Six genotypes (G01b, G05, G14, G15, G22 and G26) appear to be predominately shared among the children (child-to-child transmission). In contrast, 4 genotypes (G09, G11, G18, and G50) were predominately shared within households (intra-familial transmission). However, these differences were not statistically significant by multiple Chi Square tests (data not shown).

3.3. Multilocus Sequence Typing

Following MLST for 13 newly identified genotypes, 7 new alleles and 7 new ST were identified (ST 184–190) in this study for the rep-PCR representative Library strains (Table 4). The 7 new alleles were added to Genbank (Accession numbers KU216130-KU216136). All new ST and alleles were added to the PubMLST database. Nine of the 13 new ST were found to be unique. The other 4 strains matched previously reported ST for other Library or clinical strains (NL01b matched L5; NL03 matched L18, and NL26 matched L22, and NL27 matched G13-4).

4. DISCUSSION

4.1. Genetic Diversity

Several studies using a variety of methods have previously evaluated the genetic diversity of *S. mutans*. AP-PCR has been employed most frequently; however, this methodology raises concerns of reproducibility between laboratories and variability of interpretation approaches (Bidet et al., 2000, van Belkum, Tassios, Dijkshoorn, Haeggman, Cookson, Fry, Fussing, Green, Feil, Gerner-Smidt, Brisse and Struelens, 2007). In the present study, the genotypic diversity of *S. mutans* using the DiversiLab rep-PCR approach, which has been found to be highly reproducible between laboratories, is summarized.

In this study 34 unique *S. mutans* rep-PCR genotypes from 13,906 isolates are reported (Fig. 1). The diversity of genotypes observed in this study is generally similar to other large-scale studies of *S. mutans* using AP-PCR (Lynch, Villhauer, Warren, Marshall, Dawson, Blanchette, Phipps, Starr and Drake, 2015, Palmer, Nielsen, Peirano, Nguyen, Vo, Nguyen, Jackson, Finlayson, Sauerwein, Marsh, Edwards, Wilmot, Engle, Peterson, Maier and

Machida, 2012). While some studies have reported more amplicons with AP-PCR from smaller sample pools, it should be noted in all gel based genotyping studies there is a degree of variability due to interpretation, techniques and conditions employed. This inconsistency makes direct comparison between studies difficult.

The present study is unique in its scale with over 13,000 isolates evaluated. However, due to improved reproducibility with the rep-PCR method, only 34 genotypes were identified, which resulted in extensive commonality of isolates among children and household family members that may not be seen with other methods. This commonality, although improving consistency, complicated the ability to evaluate if transmission was exclusive for genotypic matching of individuals, whether intra- or extra-familial. It is important to note that the results of rep-PCR here are based on similar banding patterns and not percent similarity. For instance, 2,110 isolates are reported for G18 and all of these isolates are uniform in their rep-PCR gel images. In addition the same ST (166) has been found for all isolates tested for G18 with MLST in other studies (Momeni et al., 2015). However, due to the allowance for up to 2 minor band differences, other genotypes, such as G12, demonstrate subtle genetic drift as more isolates are evaluated. In order to gain meaningful data, the 3 minor bands, 1 major band guideline was necessary to allow for some grouping of isolates. Without this guideline, most isolates would have been unique genotype and no valuable conclusions could be made. Thus the balance of commonality and diversity of genotypes should be considered.

Some studies have reported that having multiple genotypes is not significantly associated with dental caries using AP-PCR and random fragment length polymorphism (Kreulen et al., 1997, Lembo, Longo, Ota-Tsuzuki, Rodrigues and Mayer, 2007, Liu, Zou, Shang and Zhou, 2007, Mattos-Graner et al., 2001). However, our finding that children with multiple genotypes were about 4.5 times more likely to have caries than children with a single genotype (Table 2) is consistent with more recent reports using AP-PCR and multilocus enzyme electrophoresis (Alaluusua and Matto, 1996, Gamboa, Chaves and Valdivieso, 2010, Napimoga, Kamiya, Rosa, Rosa, Hofling, Mattos-Graner and Goncalves, 2004, Pieralisi, Rodrigues, Segura, Maciel, Ferreira, Garcia and Poli-Frederico, 2010, Zhao, Li, Lin, Chen and Yu, 2014, Zhou, Qin, Qin and Ge, 2011). This is the first report of this association using rep-PCR and combined with the findings of the recent studies would seem to suggest that having multiple *S. mutans* genotypes may be an important factor in dental caries development. Even when multivariable analysis was performed to account for the number of isolates analyzed per child and sex, these children with multiple genotypes remained 2.3 times more likely to have dental caries. However, the results of the present study are only an association. Further study is planned to evaluate this association in more detail using a longitudinal approach to the data, as well as, in vitro and in vivo studies evaluating the potential for additive or synergistic effects of multiple genotypes (e. g., biofilm and metabolomics studies).

