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# Characterizing Severe Early Childhood Caries Microbiota through Culturomics

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## Abstract

Severe early childhood caries (SECC) is a common widespread caries affecting multiple deciduous teeth in children, which seriously affects children's physical and mental health and growth. Investigation of their pathogenic flora is essential. Two children with SECC and two caries-free children of the same age were included in this study. Using cultureomics techniques, that is, microorganisms in oral dental plaque were isolated and cultured by setting different conditions (temperature, oxygen conditions, culture medium). Species were identified by 16S rRNA sequencing, and strain function was analyzed in depth in combination with clinical information. A total of 37 species were identified in this study. 4 species had never been associated with the oral cavity before, and 3 of them had never been isolated from clinical specimens. This pilot study was able to provide some new information on the complexity of the microbiota in SECC patients versus healthy children of the same age.

## Keywords

SECC, cultureomics, oral microbiota

## 1. Introduction

Dental caries is among the most common chronic childhood diseases, affecting over 560 million children worldwide [1]. Dental caries in primary teeth is called ECC (early childhood caries) and its severe form SECC (severe early childhood caries) is defined using a combination of the child 's age and the number of teeth that are cavitated, missing (due to caries), or filled. For example, in a 4-year-old child, SECC can be diagnosed if 5 or more teeth in primary teeth are affected by caries [2]. The composition of the oral microbiome is unique to everyone and reflects various endogenous and exogenous influences (behavioral, immunological, genetic and environmental factors). The microbiome analysis in combination with related factor assessment can improve the accuracy of existing methods for SECC risk prediction and provide greater predictability for the occurrence of SECC.

Oral microorganisms are an important part of changing the balance between oral and even systemic health and diseases [3]. Based on modern gene high-throughput sequencing technology, more than 700 different microbial species such as bacteria, viruses, fungi, mycoplasmas and chlamydiae have been found on the surface of soft and hard tissues such as teeth, sulcus, buccal tongue, and soft and hard palate, which are collectively referred to as the oral microbiome [4]. These non-culture methods have found a large number of uncultured strain information, and uncultured strains account for about 70%. Many species occupying a dominant position in the sample have no cultured reference strains; Some tag sequences can only be classified into very rough taxonomic hierarchies (e.g., bacterial kingdom or a phylum), suggesting the absence of the entire large taxon (e.g., a large number of candidate phylum) in the cultured species library. The lack of this information is hindering the development of the metagenomic field; For these taxons that are largely absent in culture. it is urgent to isolate and culture representative

strains and study their functions to infer the function of the flora in the corresponding environment. In addition to validation by non-culture techniques, functional validation by isolation and culture of the corresponding strains is often required and further used in intervention studies [5]. Cultureomics is a method that uses a variety of culture conditions [6]. Based on the diversification of culture conditions, it mimics the natural environment in which bacteria are located as much as possible to obtain cultures. This method is able to detect bacteria, regardless of their relative abundance, and can only identify viable microorganisms. Cultuomics can be used to identify new species. Hamad et al. used cultureomics to isolate a total of 17,800 fungal colonies from 14 fecal samples and identified 41 fungal species, 10 of which have not previously been reported in the human gut [7]. Currently, no studies have been reported on culturable microbiomes for children with SECC.

In this study, dental plaque was collected from two caries-free children and two SECC children. Combined with clinical information and 16S rRNA gene sequence identification and comparative analysis, the characteristics of culturable microbial communities in children with SECC and healthy children of the same age were analyzed.

## 2. Materials and Methods

### 2.1 Oral examination

This study was approved by the Medical Ethics Committee of Wuhan University Stomatological Hospital, and informed consent was obtained from each child and their guardians. A thorough oral examination of the tested children was performed by one examiner and the results were entered by one recorder. The oral examination of the subjects mainly includes oral soft tissue examination, hard tissue examination and dental examination. Soft tissue examination includes whether there are abnormalities in soft tissue color, shape, texture, etc. Hard tissue examination includes whether the structure and shape are abnormal. Dental examination included 4 tooth surfaces (labial, lingual, medial, and distal) for each anterior tooth and 5 tooth surfaces (buccal, lingual, medial, distal, and occlusal) for each posterior tooth. The dental tissues were mainly examined for carious defects, and the corresponding tooth positions and tooth surfaces were recorded to calculate dmft (decayed-missing-filled tooth) and dmfs (decayed-missing-filled surface). Plaque on each tooth surface was also recorded and plaque index was calculated.

