The Use of High - Sucrose Culture Media for the Identification of Oral Streptococci in Infant- Mother Pairs

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By the end of the 60's, the theory that refined carbohydrates promotes the absorption of saccharolytic Gram-positive microbial species on the tooth surfaces has become generally. Mutans streptococci (Streptococcus mutans and Streptococcus sobrinus) were key players in this theory. On agar plates, Str. mutans produces small, circular colonies, in the presence of glucose, and in the presence of sucrose large, sticky, gelatinous colonies. This gelatinous texture is due to the shell material: mutant 1 → 3 glucose polymers and dextran 1 '! 6 glucose polymers. Str. mutans are able to survive in the oral cavity with a pH lower than 5.5. That is why consecutive multiple sugar intake promotes the colonization of Str. mutans, which results in dental caries in stagnant zones. As oral pH is continuously shifted to acid, more acid-resistant bacteria appear. Our aim was to identify species in infant-mother pair gingival crevicular bacterial flora, which can be detected on high-sucrose culture media and to underline the jeopardy of vertical oral contamination from mother to infant.

Keywords: high-sucrose media, oral Streptococii, mother-infant pair

The oral cavity is the most complex, most accessible microbial ecosystem of the human body. Teeth, gingiva, tongue and oral mucosa provide different surfaces for colonization, but they do not provide uniform and identical conditions for this. Accordingly, there are several types of separate micro-environments in the oral cavity, whose ecology is highly complex and varied.

Considered individually, 20-50 bacterial species can be found in the healthy surface of a person's oral cavity. Bacterial varieties occur in a larger number of diseased surfaces, up to 200 or more. These microorganisms can not survive as a single species, but can only exist in communities [1]. On a thoroughly cleaned enamel surface, in a few minutes, a thin layer of aquired pellicle is formed from glycoproteins, minerals, immunoglobulins and proteins from gingival crevicular fluid [2]. The transformation of this pellicle into a dental plaque occurs through the colonization of microorganisms. The bacterium species involved can be divided into three groups according to the colonization sequence:

- primary colonizers - *Str. salivarius, Str. mitis, Str. sanguinis, Str. parasanguinis and Str. gordonii, Actinomyces, Veillonella, Gemella, Abiotrophia, Granulicatella*
- secondary colonizers - *Fusobacterium nucleatum, Prevotella intermedia, Lactobacillus, Capnocytophaga*
- hird colonizers - *Porphyromonas gingivalis, Campylobacter rectus, Eikenella corrodens, Actinobacillus actinomycetemcomitans, Oral spirochaetas*

Stabilization of the oral microbial flora of the infant is expected at 3-6 years of age, eruption of the teeth and the presence of tooth surfaces allow the formation of a characteristic oral microbial ecosystem. *Streptococcus mutans* have been shown to be present in children older than 26 months, it's colonization is related to the age of the child and the incidence of caries [1]. It has also been proven that the source in most cases is mother's oral microbiota due to most intimate relationship with the infant in the first two years of life. Another source of vertical transmission toothbrush can also be considered, if they are kept in the same toothbrush [3, 4].

The purpose of our study was to identify the bacteria that can be isolated from the oral cavity of a mother and her infant and to determine the role of bacteria that can be cultured on high sucrose content cultures.

Experimental part

The subjects involved in the study were mothers and their children of less than 1 year old with lower central incisors in eruption free of any caries laesions. The presence of the enamel surface was important to examine whether *S. mutans* had already colonized on the tooth surfaces at this early stage. A total of 5 maternal child pairs were examined, before clinical examination and sampling the informed consent of the subjects and legal representatives was obtained.

Before we sampled, the mother's oral condition was examined. For this purpose DMF index was determined (D = decayed, F = filled, M = missing). The medium values of the obtained DMFT index was 11±5.

Sampling was done from plaque from the labial surface of lower frontal teeth and in three gingival points for each tooth: mesial, distal and labial sulcus. On the day of sampling, subjects were asked not to perform any mechanical plaque removal procedure nor in their nor in infant's oral cavity. Sampling from labial surface was performed with sterile brushes, in gingival sulcii sterile paper points were inserted. These samples were transported in sterile test tubes in 1 ml of sterile saline. This solution served not only as a transport medium but also facilitated the dissolution of bacteria, thus creating a bacterial suspension.