No significant difference was observed between the number of genotypes and sex, which is consistent with other studies (Liu, Zou, Shang and Zhou, 2007, Pieralisi, Rodrigues, Segura, Maciel, Ferreira, Garcia and Poli-Frederico, 2010). Li and Caufield previously reported that female infants are more likely to share genotypes with their mothers than male children (Li

and Caufield, 1995). However, in the present study, no significant difference was observed between sharing of genotypes with the mother and sex. This study also reports a significant difference in the number of genotypes observed between age groups, differing from a previous report (Mattos-Graner, Li, Caufield, Duncan and Smith, 2001). However this difference may be because the age groups evaluated by Mattos-Graner *et al.* were all under 2.5 years whereas the current study evaluated infants and children with different age ranges (infants and children).

4.2. Evidence for Transmission

Evidence for transmission by rep-PCR was evaluated by querying whether a given genotype was shared with household family members. (Tables 3, S1, S2) The goal was to estimate, in broad terms, the number of cases of potential extra-familial and intra-familial transmission as estimated by rep-PCR analysis. Generally, no weight was given to the number of isolates examined, number of household members (except as described in the results) or time of acquisition. The results of this study are intended as an overview to determine if more a detailed longitudinal analysis is warranted and to provide potential sample pools for additional transmission analysis. Further study relating the rep-PCR genotypes found in this study with transmission on an individual child basis for evaluation of longitudinal (multiple collection periods) with clinical features (e.g. genotypes related to caries) and detailed household associations are beyond the scope of the current manuscript but are planned as future studies.

The data in this study is presented in two ways: by child and by genotype. The purpose of adding the analysis by genotype is an important addition since other studies make a potentially faulty assumption that when multiple genotypes are found in a child with caries that all the genotypes isolated from that child are associated with caries. Analysis using only the child also biases the data to underreport the number of genotypes not shared, thus failing to consider other sources. For instance, many studies base their analysis on a simple match/no match with mother. By this approach if any genotype is shared with the mother, the case is considered mother-to-child transmission when the child may have other genotypes not shared that are not represented in the analysis.

As reported in Table S1, a total of 117 children and their household family members were evaluated for transmission. The total number of children was adjusted for comparison with mothers (n=105) since not all children had mothers participating in the study and 1 child was eliminated because no *S. mutans* were recovered. The number of genotypes in this study was 1–9 per child whereas others have reported that children harbor between 1–6 genotypes (Alves, Nogueira, Stipp, Pampolini, Moraes, Goncalves, Hofling, Li and Mattos-Graner, 2009, Cheon, Moser, Whiddon, Osgood, Momeni, Ruby, Cutter, Allison and Childers, 2011, Cheon, Moser, Wiener, Whiddon, Momeni, Ruby, Cutter and Childers, 2013, Domejean, Zhan, DenBesten, Stamper, Boyce and Featherstone, 2010, Liu, Zou, Shang and Zhou, 2007, Lynch, Villhauer, Warren, Marshall, Dawson, Blanchette, Phipps, Starr and Drake, 2015, Pieralisi, Rodrigues, Segura, Maciel, Ferreira, Garcia and Poli-Frederico, 2010). The average number of genotypes per child in this study was 2.7 and is notably higher than what others have reported for children (range 1.5–2.0) (Cheon, Moser, Whiddon, Osgood, Momeni,

Ruby, Cutter, Allison and Childers, 2011, Cheon, Moser, Wiener, Whiddon, Momeni, Ruby, Cutter and Childers, 2013, Domejean, Zhan, DenBesten, Stamper, Boyce and Featherstone, 2010, Liu, Zou, Shang and Zhou, 2007, Lynch, Villhauer, Warren, Marshall, Dawson, Blanchette, Phipps, Starr and Drake, 2015). This finding is significant since the presence of more genotypes in children is linked with higher caries scores possibly due to inter-species competition or augmentation. (Alaluusua and Matto, 1996, Baca, Castillo, Liebana, Castillo, Martin-Platero and Liebana, 2012, Hirose et al., 1993, Napimoga, Kamiya, Rosa, Rosa, Hofling, Mattos-Graner and Goncalves, 2004, Pieralisi, Rodrigues, Segura, Maciel, Ferreira, Garcia and Poli-Frederico, 2010, Zhao, Li, Lin, Chen and Yu, 2014)

