Phenotypes of Streptococcus mutans isolates in sound and cavitated tooth surfaces and its relationship to early childhood caries in China

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Abstract

Background To compare the phenotypes of *Streptococcus mutans* (*S. mutans*) strains isolated from sites with different caries susceptibility of a single subject. **Methods** This study was carried out in children with early-childhood caries (ECC) and caries-free (CF). The ECC subjects presented two sites: a cavitated lesion (ECC-C) and a sound surface (ECC-F). The CF subjects presented one sound surface. The following phenotypes were evaluated among these three sites: growth pattern, biofilm, exopolysaccharide (EPS), pH drop, and the expression levels of genes (*gtfB*, *gtfC*, *spaP*, *srtA*, *relA*, *ffh*, *srn225147*, and *srn821798*). **Results** All of these phenotypes were detected similarly between ECC-C and ECC-F groups. However, the capacity of biofilm formation, EPS, pH drop and the expression levels of genes (*gtfB* and *spaP*) of *S. mutans* in the CF group were lower compared to those of the ECC group. There was a relatively higher expression of *srn821798* compared to that of *srn225147* in clinical isolates, and isolates with low expression of *srn821798* had lower expression levels of predicted targets (*gtfB*, *gtfC*, and *spaP*). **Conclusions** These data indicate *S. mutans* isolated from sites with different caries susceptibility of a single subject showed similar characteristics, while the cariogenic potential of the *S. mutans* isolated from ECC individuals was relatively higher than that of CF subjects.

Background

Early childhood caries (ECC) is defined as the primary teeth of a child 71 months of age or younger with at least one decayed, missing (due to caries), or filled tooth surface [1]. ECC is one of the most common chronic childhood diseases that aggravates financial burden on the country and exerts negative effects on the family [2, 3]. In the past ten years, the prevalence of ECC has been on the rise, according to the 3rd and 4th National Oral Health Survey. For example, the prevalence of ECC for five-year-old children has increased from 66.0% to 71.9% and the mean dmft (i.e., decayed, missing, filled teeth) has increased from 2.71 to 4.23 [4]. Therefore, preventing and treating ECC in China is important and urgent.

ECC is a chronic infectious disease and *Streptococcus mutans* (*S. mutans*) is the major cariogenic bacteria [5]. *S. mutans* can adhere to tooth surfaces and metabolize carbohydrates, especially sucrose, to produce acid that causes the demineralization of the tooth surface and the onset of caries [6]. Virulence-related genes of *gtfB*, *gtfC*, *spaP*, *srtA*, *ffh* and *relA* are involved in the regulation the virulence of *S. mutans*. For example, water-insoluble glucan is an important component of exopolysaccharide (EPS) to enhance the formation of biofilm (EPS). GtfB, encoded by the *gtfB* gene, synthesizes only water-insoluble glucan. GtfC, encoded by the *gtfC* gene, produces both water-insoluble and soluble glucan [7]. In addition, *srtA* and *spaP*, associated with sucrose-independent adherence, are involved in the utilization of sucrose and enhance cariogenic properties of the biofilm [8]. The mRNAs of *ffh* and *relA* have been reported involved in acid tolerance [9, 10]. Small RNAs (sRNAs) that act by a base-pairing mechanism regulate the expression of target mRNA function in post-transcriptional level [11].

Studies have shown that the cariogenic potential of *S. mutans* isolated from individuals with ECC was higher than that in caries-free children. For example, the synthesis of water-insoluble glucan and the

capacity of biofilm formation of *S. mutans* isolated from caries-active subjects were greater than those in caries-free children [12, 13]. However, the virulence phenotypes of *S. mutans* isolated from different microenvironments (i.e., sound enamel and cavitated surfaces) of the same diseased subject remain unknown. Serotype c (70–80%) is recognized as the principal strain with the highest detection rate in the oral cavity. Thus, in this study, phenotypes and expression of virulence-related genes in *S. mutans* isolated from tooth surfaces with different caries susceptibility with c serotype between ECC and caries-free children were assessed.

Methods

Collection of dental plaque and recording of severity of ECC

The mean scores of the dmft index (i.e., the decayed, missing, filled-teeth index) and dmfs index (i.e., the decayed, missing, filled-tooth-surfaces index) were used to evaluate the severity of ECC [14]. Dental-plaque samples were collected with sterile cotton sticks from the tooth surfaces of ECC and non-ECC children aged three to five years old. The ECC subjects presented two donor sites: caries-active sites (ECC-C) and sound sites (ECC-F), whereas the caries-free subjects presented only sound sites (CF). The cotton sticks were placed in a sterile centrifuge tube with 0.9% saline and were taken to the laboratory on ice for 4 h.