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Two high-sucrose-containing media (TYC-Tryptone or TSB-Trypticase broth) of different composition were used, which can be used to selectively isolate *Str. mutans* [5]. Our expectation over the two cultures was to develop only the bacteria that ferment the sucrose or use it in any way during their metabolism. TSB was the medium that could differentiate *Str. mutans* and *Str. sobrinus*. *p*H values for both media was adjusted using the *p*H measuring instrument called inoLab Level 1, it was 7.3 ± 0.2 in the TYC medium and 6.2 ± 0.2 in the TSB medium. The sterilization of the culture media was then performed in autoclave as follows: +1 atm to 121 °C for 20-30 minutes or from +2 atm to 134 °C for 10 to 20 min. Petri cups were filled with the mediums and gel-like cultures were obtained at 45 °C.

The bacterial colonization was done by plating. Bacterial suspensions were prepared. The samples were extracted in 1 ml of sterile saline and then at a high speed mixing was performed on an axial mixer (Star Lab, Velp Scientifica, Wizard Advanced Vortex Mixer). From bacterial suspensions, 1 mL of sample was applied to each culture medium with a pipette and uniformly scattered on the surface of the culture medium with a glass stick. A total of 240 inseminations were performed, 120 were TYCs and 120 were from TSB media. Incubation of the samples was carried out under aerobic and anaerobic conditions at 37°C for 48 h (Anaerocult, Merck).

The more common and distinct strains of the colony morphology and the ones for which the streptococcal colony morphology has been described have been selected and transplanted into the liquid medium. They were then incubated for 48 h under aerobic and anaerobic conditions.

The broth mediums that showed bacterial growth were selected. The bacterial growth was indicated by the uniform, ring-like, or plaque-like dissolution of the soup. Transluctions are required to obtain pure cultures for molecular biological assays. After performing catalase or oxidase probes, smears were prepared from the microorganism suspended in the test, then stained with Gram-staining in four steps. DNA isolation was perfprmed using the AccuPrep Genomic DNA Extraction Kit. The bacterial strains were identified by the 16S rDNA sequence analysis. After DNA isolation, agarose gel electrophoresis method was used for controll, the agarose concentration was 1%, which is also suitable for most plasmids and PCR products. The effectiveness of the PCR reaction was assessed on the basis of the gel image, the DNA fragments appearing on the agarose gel electrophoretic image were analyzed by size. After purification of the DNA, the resulting chromatograms (sequences) were processed using Chromas Lite and Mega 4.0 Alignment Explorer, and compared the base sequence with data from the database of EzTaxin Internet genebank.

Results and discussions

After processing the sequencing results, the bacteria were grouped by maternal and child pairs and frequency.

The collected samples, especially the coccus, were more likely to detect streptococci, due to differentiating media and high sugar content [6]. Oral *streptococci* belong

TYC	TSB
Deionised water 1000 ml	Deionised water 1000 ml
Tryptone: 15 g/litre	Trypticase broth: 30 g/litre
Yeast extract: 5 g/litre	Yeast extract: 10 g/litre
L-Cystine: 0.08 g/litre	Agar: 11 g/litre
Sodium sulphite: 0.1 g/litre	20 % Sucrose: 200 g/litre
Sodium chloride: 0.4 g/litre	Bacitracin: 0.2 u/ml
Disodium phosphate anhydrous: 0.8 g/litre	
Sodium bicarbonate: 2 g/litre	
Sodium acetate anhydrous: 12 g/litre	
Sucrose: 50 g/litre	
Agar: 12 g/liter	
pH: 7.3 ± 0.2	pH: 6.2 ± 0.2

Table 1
COMPARATIVE COMPOSITION OF TYC (TRYPTICASE YEAST-EXTRACT CYSTINE AGAR MEDIUM) AND TSB (TRYPTIC SOY BROTH) CULTURE MEDIA

Identified species	n	Identified species	n
Streptococcus tigurinus	3	*Streptococcus oligofermentans*	1
Streptococcus dentisani	1	*Streptococcus anginosus*	1
Streptococcus sanguinis	4	*Staphylococcus hominis*	4
Streptococcus cristatus	1	*Staphylococcus epidermidis*	3
Streptococcus gordonii	1	*Staphylococcus warneri*	1
Streptococcus mutans	1	*Staphylococcus pneumoniae*	1
Streptococcus mitis	4	*Veilonella parvula*	3
Streptococcus salivarius	5	*Veilonella dispar*	1
Streptococcus intermedius	2	*Bacillus circulans, B. cereus, B. tequilensis*	8

