Profil Proporsi *Streptococcus mutans* pada Beberapa Lesi Karies dengan Tingkat Keparahan Berbeda

(Streptococcus mutans Proportion Profile in Several Caries Lesions with Different Severity Levels)

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Abstrak

Karies gigi adalah penyakit menular bakteri umum yang memengaruhi struktur gigi, dengan Streptococcus mutans dikaitkan dengan tingkat keparahannya. Penelitian ini bertujuan untuk menilai distribusi proporsi S. mutans di berbagai tingkat lesi karies sebagai faktor prognostik potensial. Desain kelompok kontrol pasca-tes saja dilakukan pada 33 pasien yang terdaftar di RSGM UMY yang memenuhi kriteria sampel dengan gigi bebas karies, gigi berlubang berat, pulpitis reversibel, dan nekrosis pulpa. Sampel DNA diekstraksi dan dianalisis menggunakan teknik polymerase chain reaction (PCR) dan analisis densitometri semi kuantitatif menggunakan Image-J. Analisis statistik dilakukan untuk membandingkan proporsi S. mutans di berbagai kategori lesi. Studi ini menemukan variasi yang tidak signifikan dalam proporsi S. mutans (p>0,05) di antara kelompok yang diamati, dengan tingkat populasi tertinggi diamati pada kelompok ICDAS V (34,53±23,09), diikuti oleh kelompok nekrosis pulpa (24,41±15,48), kelompok pulpitis reversibel (11,14±1,36), dan kelompok gigi bebas karies (6,95±2,57). Sebaliknya, proporsi populasi terendah ditemukan dalam kondisi pulpitis bebas karies dan reversibel. Meskipun S. mutans dikaitkan dengan karies, keberadaannya saja tidak sepenuhnya menjelaskan dan berkorelasi dengan tingkat keparahan lesi.

Kata kunci: Karies, Karies bebas, Proporsi bakteri, *Streptococcus mutans*

Abstract

Dental caries is a common bacterial infectious disease that affects tooth structure, with Streptococcus mutans being associated with its severity. This study aimed to assess the distribution of S. mutans proportions across various levels of carious lesions as a potential prognostic factor. A post-test-only control group design was conducted on 33 patients registered at RSGM UMY who fulfilled the sample criteria with caries-free teeth, severe cavities, reversible pulpitis, and pulp necrosis. DNA samples were extracted and analyzed using the polymerase chain reaction (PCR) technique *and semi-quantitative densitometry analysis using Image-J. Statistical analysis was performed to compare S. mutans proportions across different lesion categories. The study found non-significant variations in S. mutans proportions (p>0.05) among the observed groups, with the highest population levels observed in the ICDAS V group (34.53±23.09), followed by the pulp necrosis group (24.41±15.48), reversible pulpitis group (11.14±1.36), and the caries-free teeth group (6.95±2.57). In contrast, the lowest proportions of the population were found in caries-free and reversible pulpitis conditions. Although S. mutans is associated with caries, its presence alone does not fully explain and correlate to the lesion severity.*

Keywords: *Bacterial proportion, Caries, free caries, Streptococcus mutans*

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Dental caries is a widespread global oral health issue that affects a significant coverage within the population, with prevalence rates of 80-90% in various communities[1]. In Indonesia, the prevalence of dental caries is notably high, reported at 88.8%, according to the 2018 Basic Health Results published by the Ministry of Health[2]. Dental caries is a chronic bacterial infectious disease that involves the tooth structure, affects both hard and soft tissues, and can progress gradually to destroy the integrity of the tooth[3]. The development of caries involves complex interactions among host factors, dietary habits, time, and microorganisms. Tooth decay is a dental condition where the hard tissues of the teeth break down due to bacteria, substrates, and host factors[4]. The term 'caries,' which means decay, is affected by various factors like the presence of decay-causing bacteria such as *Streptococcus mutans*, the intake of sugary foods, and how long they are in contact with the teeth[5]. Cavities may develop when a sticky film of bacteria, called plaque, builds up on the teeth and interacts with sugars to create acid, which damages the teeth by removing minerals. If the loss of minerals continues without enough repair, holes in the teeth, known as cavities, can develop and cause permanent harm to the tooth structure[6].