Isolation of clinical *S. mutans* isolates

The centrifuge tube with the plaque sample was mixed for 30 sec and 50 ul of saline was plated on Trypticase Yeast-Extract Cysteine Sucrose Bacitracin (TYCSB) agar. Three colonies were selected randomly from each dental-plaque sample according to the morphology of *S. mutans* UA159 (ATCC 700610) after culturing for 48 hours at 37°C under 5% CO₂. Clinical *S. mutans* were confirmed according to their reaction to mannitol, sorbitol, raffinose, melibiose, aesculin, and hydrolyse arginine [15].

Identification of c serotypes

All of the isolates were cultured overnight and DNA was extracted from clinical *S. mutans* isolates by Bacterial Genome DNA Extracton Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The confirmation of the c serotype was carried out by polymerase chain reaction (PCR) using serotype-specific primers [16]. The reaction condition of PCR for c serotype included the following: denaturation at 96°C for 2 min; 25 cycles consisting of 15 s of denaturation at 96°C; 30 s of annealing at 61°C; and 1 min of extension at 72°C. The amplification products were then observed through electrophoresis in agarose gels. *S. mutans* UA159 was used as a positive reference.

Growth monitoring and pH changes at the early stationary phase

All of the *S. mutans* clinical isolates (n = 28) with the c serotype from caries-free children were selected to form the caries-free group (CF). Selected ECC individuals contained at least one *S. mutans* isolated from

both caries-active and caries-free tooth surfaces at the same time in order to reduce error. *S. mutans* clinical isolates with the c serotype from caries-active and caries-free tooth surfaces from 10 children with the top-10 dmfs scores were selected to form the caries-active group (ECC-C; n = 23) and caries-free group (ECC-F; n = 23) of children with ECC. Each of the clinical *S. mutans* isolates was incubated in brain heart infusion (BHI) broth and growth was monitored at the optical density (OD) of 600 nm every 6 h. The pH value at 12 h, which represents the early stationary phase, was tested using a pH meter.

Biofilm formation and final pH detection

The biofilm assay was performed according to Zhou et al., (2018) with a minor adjustment [41]. Two-hundred μL of BHI broth of each isolate with 1×10^7 CFU/mL *S. mutans* and 1% sucrose were added in 96-well plates. After culturing at 37°C under 5% CO₂ for 24 h, the supernatant was removed for pH assessment and 100 μL of 0.05% crystal violet (CV) was added to the well for staining for 15 min. Then, the stained biofilm was washed twice with 0.9% saline and was de-stained with 100% ethanol. Lastly, 100 μL of each sample was transferred to a new 96-well plate and was quantified at 570 nm.

Production of EPS

The production of EPS was evaluated by the congo red (CR)-binding assay [17, 18]. Fifty μL of CR along with 100 μL of fresh BHI broth was added to each well that was cultured with 24-h biofilm, as well as to a blank well (blank CR). The incubation medium was then transferred to 200- μL microcentrifuge tubes and centrifuged at 10,000 g for 5 min after incubating for 2 h. The supernatants were transferred to empty wells in a microtiter plate and were detected at 490 nm as follows: OD bound CR (EPS production) = OD blank-OD supernatant.

Quantitative real-time PCR

In each group, 10 isolates were selected randomly for mRNA detection. *S. mutans* biofilms were grown on 6-well plates at 37°C under 5% CO₂ for 24 h. The supernatant was removed and the biofilm was rinsed twice with sterilized saline to dislodge nonadherent bacteria. The total RNA was extracted and purified using the miRNeasy Mini Kit (Qiagen, German), and was then subjected to reverse-transcription PCR. For mRNAs (*gtfB*, *gtfC*, *spaP*, *srtA*, *ffh*, and *relA*), the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan) was used for reverse-transcription PCR and real-time quantitative-PCR (RT-qPCR) was performed with TB Green™ Premix Ex Taq™ II (Takara, Japan). Specifically, the mRNAs of *gtfB*, *spaP*, *srtA*, *ffh* and *relA* were potential targets for *sm225147*, and the mRNAs of *gtfB*, *gtfC*, *spaP*, *srtA*, and *ffh* were predicted targets for *sm821798* according to target prediction by IntaRNA (<http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp>) and RNAPredator (<http://nibiru.tbi.univie.ac.at/RNAPredator/introduction.html>) [19-21]. For sRNAs (*sm225147* and *sm821798*), Mir-X™ miRNA First Strand Synthesis Kit (Takara, Japan) was used for reverse-transcription PCR, and RT-qPCR was performed with Mir-X™ miRNA qRT-PCR TB Green™ Kit (Takara, Japan). The PCR reaction conditions for mRNAs included the following: denaturation at 95°C for 30 s; 40 cycles consisting of 5 s of denaturation at 95°C; and 30 s of annealing at 55°C. The PCR reaction conditions for *sm225147*

