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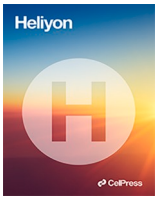

Research article

Comparison the salivary *streptococcus mutans* levels between caries-active and caries-free children from Birjand, Iran: A case-control study

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ABSTRACT

Objective: Dental plaque bacteria, including *Streptococcus mutans* (*SM*), play a role in the pathogenesis of the dental caries. There are conflicting results regarding the association of salivary *SM* level and dental caries susceptibility. Our aim was to compare salivary *SM* levels in colony-forming units (CFU) between children with active caries and caries-free children in Birjand, Iran.
Methods: This case-control study included 61 six-year-old children referred to health centers in Birjand city, Iran, in 2022. The children were divided into two groups: case (dmft/DMFT>0 with active caries) (including 31 children) and control (dmft/DMFT = 0 [caries-free]) (including 30 children). Demographic information and dental history were recorded. Oral examinations were also performed by the dentist. Unstimulated saliva samples were collected from children. The number of salivary *SM* colonies was determined using the microbial culture and confirmed using the polymerase chain reaction (PCR). The data were analyzed using Chi-square and T-tests at a significance level of $p < 0.05$.
Results: The mean number of *SM* colonies was 126.24 ± 92.78 CFU/ml and 92.38 ± 75.34 CFU/ml in case and control groups, respectively. No significant difference was found in salivary *SM* levels between case and control groups (P = 0.125). No significant association was observed between caries experience with gender (P = 0.363), type of school (public/private) (P = 0.296), receiving oral health education (P = 0.072) and frequency of tooth brushing (P = 0.935). The mean gingival index (P = 0.001) and plaque index (P = 0.025) in case group were significantly higher than control group.
Conclusion: There is no significant difference in salivary *SM* levels between caries-active and caries-free children in Birjandi children.

1. Introduction

Dental caries is one of the most common chronic infectious diseases [1,2] which occur as a result of an imbalance between demineralization and remineralization [3,4]. Dental caries is still one of the most challenging topics in the field of children's oral

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health and children are considered as high-risk groups for tooth decay [5].

Biological and environmental factors like dental plaque, saliva factors, fluoride intake, oral hygiene and type of diet are considered as risk factors for dental caries [6,7]. Many bacteria play a role in dental caries development including *lactobacillus casei* and *Streptococcus mutans* (*SM*). *SM* is a gram-positive bacterium belongs to the viridans streptococci group, which is often found as a normal flora in the mouth and throat, and various studies have determined its role in dental caries; it is believed that the acidogenic properties of this bacterium is effective in developing caries [8,9]. The results of some studies indicate that the presence of *SM* in the saliva of caries-free individuals significantly increases the risk of caries in the future [10].

The prevalence of dental caries and their associated risk factors vary in different geographical regions of the world from country to country. There is also some ethnic difference in the prevalence of caries. Although the *SM* is considered a strong etiologic factor of dental caries, ethnic differences in acquisition of *SM* may justify this ethnic difference in the prevalence of dental caries [6,11].

The results on the association between salivary *SM* level and caries experience are contradictory. While some studies are in favor of the existence of such an association [12–16], the others believe that there is no such a relationship [17–20].

So, considering the possible above-mentioned ethnic and geographic differences, as well as the conflicting results of existing studies, this study was conducted to compare the salivary *SM* levels between caries-active and caries-free children from Birjand, east of Iran.

2. Materials and methods

2.1. Sample size calculation

According to similar studies [15–17], taking into account the prevalence of salivary *SM* in two groups with caries and without caries equal to 60 % and 23 % respectively, with 95 % confidence interval and 80 % power, the sample size in each group (case and control groups) was estimated to be 27 people. Taking into account sample dropout, the final volume in each group considered 30 people. The details of the calculation are as follows:

Analysis A priori: compute required sample size.

Input:

Tail(s) = two

Proportion p1 = 0.6.
Proportion p2 = 0.23.
A err prob = 0.05.
Power (1-β err prob) = 0.80.
Allocation ratio N2/N1 = 1.
Output:
Critical z = 1.9599640.
Sample size group 1 = 27.
Sample size group 2 = 27.
Total sample size = 54.
Actual power = 0.8057151.

2.2. Study population

In this case-control study, the study population included sixty one 6-year-old children. The children were selected by the random sampling method [15,17,18] from a sample of 600 children referred to health centers of Birjand city, east of Iran for a school-oriented survey conducted in 2022. The children in case and control groups were sex-matched; they were also matched for school type (private or public) as a component of socioeconomic status.

