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Oral *Klebsiella* sp. Involved in Dental Caries: A Case of Individuals Gargling with Peatwater

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Most of the microbes inhabiting the oral cavities are harmless but can cause disturbance and discomfort if the microbial population increases significantly. One of the oral disorders that is often experienced by children, is dental caries. Dental caries is causedby various factors, including eating and brushing habits. The people of Central Kalimantan who live in the watershed use peat water in their daily activities, including for gargling and brushing their teeth. The acidic characteristics of peat water and the high content of organic substances trigger the development of microbes that cause dental caries.This study used dental caries swab samples of patients who rinsed their mouths with peat water. Bacteria were identified molecularly with 16SrRNA markers with primers 63F and 1387R. The similarity of nucleotide sequences was analyzed with BLAST on GeneBank. A phylogenetic tree was built with Maximum Likelihood. Both isolates have a fairly high level of similarity to *Klebsiella pneumoniae*, namely 91% and 93%. Both isolates are in the same clade, *Klebsiella* spp., and are close to various commonly found bacteria in the oral cavity, such as *Streptococcus mutans* and *Lactobacillus salivarius*.

Key words: dental caries, *Klebsiella*, peat water, *Streptococcus*, water-borne diseases

INTRODUCTION

The oral cavity is inhabited by more than 700 species of bacteria. While the majority of these microorganisms are benign, in some cases, they can lead to oral illnesses such as periodontal disease or dental caries (Forssten *et al.* 2010). One of the earliest and most prevalent infections in humans is dental caries (Lamont and Egland 2015). Because dental caries can result in tooth decay, it is regarded as a global oral health burden (Merchan and Ismail 2021). Dental caries is a major concern for medical professionals even though it rarely poses a life-threatening risk.

Dental caries can appear in individuals of any age group; it can appear early in life, but is rare in children under 2 years old (Matsumoto-Nakano 2014). A white spot lesion is the first indication of dental caries. A hollow or hole may form in the white spot if demineralization persists. High concentrations of cariogenic bacteria, frequent sugar consumption, insufficient salivary flow, inadequate fluoride exposure, poor oral hygiene, and impoverished conditions are risk factors for caries (Tinanoff *et al.* 2019).

Dental caries is caused by a combination of food debris and microorganism activity. The common bacteria that cause dental caries is *Streptococcus mutans*. This bacterium damages the hard tooth structure (enamel) by producing acid and making a nest on the tooth's surface. Dental caries starts when a biofilm of food particles, salivary polymers, and millions of bacterial cells forms on the surface of the tooth. The resulting biofilm has the potential to serve as a site for bacterial adhesion and colonization. Apart from *S. mutans*, bacteria that are also responsible for the incidence of dental caries are *Streptococcus sanguinis, Streptococcus sobrinus*, and *Actinomyces viscosus* (Forssten *et al.* 2010).

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Osmolarity, carbon supply, pH, and other environmental element factors affect the production of biofilms by bacteria. The fast fermentation of sucrose into acidic metabolites upsets the equilibrium of bacteria in the mouth when carbohydrates like fructose and sucrose are present (Ardenghi *et al.* 2013). Acidic conditions in the mouth are not only obtained from food, but also from the environment. For example, the use of water to rinse the mouth or brush the teeth.

Some communities in the Kalimantan region use peat water for their daily activities. Peat water is blackish brown in color and has a very low pH value (Said *et al.* 2019). Although there is no scientific evidence to support that rinsing the mouth with peat water directly causes dental caries, the low pH of peat water may be one of the factors that trigger increased acid production in the oral cavity. Determining the type of bacteria responsible for dental caries in those who rinse their mouth with peat water can aid in determining the degree of dental caries risk. The purpose of this work is to detect bacteria molecularly using the 16S rRNA gene and to separate bacteria from dental cavities in people who gargle with peat water.

MATERIALS AND METHODS

Sample Source. Samples were taken from dental caries patients who were accustomed to rinsing their mouth with peat water. Samples were obtained from the Pahandut Health Center, Pahandut Seberang Village. Samples were collected using the swab technique on dental caries both profunda and media caries. Swab samples were stored in sterile distilled water.

Isolation of Bacteria from Dental Caries. Dental caries swab samples were streaked on Blood Agar media. Colonies that grew on Blood Agar media were characterized, then continued Gram stain testing. Pure bacterial colonies were grown on Nutrient broth media, then incubated at 37°C.