Analysis of genotypes resulted in a total of 157 cases where a child presented a genotype with no matching household family members (Table S2). This is the largest known reported number of extra-familial horizontal cases. Other studies have reported horizontal transmission between children at lower rates, and these studies typically focused on cohorts of children (in nurseries or classes) without looking at households (Alves, Nogueira, Stipp, Pampolini, Moraes, Goncalves, Hofling, Li and Mattos-Graner, 2009, Domejean, Zhan, DenBesten, Stamper, Boyce and Featherstone, 2010, Liu, Zou, Shang and Zhou, 2007, Mattos-Graner, Li, Caufield, Duncan and Smith, 2001). For instance, Alves et al. reported 14 pairs of children in 7 nurseries shared an AP-PCR amplitype, although only 4 were confirmed by RFLP. In contrast, the present data is more compelling since it evaluates the commonality of the genotypes among children with more than 1 household family member for 76% of children (89/117).

The 27 children (23%, 27/117) and 157 cases of genotypes where the child had a genotype not shared with any household members indicates that the source of transmission is probably not within the household (Tables S1 and S2). Thus, given the high frequency of commonality of genotypes among children in this study, child-to-child transmission with classmates was considered as a probable alternative source. Considering minor versus established genotypes, the 53 cases of minor genotypes (1 isolate only) may be less significant as these bacteria occur in low numbers, indicating these isolates are likely transient and may not be clinically relevant to established caries infections. Although longitudinal analysis was not a part of the current analysis, transience was assumed since only 1 isolate was observed, though as many as 7 periods were collected for most children. Assuming these strains are not clinically relevant suggest that at least 33% of strains (potentially more if considering the borderline group) may be confounding the search for strains related to disease. Typical analysis in genotyping studies includes all isolates, however the current findings seem to indicate that an adjusted analysis should also be performed. It further demonstrates that *S. mutans* strains are highly transmissible but may not become established strains due to bacterial competition or host immune factors.

In contrast to the transient isolates observed above, the 47 cases where 10 isolates were found for children with no matching household members seems to be strong evidence consistent with child-to-child transmission. These genotypes were considered established genotypes since more than 10 isolates indicates these genotypes were generally observed for at least 2 periods since only 10 isolates were evaluated per period. The strength of this data is further supported by the evaluation of multiple household members, in particular for those

children with 10 isolates where 37 of the 47 children had more than 1 household member tested. The 4 cases where children had genotypes that did not match household members or other children are considered independent cases that would suggest another reason (i.e., source of genotype was beyond the household members or classmates evaluated in this study).

For children that shared a genotype with their household, it was found that these children most commonly shared genotypes with their mothers (54%; 85/158) and their siblings (46%; 72/158) (Table S3, Fig. 2). The next highest group was 23% (37/158) who also shared a genotype with cousins. Interestingly, of the 73 cases that did not share the genotype with the mother, 37 (51 %, 37/73 or 23%, 37/158) shared the genotype only with sibling(s) and/or cousin(s). This data is consistent with the 58% strain homology for children and siblings reported by Thedjosasongko and Kozai (2002). It also suggests that children may be acquiring genotypes from other children within the household who likely acquire the genotype through their classmates or other outside interactions since no adults within the household demonstrated the genotypes. However, further analysis is needed to determine which direction the genotype is shared within households since the addition of a time of acquisition component would be required.

The high number of cases (58%; 87/158) in which a child shared a genotype with more than one household family member is consistent with intra-familial transmission by rep-PCR. Since the commonality of genotypes is frequent in this study, child-to-child transmission for these children cannot be ruled out entirely; however for the purpose of this paper, it is logical to hypothesize that when a child shares the genotype with more than one family member it is more probable that the child acquired the genotype within the home.

4.3. Limitations and Future Directions

While every effort was made to recruit all household family members residing in the home, in rare instances, some household members choose to not to participate. In particular; fathers, grandparents, aunts and uncles were available less often typically because they were absent from the household or less frequently, because they were unavailable for sampling. *S. mutans* were collected by standard plate count method, which did not always result in recovery of *S. mutans* isolates from every household member. Analysis of standard plate counts are beyond the scope of the current study but partial data has been published elsewhere and a more comprehensive report is planned (Childers et al., 2011). No weight was given to the total number of isolates per individual, which is an acknowledged bias and will be considered in future studies. However the purpose of the current analysis was to provide a broad overview of genotype diversity and possible transmission so as to determine if a more detailed longitudinal analysis of children and household members is warranted. Fewer isolates were typically collected for household members (average isolates per child 47, median 31; average per family member 13, median 13). Also, rep-PCR is based on banding similarity, which allows a degree of variability within rep-PCR genotypes. However, closer examination of genotypes shows sub-groupings within the genotypes that may be further distinguished using an alternate typing method such as MLST. Thus, the 157 cases of possible child-to-child transmission observed by rep-PCR analysis in this study may

be overestimated in some cases. Results will be better refined through future additional analysis with MLST.