The International Caries Detection and Assessment System (ICDAS) is a globally recognized system for assessing dental caries and tracking their frequency and severity. ICDAS provides a framework for improved clinical management of caries by classifying the condition from the healthy teeth (ICDAS code 0) to severe decay (ICDAS code 6)[7]. The system identifies various stages of caries development, including initial visual changes in enamel (D1), clear visual changes (D2), early enamel damage (D3), localized enamel damage with dentin shadow (D4), and extensive decay with exposed dentin (D5)[8]. The most severe stage, ICDAS code 6, indicates deep cavities that involve more than half of the tooth surface and may potentially reach the pulp[9].

Pulp disease encompasses various conditions that affect the dental pulp; normal pulp is classified as clinically sensitive yet asymptomatic. The most prevalent cause of pulp disease is bacterial invasion through the dentin, typically resulting from untreated caries (tooth decay). Inflammation of the pulp, known as pulpitis, can be categorized as reversible or irreversible. Reversible pulpitis is caused by mild inflammation that

resolves once the irritant is removed. Irreversible pulpitis involves persistent inflammation that cannot return to a normal state and is often accompanied by severe pain that may radiate to adjacent teeth[10]. Understanding the factors behind tooth decay is vital for creating effective strategies to prevent and treat dental problem issues.

Streptococcus mutans is a microorganism component among the most common bacterium associated with tooth decay. *S mutants* also belong to acidogenic and acidic pathogens harboring the capacity to produce organic acids through sugar metabolism, thereby contributing to the cariogenic process[11]. *Streptococcus mutans* is a gram-positive bacterium that can survive with or without oxygen and is known as a primary pathogen associated with dental caries[12]. It belongs to the genus *Streptococcus* and can be classified into different variations, with type C being the most common in humans[13]. The bacterium plays a crucial role in causing cavities by breaking down sugars into lactic acid, lowering the pH, and destructing the enamel's hard tissue. *S mutants* can also produce sugars outside their cells from sucrose, promoting biofilm formation and helping it stick to tooth surfaces[14].

The harmful effects of *S mutants* are due to specific factors like Gtf and Gbp, which help in producing sticky substances. These factors contribute to the synthesis of sticky glucans, which form a robust extracellular matrix in dental biofilms[15]. Continuous sucrose intake by the host promotes an acidic environment that favors the growth of *S mutants* while inhibiting competing bacteria. This change leads to the production of organic acids, which lowers the pH and starts the formation of cavities. Understanding *S mutant*'s role in dental caries is crucial for developing effective prevention and treatment strategies.

Although *S. mutans* is frequently regarded as the primary pathogen, its prevalence in carious lesions compared to caries-free conditions varies, with several studies reporting inconsistent prevalence rates[16,17]. Previous study by Frakgou et al. reported variations in the prevalence of *S. mutans* between caries-free and caries-active children. In this study, which included 51 caries-active and 46 caries-free children aged 3 to 13 years, *S. mutans* was detected in 66% of samples[17]. Similarly, a study by Acevedo et al. examined the prevalence of *S. mutans* in children with and without caries, reporting its presence in 10 out of 30 children with caries (33%) and 6 out of 18 caries-free children (33%) aged 2 to 19 years, with no significant difference between the groups[18]. Another finding from Lima et al., and Thimmegowda reported a low prevalence rate of *S. mutans* in non-cavity-free teeth[19,20]. All of these reports suggest that while *S. mutans* is commonly associated with dental caries, its prevalence varies across different populations and does not always correlate directly with caries status.