Bacterial DNA Extraction. In this study we selected and characterized colonies that grew on Blood Agar Media. Colonies that grew with different shapes and colours were picked up and Gram stained (microscopic data is described in another paper) Next, we randomly selected two colonies for molecular characterization. One bacterial colony, 200 µL of PBS, and 20 µL of proteinase K were added to a sterile 1.5 ml microcentrifuge tube. Following pipetting to homogenize the mixture, it was incubated for five minutes at 60°C. Then, 200 µL of GSB Buffer (Geneaid) was added. This was followed by vortexing and a further two minutes of incubation at the same temperature. After adding 96% pure ethanol, the mixture was vortexed for ten seconds. After pouring the entire liquid into a spin column, it was centrifuged for one minute at 14,000 g. A new collection tube was installed in place of the one that was discarded beneath the spin column. After adding 400 µL of buffer W1 and centrifuging at the same speed for 30 seconds, the liquid was disposed of. After adding 600 µL of Geneaid wash buffer and centrifuging for 30 seconds, the liquid was removed once again. After three minutes of centrifugation, the spin column was disposed of 100 µL of elution buffer was added to the sterile microcentrifuge tube that was positioned at the bottom of the spin column. It was centrifuged for 30 seconds at the same speed after standing for three minutes. In order to use it as a PCR template, the resultant liquid that contained DNA in the microcentrifuge tube was kept at -40°C. The quantification of extracted DNA was conducted at the Hasanudin University Medical Research Center using spectrophotometer.

Gene Amplification (16S rRNA). The 16S rRNA gene was amplified using the following primer sets: 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3'). (Aris *et al.* 2013). PCR mix was mixed into the PCR tube with a composition consisting of 0.25 µL Hostart DNA polymerase, 1 µL reverse primer (20 pmol), 1 µL forward primer (20 pmol), 1 µL of 5 mM dNTP, 2 µL of 25 mM $MgCl_2$, 5 µL of 10X PCR buffer, 5 µL DNA sample, and 34, 7 µL ddH_20. The process of amplification was done with a PCR apparatus (DNA thermal cycler). Denaturation at 94°C for 2 minutes, followed by 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1.5 minutes for 30 cycles are the steps involved in 16sRNA gene amplification. The last step is extension at 72°C for 10 minutes and storage at 12°C ±30 minutes.

Two grams of agarose (BioRad) were dissolved in one liter of 100 milliliters of 10 Tris borate EDTA (100 milliliters of Tris base, 27.5 grams of boric acid, and 20 milliliters of 0.5 M EDTA, pH 8.0) then brought to a boil before dissolving. Then, a gel printer equipped with a comb was filled with 1 µL of ethidium bromide (0.2 ug/ml). The agarose is placed into the electrophoresis tank holding the 0.5x TBE solution when it has solidified, which takes around 30 minutes. After mixing the sample DNA with liquid "loading dye" in a 2:1 ratio, add the 100 bp marker to the well once the full sample has been added. PCR and electrophoresis (visualization of PCR results) were performed at Hasanudin University Medical Research Center.

Sequencing. DNA sequencing was performed using the one-directional sequencing method (Sanger Sequencing). This process was carried out at Hasanudin University Medical Research Center. Mutation analysis by direct sequencing was carried out at 1st Base Laboratory Malaysia. To determine whether the 16sRNA gene had undergone mutation, the PCR product's sequence findings were analyzed using "Bioedit" software and compared with information from the NCBI database's "Gene Bank" using the Basic local alignment search technique (BLAST) approach. Bioedit Sequence Alignment Editor software version 7.0.5.1 was used to analyze the electropherograms, or DNA sequencing data, after they had been aligned with normal sequences obtained from the Gene Bank.

Sequence Analysis. The Basic Local Alignment Search Tool (BLAST) in Genebank on the NCBI website was used to examine sequence similarities. The MEGA X software's Maximum Likelihood was used to build phylogenetic trees. To construct the evolution distance, a 1,000 bootstrap replication was employed. The obtained 16S rRNA sequences were compared with some commonly found bacteria in the oral cavity, such as *Streptococcus mutans* strain ATCC 25175 (NR042772), *Streptococcus sanguinis* SK1 strain NCTC 7963 (NR024841), *Streptococcus mitis* strain NS51 (NR028664), *Streptococcus sobrinus* strain ATCC 33478 (NR042773), *Levilatobacillus brevis* strain Tt *L. brevis* (MZ061639), *Lactobacillus acidophilus* strain L (MT152632), *Lactobacillus salivarius* L-ID15 (EF140741), *Klebsiella pneumoniae* strain SAVQ3 (OM877351), and *Klebsiella pneumoniae* subsp. rhinoscleromatis strain GB6-RFS1 (MW629889).