were as follows: denaturation at 95°C for 10 s; 40 cycles consisting of 5 s of denaturation at 95°C; and 20 s of annealing at 55°C. The PCR reaction conditions for *smn821798* were as follows: denaturation at 95°C for 10 s; 40 cycles consisting of 5 s of denaturation at 95°C; and 20 s of annealing at 50°C. The primers for *16S rRNA*, *gtfB*, *gtfC*, *spaP*, *srtA*, *ffh*, and *relA* were synthesized according to previous studies [8, 22-24].

The primer sequences are listed in Table 1.

Statistical analysis

IBM SPSS 20.0 software (IBM, Armonk, NY, USA) was used to analyze the data. Chi-square tests were used for qualitative data. One-way analyses of variance (ANOVAs) followed by Tukey's HSD tests or Kruskal–Wallis tests were used for quantitative data. Spearman-rank correlation coefficients were used to evaluate correlations. A *p* value less than 0.05 was considered statistically significant.

Results

The characteristics of isolates under planktonic status

There were 137 *S. mutans* clinical strains with c serotype isolated from 31 children with both ECC-C and ECC-F sites, and a total of 28 *S. mutans* clinical strains were isolated from 16 CF children. For this part of the study, all of the *S. mutans* clinical isolates (*n* = 28) with the c serotype from 16 caries-free children were selected to form the CF group. *S. mutans* clinical isolates with the c serotype from 10 children with the top-10 dmfs scores were selected to form the ECC-C group (*n* = 23) and ECC-F group (*n* = 23). Virulence characteristics including growth patterning, biofilm formation, pH changes, and EPS formation were evaluated among the three groups. A similar growth pattern of *S. mutans* isolated from the three groups (ECC-C, ECC-F, and CF) was found under planktonic status (Fig. 1a). All of the isolates reached the early stationary phase at 12 h. There was no statistical difference in the pH value at 12 h among these three groups (*p* = 0.066; Fig. 1b).

The characteristics of isolates under biofilm status

No significant difference in the capacity of biofilm formation was observed between the ECC-C and ECC-F groups (*p* = 0.947), while the capacity of biofilm formation was higher in both the ECC-C and ECC-F groups compared to that in the CF group (*p* = 0.018 for ECC-C versus CF; *p* = 0.043 for ECC-F versus CF; Fig. 2a). There was no difference in the pH value between the ECC-C and ECC-F groups (*p* = 0.928). However, the pH value was lower in both the ECC-C and ECC-F groups compared to that in the CF group (*p* = 0.017 for ECC-C versus CF; *p* = 0.046 for ECC-F versus CF; Fig. 2b). The production of EPS in both ECC-C and ECC-F groups were higher than that in the CF group (*p* < 0.001 for ECC-C versus CF; *p* = 0.006 for ECC-F versus CF), and that of the ECC-C group was higher than it in the ECC-F group (*p* = 0.048; Fig. 2c).

The relative expression of mRNAs and sRNAs under biofilm status

The expression level of *gtfB* of one isolate in the ECC-C group was defined as an expression level equal to 1.0. The relative expression levels of four genes (*gtfB*, *gtfC*, *spaP*, and *srtA*) were associated with the production of EPS and the levels of two genes (*ffh* and *relA*) were related to acid production. The expression levels of these six genes were evaluated in this study. The relative expression levels of *gtfB* and *spaP* in the ECC-C group were higher than those in the CF group ($p = 0.040$ for *gtfB*; $p = 0.011$ for *spaP*), and both genes were positively correlated to the production of EPS ($r = 0.700$ and $p < 0.001$ for *spaP*; $r = 0.638$ and $p < 0.001$ for *gtfB*; Fig 3). No significant difference was found in the expression levels of *gtfC*, *srtA*, *ffh* and *relA* among the three groups ($p = 0.077$ for *gtfC*, $p = 0.155$ for *srtA*, $p = 0.077$ for *ffh*, and $p = 0.971$ for *relA*). Next, the mRNAs of *gtfB*, *spaP*, *srtA*, *ffh*, and *relA* were potential targets for *sm225147*, and the mRNAs of *gtfB*, *gtfC*, *spaP*, *srtA*, and *ffh* were predicted targets for *sm821798*, according to target prediction by IntaRNA and RNAPredator. The relative expression level of *sm225147* in the ECC-C group was higher than that in the CF group ($p = 0.009$ for ECC-C versus CF; $p = 0.018$ for ECC-F versus CF; Fig. 4a). No significant difference was found in the expression of *sm821798* among the three groups (Fig. 4c). However, the expression level of *sm821798* was higher than *sm225147* in the 30 isolates ($p = 0.002$). In addition, the expression levels of *gtfB*, *gtfC*, and *spaP* in isolates with low expression of *sm821798* ($n = \text{end } 10$) were lower than those in isolates with high expression of *sm821798* ($n = \text{top } 10$; $p = 0.039$ for *gtfB*; $p = 0.016$ for *gtfC*; $p = 0.022$ for *spaP*; Fig. 4d), while, no significant difference was found for *sm225147* (Fig. 4b).