Differences in the proportion of *S. mutans* in carious lesions are believed to influence the severity of dental caries. Some reports indicate that since *S. mutans* possesses cariogenic properties, caries still can develop in its absence; on the other hand, *S. mutans* can also be present without any tooth decay development[21]. Therefore, this study aims to evaluate the pathogenic proportion of *Streptococcus mutans* at several levels of carious lesion severity as a pathogenic prognostic factor. The study will involve sampling from different carious lesion conditions, including active carious lesion (ICDAS V), necrotizing caries, reversible pulpitis, and caries-free states, to observe the proportion of *S. mutans* for dental caries development.

METHODS

This research is a study that observes subjects in a controlled environment using a post-test-only control group design. The study included patients with specific dental conditions, such as caries-free teeth, teeth with severe cavities, reversible pulpitis, and pulp necrosis, registered at RSGM UMY. The research protocol was approved by the Ethical Committees of the Faculty of Medicine and Health Sciences Universitas Muhammadiyah Yogyakarta 090/EC-KEPK FKIK UMY/II/2024. Samples were collected intentionally to ensure a balanced representation, resulting in 33 samples evenly divided based on lesion criteria. The sampling process involved using a swab to collect *Streptococcus mutans* bacteria from the surfaces of both caries-free teeth and teeth with cavities.

The inclusion criteria for this study comprised subjects with caries-free teeth, caries classified as code 5 according to the International Caries Detection and Assessment System (ICDAS), caries with reversible pulpitis, and caries with pulp necrosis. Additionally, only patients who provided informed consent and those attending their first visit for caries treatment were included. The exclusion criteria encompassed patients with a poor Oral Hygiene Index-Simplified (OHI-S) score (3.1–6), individuals with disabilities, those attending follow-up visits after their initial consultation such as cases where caries removal had already been performed and patients who had completed their first-visit treatment.

The analysis process was conducted at a laboratory. It involved amplifying DNA using a Polymerase Chain Reaction (PCR) technique to detect the presence of *S. mutans*, followed by gel analysis and documentation to visualize the results.

Bacterial DNA were extracted using TRNzol Universal Reagent (TIANGEN BIOTECH) according to the manufacturer's protocols and used as templates for downstream analysis. Bacterial samples were homogenized in TRNzol Universal Reagent, followed by the addition of chloroform and centrifugation to separate the aqueous phase. The DNA was precipitated using isopropanol, washed with 75% ethanol, and resuspended in nuclease-free water. The quality and concentration of the extracted DNA were assessed using a NanoVue Plus (Biochrom Ltd., Cambridge, UK) before further analysis.

For gene detection analysis, 5 µL DNA template was mixed with 25 µL 2x MyTaq HS Red Mix (Bioline, UK) and 18 µL nuclease free water (NFW) along with the indicated specific primers (Table 1)[22], following the conditions listed in Table 1.

Table 1. List primer and PCR setting for *S. mutans*

Primer	Temperature (°C)	Time	Cycle
Forward 5'-ACTACACTTTCGGGTGGCTTGG -3'	95	4 min	1
Reverse 5'-CAGTATAAGCGCCAGTTTCATC -3'	95	1 min	
	55	1 min	35
	72	1 min	
	72	10 min	1
	4	Hold	1

Amplification products were visualized by electrophoresis and densitometry analysis according to Adiningrat et al., with slight modifications in volume[23]. 8 µL PCR products from the gene detection analysis were mixed with 2 µL Novel Juice (dye-containing loading buffer, GeneDireX, USA) and loaded into a 1% agarose gel with 1 µL GelRed (Biotium, Canada). Electrophoresis gel analysis was then carried out at 50 V for 50–60 min. The gel was visualized with a UV Transilluminator (Genedirex, USA) after electrophoresis.