RESULTS

Gram Stain. Gram staining of the selected colonies showed that the cell colour was stained red, which means the bacteria were Gram negative. The coloured cell shape is rod-shaped as shown Figure 1.

Hemolytic Testing. Growth of dental caries samples streaked on Blood Agar media showed γ hemolytic results. γ hemolytic is characterised by the absence of lysis or breakdown of blood cointained in the media (Figure 2).

DNA Amplification. After staining, selected colonies were extracted and amplified by PCR method. PCR results visualised by electrophoresis were compared with marker DNA. The target DNA band was 1,200 base pair (Figure 3).

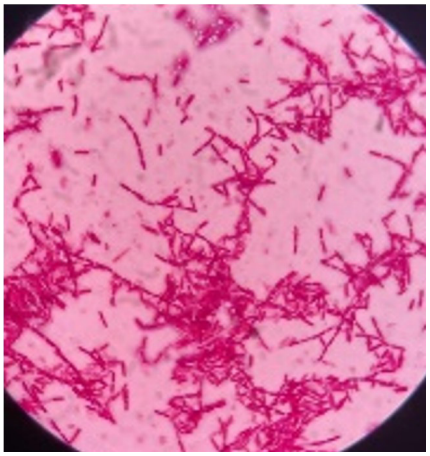

Figure 1. Micromorphology observation: cell shape and gram staining

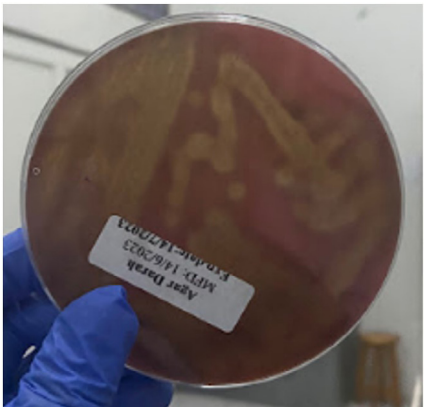

Figure 2. γ hemolytic testing in blood agar media

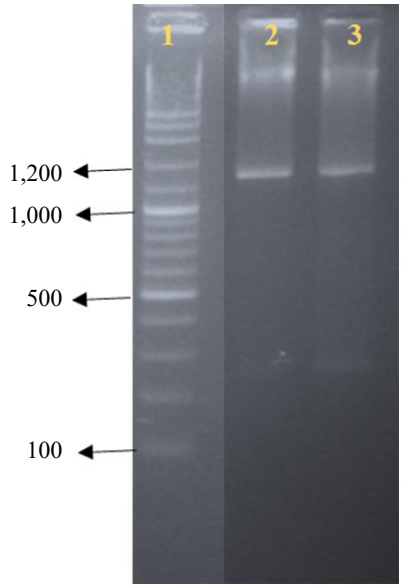

Figure 3. Visualization of the amplification product of the 16S rRNA gene: column 1) marker: 100 bp DNA ladder, 2) isolate S9.4, 3) isolate S13.2.

DISCUSSION

Colonies that grow on Blood Agar media have characteristics: small colonies, circles, and γ hemolytic (Figure 2). The results of Gram staining confirmed that isolates S9.4 and S13.2 were Gram-negative (Figure 1) with a chain coccus cell shape. Other characteristics from these samples (data on other publications) show *Streptococcus* sp. and (Endriani *et al.* 2021) found *Streptococcus* sp. in the largest dental caries patients. Similar results were shown by Karpinski and Szkaradkiewi (2013) discovered that cariogenic bacteria especially lactic acid bacteria like *Lactobacillus* spp. and oral streptococci such group mutans are essential to the development and progression of dental caries. *Streptococcus mutans* is widely regarded as the primary instigator of caries, particularly enamel decay. Additionally, *Lactobacillus* species contribute significantly to the advancement of caries, particularly within dentin. The carious process is further exacerbated by the fact that lactobacilli and mutans streptococci can both grow in acidic environments and quickly convert dietary carbohydrates into organic acids, particularly lactic acid.