2.3. Inclusion and exclusion criteria

The inclusion criteria included: 6 years of age, living in Birjand city, having informed consent, not having a systemic disease [15] and health of children at the time of entering the study [15].

The exclusion criteria included: non-cooperation of the child, use of space maintenance or orthodontic appliances [12,16] and antibiotic use at the last three months [15,16,18].

2.4. Study design

This case-control study was conducted in 2022 in Birjand city, Iran. Demographic information and dental history (frequency of tooth brushing per week, receiving oral and dental health education) of the children were collected from participants.

A qualified dentist who had at least 5 years of dental work experience and had a history of cooperation in at least ten research projects, especially in the fields related to the epidemiological projects of oral health, did the oral and dental examination of children.

In order to ensure consistency in intra-observer examinations, calibration was done according to the standards of the World Health Organization by the supervisor of the research project. The oral examination was performed in a supine position under an appropriate light source from the dental chair using a dental mirror and a probe.

According to world health organization (WHO) guideline and based on the sum of the number of decayed, missing due to caries, and filled teeth in the primary teeth (dmft index) or permanent teeth (DMFT index), the children were divided into two groups: case (dmft/DMFT>0 with one or more than one non-filled cavitated lesions [active caries]) and control (dmft/DMFT = 0 [caries-free]) [12,17,21].

The plaque index (PI) and gingival index (GI) of children was recorded according to the Silness and Loe plaque and gingival indices [22,23].

All children were asked to avoid from eating, drinking and brushing their teeth 2 h before sampling. 5 ml of unstimulated whole saliva samples were collected from children in sterile sample containers. The saliva samples were immediately sent to the microbiology laboratory.

2.5. Microbial analysis and polymerase chain reaction (PCR)

Salivary *SM* level was determined by culture-based method, morphological identification and PCR-based confirmation.

In the laboratory, after complete vortexing of the saliva sample, ten-fold serial dilutions of the homogenized samples prepared in

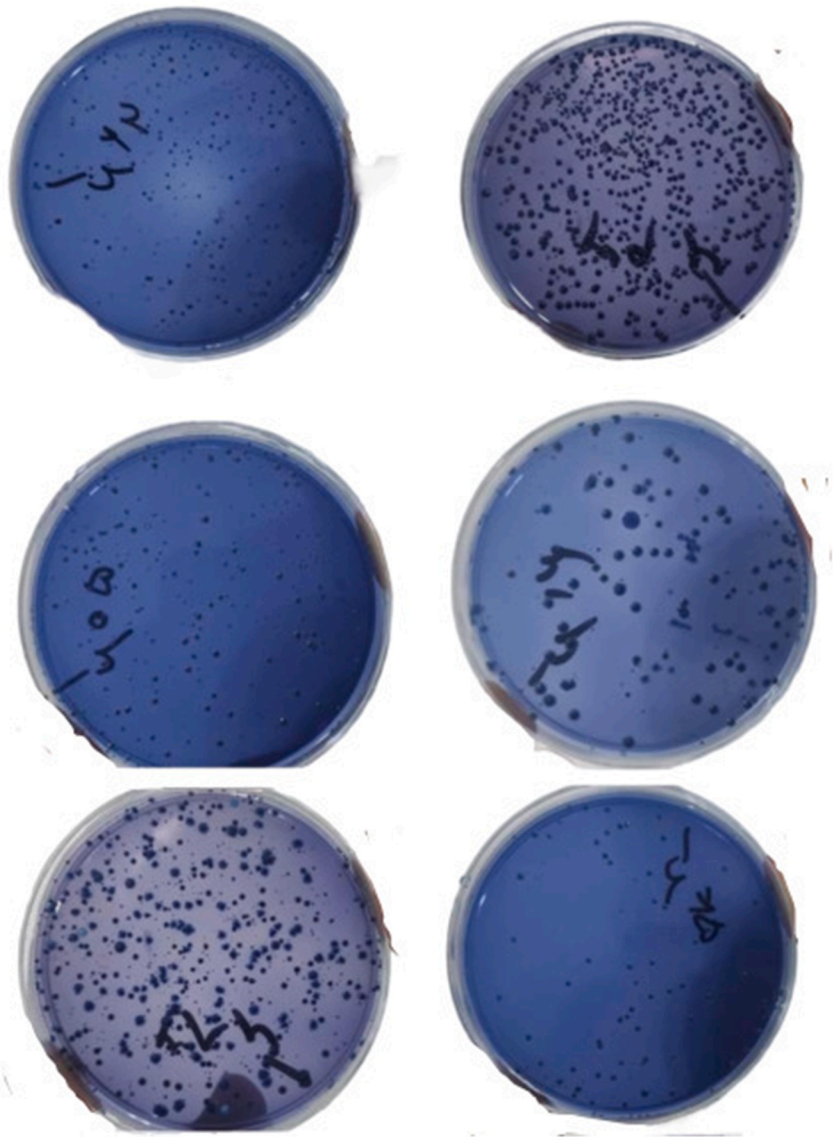

Fig. 1. Images of bacterial colony culture from the saliva samples of the studied children.