4.4. MLST Library Update

Previously, we used MLST to validate the uniqueness of rep-PCR genotypes reported for 22 representative Library strains (Momeni, Whiddon, Moser, Cheon, Ruby and Childers, 2013). In that study, all 22 stains were found to have unique MLST ST, which was anticipated since MLST is typically a more discriminative method. Here another 13 different representative Library rep-PCR genotypes have been identified (Table 4). As in the first report, 9 additional Library strains were confirmed as unique by MLST. However, in contrast to the previous findings, 4 of the new Library strains (NL01b, NL03, NL26 and NL27) in this study matched previously reported ST, 3 of which were previous library strains. This finding indicates that these strains have identical housekeeping genes but differ within the repetitive extragenic elements. This finding offers more support of the recent report that rep-PCR can be more discriminative than MLST in some cases when evaluating *S. mutans* (Momeni, Whiddon, Cheon, Moser and Childers, 2015). It should also be noted that one of the previous library genotypes (L16) was reassigned to L23 due to similarities in rep-PCR profiles. Thus the current total of Library strains evaluated with MLST agrees with the number of rep-PCR genotypes at 34.

5. CONCLUSIONS

In summary, this study presents a DiversiLab rep-PCR reference library for *S. mutans* for epidemiological surveillance. This study highlights the effective use of the Diversilab rep-PCR approach as a cost-effective screening tool for large-scale epidemiological study of bacterial isolates. This is the first known study to evaluate transmission by all three possible routes of transmission in the same study (unrelated child-to-child, mother-to-child, and interfamilial including extended family) within the same study. Although this study's findings support mother-to-child transmission, it also provides compelling evidence that child-to-child transmission is observed in 37% of children and for 50% of genotypes of *S. mutans* in this population. The high degree of child-to-child transmission reported warrants further study with an alternate method such as MLST to validate possible routes of transmission. The significance of age group and caries status related to genotype diversity reported here indicates that a more thorough, longitudinal analysis is justified. These findings advocate the importance of considering child-to-child, as well as intra-familial, acquisition of *S. mutans* in risk assessments and prevention strategies for dental caries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Lapirattanakul directed the confirmation and registration of isolates with PubMLST; Dr. Ping Zhang and Kazuhiko Nakano generously donated some controls strains.

Abbreviations

AP-PCR	arbitrarily primed PCR
PFGE	pulse field gel electrophoresis
rep-PCR	repetitive extragenic palindromic PCR
MLST	multilocus sequence typing
ST	sequence type

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Highlights

- A DiversiLab rep-PCR reference library for *Streptococcus mutans* is described and made available upon request.
- Children having multiple *S. mutans* genotypes were 2.3 times more likely to have dental caries.
- Analysis for transmission performed from two perspectives (by child and by genotype) indicating 63% of children shared at least 1 genotype with their mother, but 72% of children had at least 1 genotype not shared with any household family members.
- This is the first study to investigate transmission of *S. mutans* by evaluating multiple transmission routes including transmission among unrelated interacting children (school-mates) as well as with household family members (including extended family).

Fig. 1. Summary rep-PCR dendrogram of 34 representative *S. mutans* genotypes. Total number of isolates and individuals with similar rep-PCR genotypes are indicated. MLST ST is for representative isolate only (other isolates with the same rep-PCR genotype may have different ST). Best match rep-PCR genotypes for prototype strains are indicated. Prefix “G”; rep-PCR genotype.

Fig. 2.

Distribution of minor, undetermined, and established genotypes among children who did not share the genotype with any household family members.

Fig. 3. Number of genotype cases for intra-familial transmission (N=158) by family members where a child shared a genotype with household members. Totals will not equal 158 since children may share genotypes with multiple family members.

Fig. 4.
Comparison of child-to-child and intra-familial transmission among children by evaluated by rep-PCR genotype. Prefix “G”; rep-PCR genotype.

Table 1

Distribution of children with single or multiple *S. mutans* rep-PCR genotypes by sex, age group, and caries experience.