The dominance of *Lactobacillus* in caries patients was also found by (Obata *et al.* 2014), clustering of bacterial communities resulted population of *Lactobacillus* being the most prevalent, followed by *Olsenella, Propionibacterium, Atopobium*, and *Prevotella* obtained from adult patients over 30 years old. The significant capacity of *Lactobacillus* species to generate organic acids implies their involvement in dentin deterioration. Conversely, the specific capability of *Lactobacillus* to degrade organic compounds, particularly collagen, remains unexplored. Recent proposals suggest that host matrix metalloproteinases (MMPs) may be crucial in breaking down the organic matrix of demineralized dentin, possibly activated by bacterial acids. Slightly different from infants, (Zeng *et al.* 2020) reported that there are several species of bacteria in the oral cavity of children, including *S. mutans, Staphylococcus epidermidis, Klebsiella quasipneumoniae, Klebsiella pneumoniae, Enterobacter kobei, Enterococcus faecalis, Staphylococcus hominis, Streptococcus anginosus*, and *Phytobacter*. In addition to being frequently detected in children's oral cavities, *E. faecalis, E. coli, Enterobacter cloacae, K. pneumoniae, S. epidermidis,* and *S. haemolyticus* are typical microorganisms in an infant's gastrointestinal system.

The most prevalent condition in kids is called Early Childhood Caries (ECC). According to (Neves *et al.* 2017) *L. casei* and *Bifidobacterium* spp. are commonly found in ECC lesions, where they play important roles in lowering pH levels in active lesion habitats and flourishing in acidic carious circumstances. When children with deeper dentinal lesions at any other place, *Veillonella* sp. was discovered in addition to *L. casei* and *Bifidobacterium* spp. Propionic and acetic acids, which are both weaker than lactic acid and less damaging to dental enamel, are produced by Veillonella using lactic acid produced by other species. Additionally, by acting as a reservoir for the lactic acid generated, Veillonella has been demonstrated to improve glycolysis in *S. salivarius*. According to (Becker *et al.* 2002), these results imply that Veillonella's use of lactic acid may be essential to the caries process, shielding the acid-producing bacteria.

Another study conducted by Rani *et al.* (2016) shows that the cause of dental caries is not only caused by one microbe but by several microbes that affect each other, including *E. hormaechei, Enterobacter sp., M. luteus, A. radioresistens, Exiguobacterium sp., W. confusa, S. sciuri, K. pneumoniae, B. cereus*, and *B. subtilis*. Because there are so many species of bacteria in dental caries, in this study researchers compared *Klabsiella* with other species that cause dental caries (*S. sanguinis, S. mitis, S. salivarius, S.mutans, S. sobrinus, L. salivarius, L. acidophilus, L. brevis, Veillonella parvula, B. dentium,* and *B. adolescentis*). This was done to determine the similarity between the species that cause dental caries in general compared to the species that cause dental caries in patients who gargle with peat water.

The results of molecular tests with 16S rRNA gene markers show that both test isolates have high similarity to *Klabsiella pneumoniae*, which is 90.83-93.18% (Table 1). This result is different from the suspected species based on morphological tests. When an isolate shares more than 99% of its sequence with similar strains in GenBank, it is generally considered to be of the same species (De Silva *et al.* 2016); (Malviya *et al.* 2000). More research is required

Table 1. Analysis of sequence similarity accessed from GenBank database

Isolate	Nucleotide length	Identical species	Query cover (%)	Max score	Similarity (%)
S9.4	873	*Klabsiella pneumoniae* subsp. *Rhinoscleromatis* strain GB6-RSF1_C01.ab1	19	193	93.18
S13.2	873	*Klabsiella pneumoniae* strain SAVQ3	87	464	90.83

because the sequence similarity was less than 97% (Rani *et al.* 2016) discovered 99% commonality between *Klabsiella pneumoniae* from caries samples. In addition, *K. pneumoniae* was also found together with *Staphylococcus sciuri* and *S. albus*.

Opportunistic microorganisms that have the ability to cause acute infections can reside in human oral and nasal passageways. The *Klebsiella* genus stands out among these pathogens due to its frequent correlation with nosocomial infections and multidrug resistance. All of these data suggest that oral opportunistic pathogens, such as *Klebsiella* species, have a greater capacity to tolerate longer periods of time than other oral and nasal microbial populations when they are starved. Numerous studies on oral *Klebsiella* as an opportunistic pathogen have been carried out in mice, showing that oral *Klebsiella* species from both humans and mice can move to the mouse gut and cause a variety of inflammatory reactions, including colitis (Atarashi *et al.* 2017; Kitamoto *et al.* 2020).