Discussion

Studies has shown that the microbial flora in tooth surfaces with different caries susceptibility or saliva from caries-active and caries-free children or adults are different from one another [12, 25-27]. There are more than 700 bacterial species colonized in the oral cavity. Among them, *S. mutans* is identified as the most important pathogenic bacteria for dental caries [28, 29]. Recent studies suggest that the virulence of *S. mutans* isolated from caries-active subjects is high in comparison to that in caries-free subjects [12, 13]. However, the characteristics of *S. mutans* isolated between the caries-active and sound tooth surfaces from a single individual, as well as from the sound tooth surfaces between ECC and non-ECC individuals, have remained unknown. Thus, exploration of these characteristics may play important roles for future clinical practice.

Only ECC children that had the *S. mutans* isolated from the caries-active and sound tooth surfaces were included in order to exclude other factors, such as different dietary habits or oral-hygiene behaviors that may affect the occurrence of dental caries [30, 31]. Moreover, the *S. mutans* were isolated from children living in the same district. The growth pattern of *S. mutans* isolates under the planktonic status were similar, and similar models were found on phenotypes of *S. mutans* under the biofilm status between the groups of ECC-C and ECC-F. These results remind us that the prevention of dental caries is important for every tooth because the virulence of *S. mutans* colonized in dental plaque of a single subject is similar, such that it could disperse and colonize at other tooth surfaces. Conversely, virulent phenotypes were high in the ECC-F or ECC-C groups compared to that in the CF group. This result is consistent with a previous report, in spite of different details of the study [32]. In that study, the bacterial activity (e.g.

lactobacilli) evaluated in the ECC-C group was higher than it in ECC-F group, and a saccharolytic fusobacteria was most active in the CF group and was less active in the ECC-C and ECC-F groups. These results suggest that *S. mutans* colonized in the tooth surface of individuals with caries might have the same cariogenic potential, while the cariogenic potential of the *S. mutans* colonized in the tooth surface of sound individual is relatively lower.

Genes of *gtfB*, *gtfC*, *spaP*, and *srtA* have been reported to be virulent factors involved in the regulation of EPS synthesis and biofilm formation in *S. mutans* [33, 34]. The relative expression levels of *gtfB* and *spaP* in the ECC-C group were higher than those in the CF group, and they were positively correlated to the production of EPS. These results are in accordance with previous studies that have shown that the virulence gene *gtfB* contributes to the adherence, synthesis of EPS and to biofilm formation under a sucrose environment, and that *spaP* is also involved in such adherence [35, 36]. Importantly, sRNAs coordinate adaptation processes in response to environmental changes in bacteria by regulating target mRNA expression [37-41]. Specifically, *srn225147* and *srn821798* were sequenced in our previous studies [22, 23]. Virulence-related genes selected in our present study were predicted targets for these two sRNAs according to IntaRNA and RNAPredator. Therefore, the relative expression of these two sRNAs were tested. The expression level of *srn821798* was higher than that of *srn225147* in the 30 isolates, and the expression levels of *gtfB*, *gtfC*, and *spaP* in isolates with low expression of *srn821798* were lower than those in isolates with high expression of *srn821798*. Thus, we speculate that *srn821798* may be important in maintaining homeostasis and adherence of *S. mutans*.

Although this study provided practical information, there are some limitations. Only the *S. mutans* isolated from children with high dmfs scores were selected to compose the ECC-C and ECC-F groups. The phenotypes of *S. mutans* between the groups of ECC-C and ECC-F weren't similar, but whether *S. mutans* isolated from different sites of a single subject are closely related isolates is still unknown. Multilocus sequence typing of these isolates will be needed in the future.