Semi-quantitative densitometry analysis using ImageJ was performed following the method described by Adiningrat et al. The analysis began by importing the gel electrophoresis results and converting the image to grayscale format. The Rectangle Tool was then used to select the band area for analysis, ensuring that all bands within the same gel were measured using a consistent Region of Interest (ROI) to maintain accuracy. The Measure feature (Analyze → Measure) was utilized to obtain the area under the curve value, which represents band intensity. Band intensity was then normalized against the control band. Finally, data interpretation was conducted by comparing intensity values between samples to assess the relative amount of the target, with further statistical analysis performed using Excel[23].

The normality of the data will be assessed using the Shapiro-Wilk test. If the data are normally distributed, an independent t-test will be performed to identify differences in *S. mutans* proportions at different levels of caries severity. Statistical analysis will be conducted using IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA) to ensure the validity and reliability of the results.

RESULTS

All samples used in this study underwent semi-quantitative PCR testing with densitometry to determine the presence of a DNA fragment and to measure the intensity of the DNA band. The results of this test are expressed as optical density (OD), as shown in Figure 1 and Table 2.

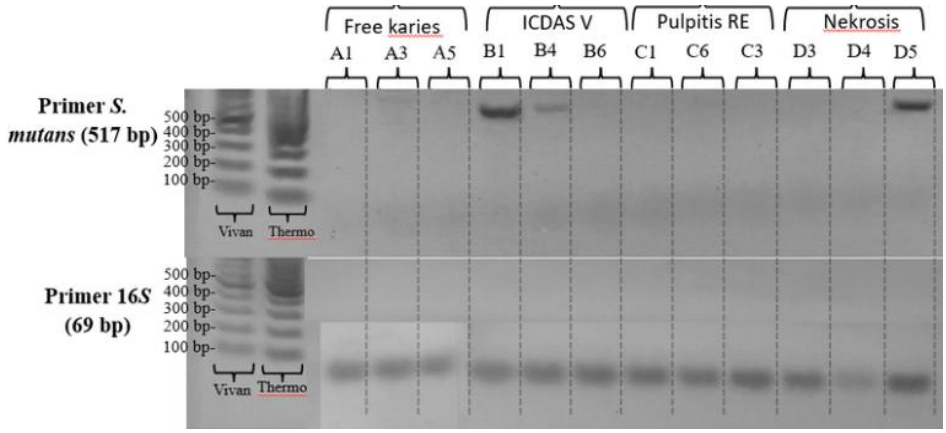

Figure 1. Gel analysis visualization of PCR products from 12 samples with *S. mutans* primer (517 bp) and 16s primer (69 bp)

Table 2. Semi-quantitative PCR test result

Sample	Repetition	Percentage%	Mean	St.Dev	SE
	A1	2.37			
FREE CARIES	A3	11.25	6.95	4.446617	2.567255
	A5	7.23			
	B1	80.55			
ICDAS V	B4	14.81	34.53	39.98677	23.08637
	B6	8.24			
	C1	11.64			
P. REVER	C6	13.20	11.14	2.350234	1.356908
	C3	8.58			
	D3	9.96			
NEKROSIS	D4	7.92	24.41	26.81423	15.4812
	D5	55.35			