K. pneumoniae has been reported to be present in human oral cavities on multiple occasions. Whether *K. pneumoniae* is a part of the typical oral flora is still unknown, though. 10.0% of the 30 saliva samples had *K. pneumoniae* subsp. pneumoniae present (Tsuzukibashi *et al.* 2023). Pneumonia infections are closely associated with *K. pneumoniae* virulence factors. Most of the eight non-*S. mutans* bacteria that were recovered from Mitis Salivarius Bacitrasin (MSB) are thought to be migratory species, potentially impacted by the distinct features of newborn innate immunity (Zeng *et al.* 2020). The significant reduction in *K. pneumoniae* growth is observed when H2 generation is inhibited. These findings imply that H2 generation and/or the mitigation of oxidative stress play crucial roles in the survival and proliferation of *K. pneumoniae* within the oral cavity (Kanazuru *et al.* 2010). If further research isto be done to characterize *K. pneumoniae*, this H2 reductation test can be performed.

In this study, both similarity and distance parameters placed the isolates S9.4 and S13.2 in the same clade with *Klebsiella pneumoniae* (Figure 4). This shows their phenotypic similarity, both also have similarities with commonly found bacteria in the oral cavity, such as *Streptococcus mutans, Streptococcus sanguinis, Streptococcus mitis, Streptococcus sobrinus, Levilatobacillus brevis, Lactobacillus acidophilus*, and *Lactobacillus salivarius*.

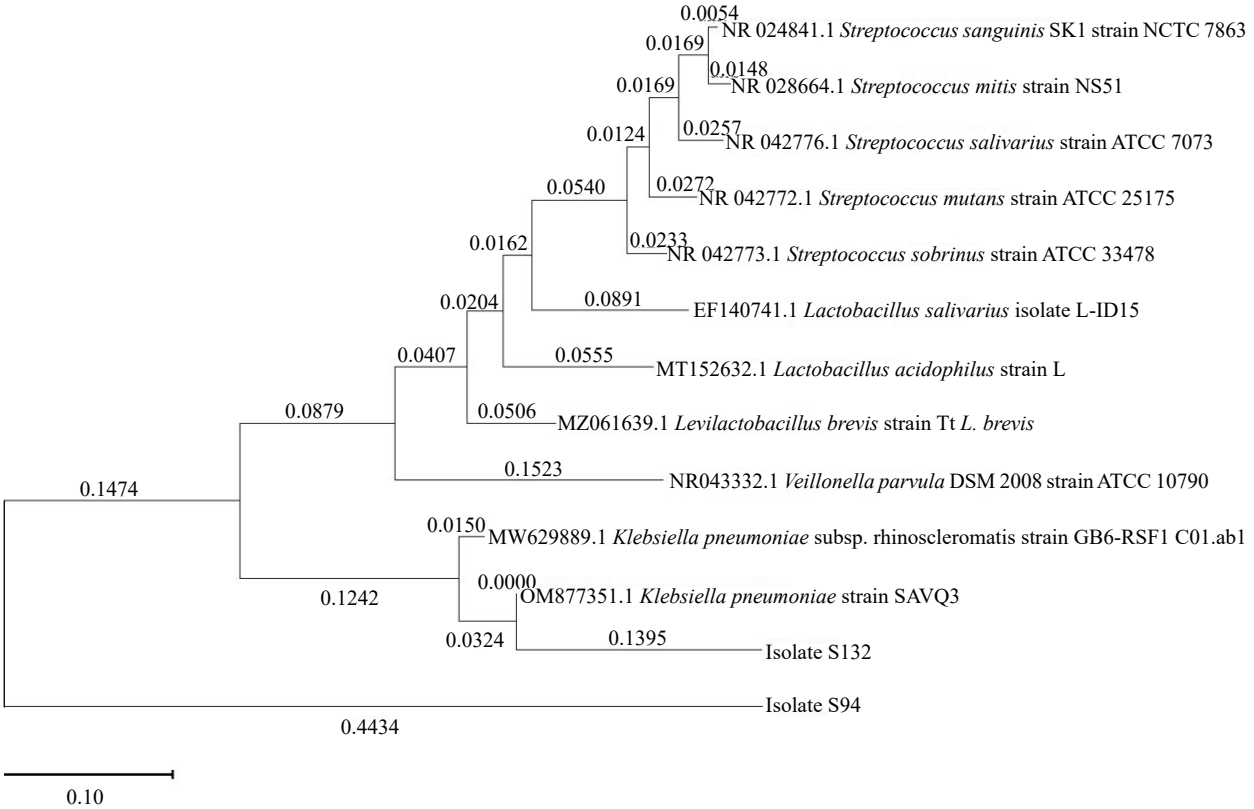

Figure 4. Similarity index of isolate S9.4 and S13.2 with other commonly found bacteria in oral cavity. Using 1,000 bootstrap replication and the Maximum Likelihood technique in MEGA X, the phylogenetic tree was built

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