sterile normal saline and 10 μl of them were cultured on the Mitis salivarius agar medium supplemented by bacitracin (0.2U/ml) and potassium tellurite, using the spreading method, duplicately (Fig. 1); the conditions for the growth of *SM* became specific to a large extent. Cultured plates were incubated in microaerophilic conditions for 24–48 h at 37 °C.

Because we encountered four different types of colonies in cultured plates, PCR test by specific primers for htrA genes of *SM* was used to confirm *SM* colonies. For this purpose, DNA extraction was done from the 4 desired colonies separately and also SM ATCC35668 as positive control by high pure polymerase chain reaction template preparation kit (Cat.no: 11796828001, Roche Diagnostics GmbH, Germany). Each PCR reaction contained 12.5 μl of 2× Taq DNA polymerase Master Mix Red (Amplicon, Denmark), 1 μl of each 10 pM/μl forward and reverse primers (Table 1), 1 μl of bacterial extracted DNA or *SM* ATCC35668 DNA as positive control or Double Distilled water (DDW) as negative control, and DDW up to 25 μl final volume. 2 × universal gradient thermocycler (PEQLAB, Germany) was used for DNA amplification, starting with the initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s the final extension was completed at 72 °C for 10 min [24]. The PCR products were seen under UV light following 1 % agarose gel electrophoresis. The PCR product size (bp) was determined by comparing it with 1 kb DNA ladder (Fig. 2 and s).

According to the PCR performed, dark blue colored colonies were confirmed as *SM*. In the following, in order to further investigate the 10 targeted colonies (dark blue), DNA extraction was performed and PCR test was performed on them using a specific primer for the htrA gene (Figs. 3 and 2s).

Then, based on morphology and color, *SM* colonies were counted using colony counter. Finally, based on the average number of bacterial colonies grown for each sample and according to the dilution factor, the salivary *SM* level for each sample was calculated in terms of colony forming units per milliliter (CFU/ml) in the saliva sample [25].

2.6. Statistical analysis

SPSS software (version 22, SPSS Inc., Chicago, USA) was used for analyzing the data. The data were analyzed using Chi-square and T-tests at a significance level of $p < 0.05$.

3. Results

In this study, sixty-one 6-year-old children referred to the health centers of Birjand city, east of Iran, were included in the study. Among them, thirty children were in the control group (caries-free) and thirty one children were in the case group. Table 2 shows a summary of the demographic information of the participants (Table 2). There was no significant difference between studied groups in terms of gender and type of school (public/private) (*P*-values >0.05). Table 3 shows comparison of studied group based on oral health factors (Table 3). There was no significant difference between the children in the case and control groups in terms of receiving oral health education and the frequency of tooth brushing (*P*-values >0.05). The gingival index and plaque index in children of the case group were significantly higher than those of the control group (*P*-values<0.05).

As mentioned in the materials and methods section, dark blue colored colonies were identified and confirmed as *SM* colonies. The number of *SM* colonies was 126.24 ± 92.78 CFU/ml in the case group and 92.38 ± 75.34 CFU/ml in control group. There was no significant difference between studied groups in terms of *the number of SM* colonies (P = 0.125).

4. Discussion

The findings of our study showed that although the number of *SM* colonies in the saliva of children in the case group was higher than in the control group, this difference was not significant.

Dental caries is a multifactorial disease and is one of the most common diseases in children that is formed through demineralization of enamel and dentine by the organic acids produced by dental plaques' bacteria as a result of metabolism of dietary sugars. *SM* is the principal bacteria present in dental plaques which initiate dental caries. High level of *SM* in plaque is associated with increased caries susceptibility. Some studies have found that ethnic differences are present in the proportion of children with *SM* and its level. These ethnic differences (in cariogenic bacteria) may justify some ethnic differences in caries's prevalence [6,7]. An association has been hypothesized between the salivary numbers of *SM* and dental plaque number of it and also the prevalence of dental caries [25].