### 2.2 Determination of Salivary pH and Salivary Buffering Capacity

Caries indicator test strips (Eyte, China) were used, a caries indicator test strip was removed during the examination, and the test strip was moistened with saliva. After the color of the test strip was stable (usually 10 s), it was compared with the standard colorimetric card on the packaging bottle, and the risk of caries replacement could be determined according to the area divided by the color card.

### 2.3 Plaque acidogenic capacity (Cariostat test)

Using a standard cotton swab, repeatedly wipe and sample the buccal side of the upper posterior teeth and the lingual side of the lower anterior teeth, place them in enrichment medium (Yuba, China) for repeated shaking, and transfer them to a 37 °C incubator for 48 hours before reading.

### 2.4 Sample Collection and Processing

Two healthy caries-free children and two SECC children were selected. Inclusion and exclusion criteria were as follows: 1. non-smokers; no systemic diseases; no history of long-term medication; no antibiotic treatment three months before sampling; 2. for healthy caries-free children, dmft was 0, and oral soft and hard tissues were normal; 3. for children with SECC, caries appeared on the smooth surface of the teeth in the mouth in children aged 3 years or younger, or dmft was greater than 4 over 3 years of age, or dmft was greater than 5 at 4 years of age, or dmft was greater than 6 at 5 years of age. Oral soft and hard tissues were normal except for caries. All children had no food or water for 2 hours before sampling.

The specific procedure for sampling was as follows. Plaques on the surfaces of six index teeth (55, 51, 63, 71, 75, and 83) were gently scrubbed with sterile cotton balls separating moisture with a sterile small brush. Samples were collected in a 1.5 mL EP tube containing 1 mL PBS and rotated for 1 minute. The EP tubes were placed in an ice box and transferred to the laboratory within 1 h for further isolation and culture.

### 2.5 Cultureomics methods

The samples were divided equally into two sterile vials which contained 2 mL Brucella broth or 2 mL Thioglycollate broth (THIO) after sufficient shaking on a vortex. These samples were then inoculated into blood culture

bottles and incubate at 37 C, 30 C and 42°C for 7 and 14 days, respectively. Following the incubation period, the samples were inoculated onto agar medium. Growth conditions used were as follows. BHI (Brain-Heart Infusion Agar, HKM, China), PYGV (PYGV medium: 20.00 ml of mineral element solution (NTA, 1 g; MgSO4 · 7H2O, 2.97 g; CaCl2, 0.252 g; Na2MoO4 2H2O, 1.267 mg; FeSO4 7H2O, 0.01 g), 0.25 g of peptone, 0.25 g of yeast extract, 15.00 g of agar, 965.00 ml of sterile water, mixed at 115 ° C for 20 min, and 10.00 ml (2.5%) of sterile glucose solution was added after cooling), CBA (Columbia Blood Agar, HKM, China) + 5% sheep blood for aerobic bacteria. BHI, PYGV, CBA + 5% sheep blood, CDC (anaerobic blood agar basal medium, Haibo, China) + 5% sheep blood for Anaerobes. The plates were incubated aerobically and anaerobically at 37°C, 30°C and 42°C for 5 days. Any colonies found on the medium were purified and re-cultured using the same medium and the same growth conditions as described above. Subsequently, all grown bacteria were analyzed by 16S gene sequence analysis, and the sequencing results were compared and analyzed by BLAST at NCBI. The specific process is shown in Fig.1

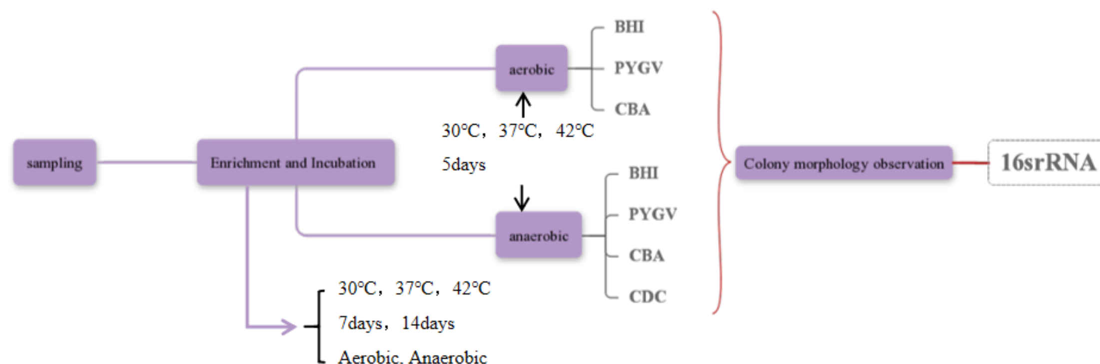


**Fig. 1. Culturomics flowchart.**

## 3. Results

The relevant information of the 4 enrolled children is shown below (Table 1, Table 2), and cultureomics analysis was performed on the dental plaque of 2 normal caries-free children and 2 SECC patients in this study. All microorganisms identified are reported in Table 3.