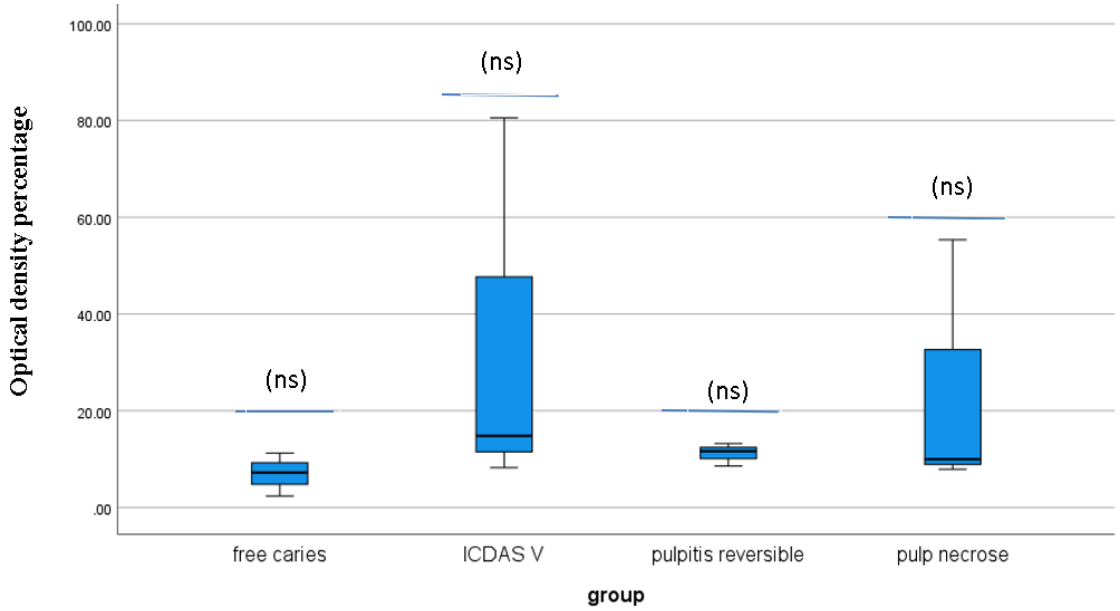

Figure 2. Percentage distribution in four groups: free caries, ICDAS V, reversible pulpitis, and pulp necrosis. ns indicates: no significant differences p>0.05; asterisks indicate statistically significant differences: p<0.05 (*), p<0.01 (**), p<0.001 (***).

The data presented in Figure 1 results from a densitometry analysis conducted on 12 samples. All samples showed the presence of bacteria when analyzed with the 16S primer (69 bp). However, specific signals were detected in only three samples when assessed with the *S. mutant 's-specific* primer (517 bp): two from the ICDAS V category and one from the pulp necrosis category. The table summarizes the results of the densitometry calculations (Table 2)

The data presented in Table 2 calculates the percentage of densitometry results, indicating that the highest rate of *S. mutans* is found in sample B1 (ICDAS V) at 80.55%, while the lowest percentage is observed in the categories of caries-free.

The boxplot, a graphical representation of the distribution of *S. mutants* across the four caries lesion categories, is shown in Figure 2. The range of data values is narrow for free caries and reversible pulpitis, with the median almost reaching 10%. The median for the ICDAS V group is around 45%, compared to about 30% for the pulp necrosis group.

To assess if the data follows a normal distribution, a normality test using the Shapiro-Wilk test will be performed on the dataset from Table 2 due to the sample size being less than 50.

The normality test results show a significance value (p > 0.05), indicating that the data follows a normal distribution. Using these results, a parametric test, such as the independent t-test, will be used to determine if there are notable variations in the population distribution across each sample category.

From Figure 2, it can be seen from the results of the independent sample t-test that the results of each test between categories are not significantly different.

DISCUSSION

The results of this study indicate a trend toward normal distribution in the statistical analysis of *Streptococcus mutans* proportions across different caries severity groups. However, no significant differences were observed between the control (caries-free) and target groups, including ICDAS V, reversible pulpitis, and pulp necrosis. These findings suggested that although *S. mutans* is commonly associated with the development of dental caries, the variable of its presence only may not be sufficient to differentiate the various carious lesion conditions. This aligns with the growing body of evidence indicating that caries development is a multifactorial process involving complex microbial interactions in a longitudinal process rather than the sole presence of *S. mutans* at a single point in time [24–26].