Lack of significant difference in *SM* colony count between caries-active children and caries-free children in our study suggests the hypothesis that there might be no significant association between the salivary *SM* level and caries experience in children of this region. In theory, plaque is more suitable for measuring *SM* levels in the mouth than saliva because tooth surfaces are the bacteria's natural habitat [17]; researches have also shown that level of *SM* is higher in plaque than in other sampling techniques including saliva [26,27]. Therefore, knowing this information, the finding of our study on the lack of significant difference in salivary *SM* between caries-active and caries-free children will not be far from expected.

Table 1
Primers for htrA gene of Streptococcus *mutans* used in the study.

Gene	primers	Amplicon size	Reference
htrA	htrA F:5′TCGCGAAAAAGATAAACAAACA-3′ htrA R:5′GCCCCTTCACAGTTGGTTAG-3′	479 bp	[17]

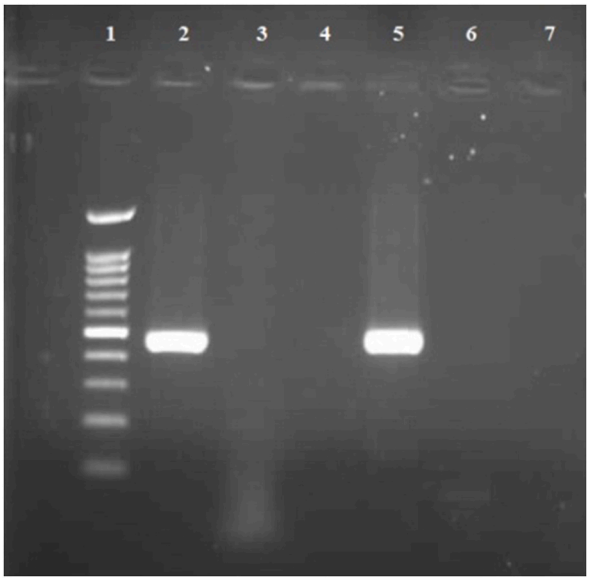

Fig. 2. Representative agarose gels from the 4 desired colonies separately; order of samples from left to right: 1) DNA marker 1 Kb, 2) *Streptococcus mutans* ATCC35668 as positive control (479 bp), 3) colony 1 (light blue), 4) colony 2 (tiny dark blue), 5) colony 3 (dark blue) (479 bp), 6) colony 4 (Flat dark blue), 7) Negative control Fig. 2s: uncropped versions of figures [2, 3].

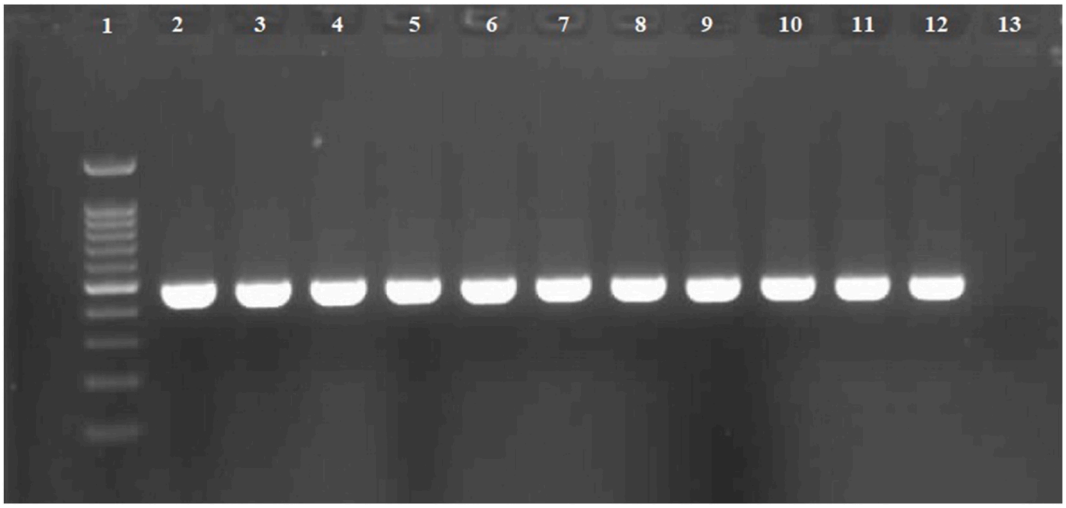

Fig. 3. Representative agarose gels from the 10 targeted dark blue colored colonies; order of samples from left to right: 1) DNA marker, 2) positive control, 3–12) ten selected clones (dark blue), 13) negative control.

Table 2
Demographic information of the study participants.