**Table 1. Clinical information**

| | Age | Salivary pH | Salivary buffering capacity | Dentition condition | Plaque status | Plaque acidogenic capacity (Cariostat assay) |
|---|---|---|---|---|---|---|
| Caries-free child 1 | 4 | 6.5-7.0 | ＜250ppm | dmfs = 0 dmft = 0 | 45.00% | Green (Machine value：1/160) |
| Caries-free child 2 | 4 | 6.5-7.0 | ＜250ppm | dmfs = 0 dmft = 0 | 35.00% | Green (Machine value：1/113) |
| SECC child 1 | 4 | ≤6.0 | ＜250ppm | dmfs = 14 dmft = 7 | 40.00% | Green (Machine value：1/216) |
| SECC child 2 | 4 | ≤6.0 | ＜250ppm | dmfs = 44 dmft = 18 | 35.00% | Green (Machine value：1/147) |

**Table 2. Oral tissue examination**

| | Oral cavity | tongue | gums | hard palate | soft palate | labial | vestibular | mucosa | Others |
|---|---|---|---|---|---|---|---|---|---|
| Caries-free child 1 | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Caries-free child 2 | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| SECC child 1 | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| SECC child 2 | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |

**Table 3. Summary of the species isolated from each patient**

| | Species Identified by Culturomics |
|---|---|
| Caries-free child 1 | *Lactobacillus pentosus, Firmicutes, Rothia aeria, Neisseria sicca, Neisseria suhflava, Neisseria elongate, Lactiplantibacillus plantarum, Actinomyces naeslundii, Neisseria oralis, Streptococcus mutans, Streptococcus sanguinis, Streptococcus oralis* |
| Caries-free child 2 | *Streptococcus cristatus, Neisseria sicca, Actinomyces viscosus, Bacillus circulans, Corynebacterium durum, Streptococcus timonensis, Neisseri suhflava, Actinomyces naeslundii, Actinomyces oral, Streptococcus infantis, Streptococcus oralis, Streptococcus mitis, Rothia aeria, Rothia dentocariosa, Neisseria mucosa* |
| SECC child 1 | *Streptococcus Gordonii, Streptococcus mitis, Streptococcus pneumoniae, Staphylococcus, Abiotrophia adiacens, Sporisorium reilianum, Candida tropicalis, Lactiplantibacillus plantarum, Actinomyces naeslundii, Neisseria oralis, Streptococcus mutans, Streptococcus sanguis, Streptococcus oralis* |
| SECC child 2 | *Streptococcus australis, Streptococcus uberis, Streptococcus mutans, Rothia mucilaginosa, Lactobacillus salivarius, Thermophilic protease-producing bacteria, Clostridium, Prevotella copri, Niallia circulans, Streptococcus uberis, Streptococcus sanguis, Streptococcus intermedius, Streptococcus infantis, Actinomyces naeslundii, Actinomyces oral, Streptococcus infantis, Streptococcus oralis, Streptococcus mitis, Rothia aeria, Rothia dentocariosa, Neisseria mucosa.* |