Conclusions

In conclusion, *S. mutans* isolated from sites with different caries susceptibility of a single subject showed similar phenotypes. However, the cariogenic potential of the *S. mutans* isolated from ECC individuals was relatively higher than that of CF subjects. These findings may improve our knowledge on *S. mutans* virulence and the consequent development of dental caries.

Abbreviations

S. mutans: *Streptococcus mutans*; ECC: early-childhood caries; CF: caries-free; ECC-C: a cavitated lesion from children with early-childhood caries; ECC-F: a sound surface from children with early-childhood caries; EPS: exopolysaccharide; dmft: decayed, missing, filled teeth; TYCSB: Trypticase Yeast-Extract Cysteine Sucrose Bacitracin; PCR: polymerase chain reaction; BHI: brain heart infusion; CV: crystal violet;

CR: congo red; RT-qPCR: real-time quantitative-PCR; sRNAs: Small RNAs; ANOVAs: One-way analyses of variance; dmfs: the decayed, missing, filled-tooth-surfaces.

Declarations

This study was approved by the Ethics Committee of [2017] KY011 of the First Affiliated Hospital of Bengbu Medical College. Informed consent for participation under 16 years old in the study was obtained from their parent or guardian.

Consent to publish

Not applicable.

Availability of data and materials

The data and materials of the present study were available from the corresponding author.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

KZ and SL conceived and designed the experiments; SL, HL, and ZG conducted the experiments; YS, QZ, and FH analyzed the data; SSL wrote the manuscript; KZ and JG revised the manuscript. All authors read and approved the manuscript.

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Tables

Table 1 Primer sequences

ID	Primer sequences
c serotype (F)	CGG AGT GCT TTT TAC AAG TGC TGG
c serotype (R)	AAC CAC GGC CAG CAA ACC CTT TAT
<i>gtfB</i> (F)	GATCAAGATGTTTCGCGTTGC
<i>gtfB</i> (R)	ACACATACTGCGGTGCCATT
<i>gtfC</i> (F)	GATCAAGAAGCGGCTGGTTT
<i>gtfC</i> (R)	ACATGACGCGTGAATCAAGG
<i>spaP</i> (F)	TCCGCTTATACAGGTCAAGTTG
<i>spaP</i> (R)	GAGAAGCTACTGATAGAAGGGC
<i>srtA</i> (F)	GAAGCTTCCTGTAATTGGCG
<i>srtA</i> (R)	TTCATCGTTCCAGCACCATA
<i>ffh</i> (F)	AAGGTAAGCAAGTCTCCCATTC
<i>ffh</i> (R)	TCCGTCAAATCACTGGAAAAC
<i>relA</i> (F)	ACAAAAAGGGTATCGTCCGTACAT
<i>relA</i> (R)	AATCACGCTTGGTATTGCTAATTG
<i>smn225147</i>	ATTGACTTTGAGTCTGTGC
<i>smn821798</i>	GATTAGTTATCAGATTTT
<i>16S rRNA</i> (F)	CTTACCAGGTCTTGACATCCCG
<i>16S rRNA</i> (R)	ACCCAACATCTCACGACACGAG

Figures

Figure 1

Growth curve and pH value of *S. mutans* under planktonic status

Figure 2

The virulent characteristics of isolates under biofilm status. (a) The biomass of the 24 h biofilm; (b) The final pH value of the 24 h biofilm; (c) The production of EPS of the 24 h biofilm. * represents p value < 0.05 , ** represents p value < 0.01 , and *** represents p value < 0.001 .

Figure 3

The relative expression levels of virulent genes and correlation between virulent genes and phenotypes. (a-f) The relative expression levels of reported virulent genes of *gtfB*, *gtfC*, *spaP*, *srtA*, *relA*, and *ffh*. The expression level of *gtfB* of one isolate in the ECC-C group was defined as 1.0. * represents p value < 0.05 ; (g-h) The correlation between virulent genes (*gtfB* and *spaP*) and EPS.

Figure 4

The relative expression levels of sRNAs and their associations with predicted targets. The mRNAs of *gtfB*, *spaP*, *srtA*, *ffh*, and *relA* were potential targets for *srn225147*, and the mRNAs of *gtfB*, *gtfC*, *spaP*, *srtA*, and *ffh* were predicted targets for *srn821798*, according to target prediction by IntaRNA and RNAPredator. The expression level of *gtfB* of one isolate in the ECC-C group was defined as 1.0. * represents p value < 0.05 and ** represents p value < 0.01 .