Group Variable		Control n (%)	Case n (%)	*P*-value
School	**Public**	22 (73.3)	19 (61.3)	0.296
	Private	8 (26.7)	12 (38.7)	
Sex	**Boy**	12 (40)	16 (51.6)	0.363
	Girl	18 (60)	15 (48.4)	

Contradictory results are present regarding the association of salivary *SM* level and caries experience. Some studies have found that salivary *SM* level is significantly higher in high caries/caries-active children than low caries/caries-free children/adults [12,14,15,21,27,28]; these results are not consistent with the results of our study; the reasons for this inconsistency may be related to different

Table 3
Comparison of studied group based on oral health factors.

Group Variable		Control n (%)	Case n (%)	*P*-value
Receive oral health education	**Yes**	27 (90)	23 (74.2)	0.072
	No	3 [10]	8 (25.8)	
Frequency of tooth brushing per week		3.53 ± 2.33	3.48 ± 2.36	0.935
Gingival index		3 ± 3.98	8.7 ± 8.29	0.001
Plaque index		0.46 ± 0.42	0.71 ± 0.43	0.025

selection criteria for studied groups, different age-groups of the studied children and different methods for colony counting and identification of *SM*. Also, *SM* levels were sometimes associated with severity of caries (dmfs score) [27].

On the other hand, some studies have observed that salivary *SM* level is not significantly different in high caries/caries-active children compared to low caries/caries-free children [17,20,29]; they concluded that high *SM* levels in the saliva do not influence the DMFS index/caries scores [29]; these results are consistent with the results of our study. The results indicated that *SM* level in plaque have much stronger association with early childhood caries (ECC) than that level in saliva [17].

In Vachirarojpisan et al. study, they found that salivary *SM* level (measured by Strip mutans test) was a significant predictor of intensity of early childhood caries (I-ECC) in rural Thai children aged 6–19 months, so that children who had higher count of *SM* also had higher I-ECC score [30].

The findings of our study showed that the gingival index and the plaque index in the case group were significantly higher than the control group (caries-free). This finding indicates an association between periodontal indices and caries experience in children of this region. In a study conducted by Hamalaw et al. [28], the mean plaque index in high caries children group was significantly higher than low caries children group, which is consistent with the results of our study.

Current evidences show that many types of oral bacteria, such as *Actinomyces*, *Prevotella*, etc., along with *SM*, have close relationship with the development and progression of dental caries and the imbalance of oral bacterial communities is an important background for dental caries. In fact, current literature supports a polymicrobial etiology, in which caries-associated bacterial communities are considered as an etiologic agent, rather than a single microorganism such as *SM* [31,32]. Therefore, it is suggested that in future studies, instead of examining only one bacterium, a set of bacteria in the oral microbial communities that are related to caries should be examined through new technologies such as next generation sequencing or high-throughput sequencing.

4.1. Limitation

We know that Next-Generation Sequencing (NGS) allows for more comprehensive and precise analysis of microbial communities in oral samples, providing a deeper understanding of the role of various bacteria, including *SM*, in dental caries development. Unfortunately, due to the unavailability of this method in our region due to the embargo of our country, Iran, as well as the high costs of doing it, it was not possible to analyze the oral microbial community of children accurately, which is one of the limitations of the present study.

Another one is the limitations of culture-based methods, such as potential underestimation of bacterial diversity and viability, which may affect the interpretation of the results.

Previous studies that collected saliva samples from children have acknowledged the fact that bacteria in the saliva come from various intraoral sites, including the buccal mucosa, tongue, and supragingival plaque. This issue points out the limitation of using saliva as a sample in assessing the associations with dental caries susceptibility. Therefore, the use of saliva samples and not using more precise samples can be considered as limitations for the present study.

5. Conclusion

The result showed that there is no significant difference in salivary *SM* levels in colony-forming units (CFU) between caries-active and caries-free children from Birjand, Iran.

Ethical statement

This study approved by ethics committee of Birjand University of Medical Sciences (ethics code: IR.BUMS.REC.1400.180). The children voluntarily participated in this study and informed consent was obtained from the children's parents or guardians.

Consent to participate

All the children voluntarily participated in this study. Also, the informed consent form was completed by the parents of the children.

Consent for publication

Not applicable.

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Data availability statement

Data included in article/supp. Material.

CRediT authorship contribution statement

Ebrahim Shafaie: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Zahra Badri:** Writing – review & editing, Investigation, Data curation. **Hamid Salehiniya:** Writing – review & editing, Writing – original draft, Software, Methodology. **Hamid Abbaszadeh:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25663.

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