	No. of isolates tested	No. of genotypes detected	1 genotype	>1 genotype
Sex				
Male (N = 91)	4024	24	29 (31.9%)	62 (68.1%)
Females (N = 77)	4014	26	29 (37.7%)	48 (62.3%)
Age Group				
Infants (Cohort 2, N = 87)	3108	24	24 (27.6%)	63 (72.4%)
Children (Cohort 1, N = 81)	4930	27	34 (42.0%)	47 (58.0%)
Caries Experience				
Caries Free (N = 41)	684	17	25 (60.1%)	16 (39.0%)
Caries (N = 127)	7354	28	33 (26.0%)	94 (74.0%)

TABLE 2

Results of bivariate and multivariate logistic regression modeling the probability of children (N = 168) having multiple genotypes.

BIVARIATE ANALYSIS				
Variable: Reference	Level (% with multiple genotype)	Odds Ratio	95%CI	Overall p-value
Age: Cohort 2	Cohort 1 (58.02%)	1.899	0.996–3.618	0.053
	Cohort 2 (72.41%)			
Sex: Male	Female (62.34)	1.292	0.683–2.444	0.431
	Male (68.13)			
HH genotype (N =125): Match	No (79.38%)	0.548	0.216–1.394	0.207
	Yes (67.86%)			
	Group1 (48.78%)			<0.001
Isolates group 2 vs. 1	Group2 (35.71%)	0.583	0.242–1.406	
Isolates group 3 vs. 1	Group3 (90.00%)	9.45	2.844–31.402	
Isolates group 4 vs. 1	Group4 (86.67%)	6.825	2.376–19.608	
Caries: Yes	No (39.02%)	4.451	2.119–9.350	<0.001
	Yes (74.02%)			
MULTIVARIABLE ANALYSIS				
Variable Reference	Odds Ratio	95%CI	Overall p-value	
Cohort 2	2.905	1.271–6.638	0.0114	
Isolates group 2 vs. 1	0.53	0.201–1.397	<0.001	
Isolates group 3 vs. 1	6.317	1.730-23.070		
Isolates group 4 vs. 1	7.397	2.129-25.695		
Caries (yes)	2.315	0.938–5.712	0.0685	

Isolate groups defined as: group 1 (0–9 isolates), group 2 (10–29 isolates), group 3 (30–82 isolates), group 4 (more than 83 isolates)

Table 3

Summary of rep-PCR genotypes observed in index children shared and not shared with household family members.

Genotype	Total Children with Genotype*	Only Child has genotype (%)	Child and Household Share Genotype (%)
G01	22	9 (41)	13 (59)
G01a	25	11 (44)	14 (56)
G01b	15	13 (87)	2 (13)
G01d	2	1 (50)	1 (50)
G02	2	1 (50)	1 (50)
G05	9	7 (78)	2 (22)
G06	19	8 (42)	11 (58)
G07	19	10 (53)	9 (47)
G08	4	2 (50)	2 (50)
G09	31	9 (29)	22 (71)
G11	6	1 (17)	5 (83)
G12	21	10 (48)	11 (52)
G13	21	10 (48)	11 (52)
G14	12	9 (75)	3 (25)
G15	10	7 (70)	3 (30)
G18	39	16 (41)	23 (59)
G19	1	-	1 (100)
G22	18	12 (67)	6 (33)
G23	20	10 (50)	10 (50)
G26	7	6 (86)	1 (14)
G49	2	2 (100)	-
G50	10	3 (30)	7 (70)
Total Cases		157	158

* Total represent children evaluated in this study (N = 117) and excludes children for whom no household members were evaluated or no *S. mutans* were observed. Children can have more than one genotype represented. Prefix “G”; rep-PCR genotype

Table 4

New rep-PCR Library Strains with MLST Sequence types.

Isolate ID	MLST ST	<i>tkt</i>	<i>glnA</i>	<i>gltA</i>	<i>glk</i>	<i>aroE</i>	<i>murI</i>	<i>lepC</i>	<i>gyrA</i>
NL01	175	1	2	1	8	4	3	1	1
NL01b	1	1	1	1	1	1	1	1	1
NL01d	184	1	1	1	2	2	3	1	1
NL03	166	3	24	5	25	1	1	30	15
NL25	185	1	2	1	1	19	3	24	1
NL26	97	3	2	1	1	21	11	24	1
NL27	161	1	1	5	24	4	3	31	1
NL30	186	1	2	34	4	2	24	1	21
NL38	187	22	2	31	4	4	3	11	1
NL42	179	1	2	15	3	1	11	11	1
NL45	188	3	2	1	23	30	3	34	1
NL49	189	2	3	20	4	5	5	1	22
NL50	190	23	8	15	8	2	5	21	1

ST= MLST sequence types. NL = New Library strains. New Sequence types and alleles identified in this study are in bold.