## 4. Discussion

In this study, caries plaque microorganisms were analyzed by culturomics method, focusing on caries-free preschool children and SECC patients of the same age. Analysis of the above oral samples showed the identification and culture of some bacterial species that had never been isolated from the oral cavity. These species include *Sporisorium reilianum, Niallia circulans, Thermophilic protease-producing bacteria, and Streptococcus timonensis.* Moreover, some of these species have never been associated with the human microbiota before, including *Sporisorium reilianum, Niallia circulans, and Thermophilic protease-producing bacteria*. Further, in our comparative analysis of healthy preschool children and children with SECC, it can be found that in addition to *S. oralis, S. mitis, S. sanguinis, S. mutans, A. oralis, A. naeslundii, L. plantarum, N. mucilaginosa* and other common oral normal flora were isolated. *Rothia aeria* was also isolated in healthy individuals. The bacterium was first isolated and identified in the Russian space station in 2004 [8] and in China in 2014. In recent years, the relationship between oral microbiota and smoking in the Chinese population, sex-specific differences in the salivary microbiome of children with active dental caries, and the analysis of bacterial characteristics of supragingival dental biofilms in deciduous and early mixed dentition children using HOMINGS sequencing technology have been mentioned [9-11]. Both *N. sicca and N. subflava* were isolated only in dental plaque of normal children. In addition, *Neisseria elongata* and *Neisseria oralis* were also isolated and cultured from the oral plaque of Caries-free child 2. Humans are the natural reservoir of *Neisseria* bacteria, and none of the *Neisseria* strains isolated above are usually pathogenic and inhabit the nasopharyngeal mucosa [12]. *Neisseria and Streptococcus spp* were common in healthy children. It is worth mentioning that *Streptococcus cristatus* isolated from oral plaque of Caries-free child 2 was regarded as a beneficial commensal. It has been documented that it attenuates Fusobacterium nucleatum induced cytokine expression by affecting Nf-κB and also inhibits P. gingivalis pathogenicity [13]. *Corynebacterium durum* is also a freshly reported oral probiotic [14].

We focused on oral bacterial composition in children with SECC. For SECC child 1, common opportunistic pathogens including *Streptococcus pneumonia* [15], *Staphylococcus* [16], *Candida tropicalis* [17] were isolated. These bacteria tend to multiply in the presence of decreased body resistance and cause acute symptoms. In addition, the more interesting species isolated from the oral plaque of this child were *Abiotrophia adiacens*, *Sporisorium reilianum.* The former is associated with a variety of inflammatory conditions including keratitis, osteoarthritis, osteomyelitis, bacteremia, and infective endocarditis [18-19]. It has been more reported to be strongly associated with the risk of lung cancer development [20]. A study showed that this bacterium can emerge from the transition from normal oral microbiota to acute endodontic infection [21]. The latter, *Sporisorium reilianum*, was first isolated and cultured from the oral cavity and has not previously been reported to be associated with human microorganisms. It mainly causes maize head smut, which overwinters in soil and invades maize roots when seeds germinate, inducing disease symptoms once it enters the meristem of apical floral organs. Because the affected site is the floral organ, the susceptible plants are granular and not received. It can spread through carrier soil, seeds, etc [22].

SECC child 2 had the most abundant bacterial composition, and cultureomics analysis revealed two bacteria that had never been isolated from humans before: *Thermophilic protease-producing bacteria* [23], *Niallia circulans* [24]. In addition, *Clostridium* [25] was also isolated and cultured in oral plaque for the first time. Pathogenic and opportunistic pathogens isolated from this patient mainly included *Rothia mucilaginosa, Rothia dentocariosa, Streptococcus intermedius, Clostridum, and Streptococcus uberis.* Selection of *Rothia mucilaginosa, Streptococcus species, and Veillonella parvula* as important distinguishing features in all models has been documented as biomarkers of risk for ECC [26]. *Streptococcus intermedius* is a member of the *Streptococcus anginosus* group of bacteria [27]. It has been reported to cause meningitis, endocarditis, and abscesses, even in immunocompetent hosts [28]. Several cases of S. intermedius liver abscess with active periodontal infection have been reported [29]. *Clostridum* have rarely been reported in the oral cavity. Most of the bacteria grow strictly anaerobically, most of which are saprophytic parasitic bacteria and a few are pathogens. It secretes exotoxins and enzymes that can cause disease in humans and animals [30]. Finally, for *Streptococcus uberis*, related reports mainly revolve around bovine mastitis, there are few reports in humans [31].

It is important to highlight some of the limitations that exist in this study. The number of study subjects may represent a major limitation of this study. It took a long time and was a heavy workload. In fact, even though culturable techniques allow for in-depth and accurate descriptive analysis of oral microbiota, small sample sizes are not conducive to exploring the possibility of any microbial association with disease, particularly for children with SECC, where there are large differences in the composition of cultured bacteria. We will continue to expand the sample size to reach more conclusions later. In addition, as demonstrated by the results of this study, cultureomics represents a suitable method for personalized diagnosis and treatment of oral diseases. Both metagenomics and cultureomics should be regarded as strategies to identify new potential pathogens. This pilot study was able to provide some new information on the complexity of the microbiota in SECC patients versus healthy children of the same age.

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