Dental caries is a complex pathological condition where cross-interactions among bacteria, substrate, host, and time contribute to the process of enamel demineralization[26,25] due to the increase of biofilm acidity. Among the various microbial factors involved in caries progression, *S. mutans* plays a crucial role due to its ability to thrive in acidic environments. Both acidogenicity and aciduric properties are important biochemical characteristics for the cariogenic capacity of *S. mutans*[27]. On the other hand, the occurrence of dynamic microbial interaction may also exhibit microbial homeostasis that may control microbiome pathogenicity. *Veillonella* is able to use the lactic acid produced by *S. mutans* as a carbon source, thus acting as an "acid sink." This may prevent the biofilm from reaching a very low pH, which could reduce the ability of *S. mutans* to survive and contribute to caries progression[16]. As probiotics, some *Lactobacillus* species can also reduce the cariogenic activity of *S. mutans*. They

compete for space and nutrients in the complex biofilm and produce antimicrobial metabolites that can inhibit the growth of *S. mutans,* thus reflecting on the internal pathogenicity control mechanism[29].

Although *Streptococcus mutans* is often linked to caries development, our study found that it did not consistently dominate in all cases, suggesting the involvement of other bacterial species during the caries development process since it is also inevitable existence. This result may relate to the previous report, which demonstrates the possible dynamic carious lesion development process by not only a single bacteria domination approach but also a collective bacterial approach within the carious lesion microbiome. For example, previous research conducted by Klein et al. revealed that other bacteria, such as *Lactobacillus spp*. and *Actinomyces spp*., may contribute to caries formation, especially under conditions where *S. mutans* is absent[30]. Other studies have shown that bacteria other than *S. mutans* play a dominant role in caries development. Species from *Veillonella*, *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Atopobium*, and *Actinomyces*, along with non-*S. mutans* acidogenic streptococci, often at higher levels than *S. mutans*[31]. Furthermore, Marsh emphasized the importance of understanding the broader microbial ecosystem within the oral cavity, where interactions among different microbial species can influence caries development, even in the absence of *S. mutans*[32]. This indicates that dental caries could be dependent not only on a single type of bacteria but also on the complex interactions among various microbes in the dental biofilm.

The non-dominance of *S. mutans* in pulp necrosis and reversible pulpitis suggested a shift in the microbial landscape, with other bacterial groups such as *Actinomyces spp*., *Staphylococcus aureus, Firmicutes,* and *Actinobacteria,* becoming more prevalent [33–37]. These findings suggested a significant shift in the oral microbiome, where diverse acidogenic bacteria may play a more prominent role in the transition from early carious lesions to pulpitis. The observed variability in *S. mutans* presence among individuals underscores the complex interactions within the oral microbial community[38]. This variability also presents a challenge in ensuring sample homogeneity which may influence data consistency.

Sample homogeneity remains a key challenge and limitation of this study. Variations in clinical conditions during the pre-analytical phase, including differences in cavity or caries status at initial sample collection, may have led to sample loss and compromised data consistency. To address this, further optimization of pre-analytical procedures is necessary to improve sample standardization and ensure data reliability. Additionally, expanding sample coverage is essential to enhance the study's representativeness and strengthen its external validity.

Although *Streptococcus mutans* is often considered as the main pathogen factor associated with dental caries, the findings from this study, corresponding with previous studies, suggested that its presence as normal oral flora did not solely and directly correlate with caries development at a single point of time[39]. The absence of significant differences in the proportion of *S. mutans* among different dental carious lesion conditions also indicated the possibility that other microbial species and environmental factors could play an important role in dynamic caries development. These underline the complexity of the oral microbiome and highlight the need for a deeper understanding of the interactions between different bacterial populations toward carious lesion development. Further investigation is essential to elucidate the multi-factorial nature of carious and to refine prevention and better treatment strategies.

From a clinical perspective, if the findings of this study support the proposed hypothesis, they could contribute to minimizing the severity of dental caries. Early detection of *Streptococcus mutans* could be incorporated as part of a screening protocol, allowing for a preventive approach to reduce potential for increased severity of caries in the future.

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