Table 2
IDENTIFIED SPECIES AFTER GENE SEQUENCE ANALYSIS (N=NUMBER OF SAMPLES)

Pair nr.	Subjects	Identified species
1	Mother (DMFT 6)	*Str. salivarius*
	Infant	*Str. salivarius*
2	Anya (DMFT 16)	*Str. oligofermentans, Str. salivarius, Str. tigurinus, Str. mitis, Staphylococcus pseudopneumoniae*
	Infant	*Str. intermedius, Str. salivarius, Str. mitis*
3	Mother (DMFT 15)	*Str. anginosus, Str. tigurinus, Str. mitis, Str. intermedius, Str. gordonii, Str. mutans, Str. anginosus Staphylococcus epidermidis, Veilonella dispar*
	Infant	*Str. sanguinis, Staphylococcus hominis, Str. epidermidis, Str. warneri*
4	Mother (DMFT 10)	*Veilonella parvula, Str. dentisani, Str. tigurinus, Bacillus tequilensis*
	Infant	*Str. intermedius, Str. mitis*
5	Mother (DMFT 13)	*Str. salivarius, Str. mitis, Str. tigurinus*
	Infant	*Str. mitis, Str. salivarius, Staphylococcus warneri, Str. epidermidis*

Table 3
IDENTIFIED SPECIES IN SAMPLED MOTHER-INFANT PAIRS

to early colonists, first colonizing on the oral mucosa and tooth surfaces, the species are: *Str. salivarius, Str. mitis, Str. sanguinis, Str. parasanguinis and Str. gordonii.* During our study, we were always working on fresh cultures due to the sensitivity of oral bacteria.

The members of the pyogen family have intracellular invasive skills, invasive infections, pharyngitis, tonsillitis, rheumatoid intestinal inflammation, acute gromerulonephritis, pneumonia, meningitis, and otitis media play an important role [6]. *Str. mutans* species play a role in the development of dental and root caries and may cause infective endocarditis [7]. Members of the mythic group also belong to the primary colonists, they have a significant role in the formation and accumulation of plaque as they provide a crosslinked structure for the other oral bacteria, thus facilitating their adhesion. They contribute to plaque, tooth decay, gingivitis, and periodontal disease [8]. *Str. tigurinus* was discovered by a Swedish research team in 2012, we do not yet know its general location and colonization capabilities. In 14 cases they were isolated from aggressive infections (infectious endocarditis, spondylodiscitis, bacteremia, meningitis) [9]. Each of the oral streptococci isolated by us was Gram positive, catalase and oxidase reactions were negative.

Various staphylococci were also isolated by this method. They are usually found in plaque, but they can be isolated from immunosuppressive conditions in various oral infections. It has been isolated several times from root canals and from treatment rezistent apical periodontal infections [10]. *Staphylococcus epidermidis, S. warneri, S. hominis* are members of normal microbial, Gram positive cocci, mainly on the skin and mucous membranes. They can cause infections, urinary tract infection, meningitis, endocarditis and gingivitis in immunosuppressive conditions.

Furthermore, we were able to isolate *Veilonella parvula* and *Veilonella dispar*. These bacteria also have a significant role in the oral cavity. They are strictly anaerobic, Gram negative cocci. These two bacteria can be isolated from individuals with mostly dental caries, but their role in the oral cavity is to decrease the highly erosive effect of lactic acid and propionic acid through various metabolic processes. In the mother-child pairs 50% of the same bacteria were detected [11].

The spread of the oral bacteria within the family varies in different nationalities. This diversity is due to the different habits and cultural differences that affect the parent-child relationship. The same genotype of oral streptococci in the United States is 71% for the parent-child pairs tested, 24% for Sweden and 31% for Japan studies and 31% is the genotype of the father's bacteria [4]. The possibility of vertical intra-family infections [5] was also mentioned in the case of *Treponema denticola*, *Capnocytophaga sputigena*, *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens, Porphyromonas gingivalis, Porphyromonas nigrescens, Prevotella intermedia, Tannerella forsythia*.

Conclusions

In more than half of our studied mother-infant pairs the same oral microbiota was identified. In infants free from caries laesions we were able to identify streptococci which can cause caries, but we isolate *Str. mutans* in only one mother subject. Each subject and each mother-infant pair has other oral bacterial microbiota. The isolated species and mother's DMFT indices are distributed proportionally. For a higher DMFT index, several types of bacteria were isolated and identified and their role was closely related to the development of dental caries.

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