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Identification and Screening of Pathogenic Microbes in Oral Smokers

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ABSTRACT

Smoking has been shown to adversely affect the oral cavity by altering its physiological conditions, thereby promoting the growth of pathogenic microorganisms and increasing the risk of oral diseases. It also reduces salivary pH, rendering it more acidic, and facilitates plaque accumulation. This study aimed to identify the presence of pathogenic microorganisms (*Staphylococcus* spp., *Candida* spp., and Entamoeba gingivalis) in the saliva and oral cavity of individuals who smoke. An analytical survey with a cross-sectional design was conducted using 40 samples. Microbial identification was carried out through culture techniques on specific media for bacteria and fungi, while *E. gingivalis* was detected via direct microscopic observation using safranin staining. 40 samples were analyzed and showed 31 tested positive for at least one of the target microorganisms: *Staphylococcus* spp., *Candida* spp., or *E. gingivalis*, from both saliva and oral swab specimens. *Staphylococcus* sp. and *Candida* sp, detected in 13 samples. *E. gingivalis* was identified in 7 samples. Saliva samples yielded more comprehensive results than oral swabs, suggesting that saliva more accurately reflects the overall microbial condition of the oral cavity.

Keywords
Microscopic Observation
Pathogenic Microbes
Safranin Staining
Saliva
Smokers

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Introduction

The oral cavity is one of the human organs, and it is under very complex conditions. The teeth, gums, tongue, and saliva in the mouth make it one of the favorite habitats for various microorganisms. The normal flora in the oral cavity is called the oral microbiome. An oral microbiome is a collection of genomes in microorganisms in the oral cavity [1]. The oral microbiome also plays a role in local and systemic inflammation, which can cause various diseases because the mouth is the main gateway for the entry and development of pathogenic microbes [2].

Smoking can lead to changes in oral conditions, increasing the prevalence of pathogenic microorganisms and oral diseases. Microbes in the oral cavity coexist with each other, both pathogenic and non-pathogenic, living together to maintain homeostasis [3]. Ingredients of cigarettes, such as tar and nicotine, can settle on the surface of the teeth, gums, and tooth roots, causing the surface to become rough and making it easier for microorganisms to attach and form plaque [2]. Periodontal disease, caries, tooth loss, gingival recession, precancerous lesions, oral cancer, and implant failure are cases that can arise due to smoking habits [4].

Cigarettes also cause the pH of saliva to become more acidic. The ability of saliva as a buffer and acid neutralizer is not optimal, so microorganisms become unbalanced between normal flora and pathogens. Recent studies have shown that smoking affects the oral microbiome, including creating an anaerobic environment, damage to host immunity, changes in oral salivary pH and oral bacterial adhesion, and the antibacterial effects of toxic substances in cigarette smoke [3]. This disruption makes it easier for pathogenic microbes to multiply. Pathogenic microbes commonly found in the oral cavity of smokers include *Haemophilus influenzae, Candida albicans, Streptococcus pneumoniae,* and organisms of non-oral origin such as *Pseudomonas aeruginosa, Staphylococcus aureus* and and *Klebsiella pneumoniae* [5]. This increase is caused by exposure to cigarette smoke in public places such as restaurants, households, government buildings, workplaces, public transportation, and even in health care facilities. Another statistic showed that person in upper 15 years start smoking [6]. It's bad for their stability microflora. Many studies report that pathogenic microbes in the oral cavity are limited to bacteria and fungi. A study reported the presence of *S. aureus* in active smokers in Paron Village, Kediri [7].

The number of smokers in developing countries has reached more than 800 million people, almost three times that of developed countries [8]. Around 80,000 teenagers in the world are addicted and addicted to cigarettes. If this pattern continues, around 250 million children living today will die from diseases caused by the effects of smoking. Indonesia is ranked 5 in world cigarette consumption. East Sak-sak is an area in Bangkalan District, East

Java, with many smokers accounting for around 80% of the population in East Sak-sak.) the number of patients who came for treatment due to respiratory complaints reached 60% complaintss of dental and oral diseases rose 29.3% and covering both reached 38% [9]. So the purpose of this study is to determine pathogenic including bacteria, fungi and protozoa in oral smokers at Sak-Sak, Bangkalan.

Material and Methods

A. Research Design

This study is an analytical survey study with a cross-sectional design. The study aims to determine several microorganisms (fungi, bacteria, and protozoa) present in the oral saliva of smokers with several specific tests for each type of microorganism. This research has been tested for ethical feasibility: No. 2319/KEPK/STIKESNHM/EC/VII/2024.

B. Population and Sample

This sampling technique used purposive sampling. The population in this study was residents of East Sak-sak. All residents of East Sak-Sak who met the inclusion and exclusion criteria were included, resulting in a final sample of 40 participants. The inclusion criteria in this study were active smokers who had smoked for more than five years, with a daily cigarette consumption of more than four cigarettes/day. It was of productive age (25-40 years). Excluded criteria were active smokers had undergone dental treatment, had consumed antibiotics, antifungal or immunosuppressive theraphy within the last two weeks, had accute oral infections or active oral lessions, had a story of systemic diseases (Diabetes Mellitus, Autoimmune disorders or immunodeficiency) based on medical examination within the past year.

C. Preparation, Isolation and Identification Sample

This study has two types of samples: oral swabs and saliva. Saliva was taken by providing a sterile sample pot and asking respondents to spit into the sample pot. While for swabs, it was carried out by team representatives swabbing the mucosa and gaps of respondents' teeth using sterile cotton buds and then placed in a sterile pot filled with 0.9% NaCl. All samples were labeled.

Oral swabs in a pot containing NaCl 0,9% were homogenized using a vortex, and then the cotton bud was swabbed on the media to be cultured. Potato Dextrose Agar (MERCK, Germany) media for *Candida* sp. and Mannitol Salt Agar (MERCK, Germany) for *Staphylococcus* sp., Protoza was conducted directly under a microscope using a glass concave object with safranin staining. Examination of all samples was carried out aseptically.

The collected saliva was homogenized using a vortex, and then 1 ml was taken. In examining Fungi and Bacteria was cultured on the media. PDA media for examining *Candida* sp. and Mannitol Salt Agar (MSA) for examining *Staphylococcus* sp. The protozoan examination

was observed directly under a microscope using a glass concave object with safranin staining. Examination of all samples was carried out aseptically.

D. Data analysis.

This study uses descriptive analysis to explain each research variable. This study uses descriptive data with tables in the form of positive and negative and morphological descriptions of the identification of microbes in oral cavity and saliva samples of smokers in Sak-Sak Timur Bangkalan

Results and Discussions

A. Respondent Characteristics

Respondent characteristics are general data describing the frequency of smokers' age, duration of smoking, number of cigarettes consumed per day, and frequency of brushing teeth (Table 1). Based on the data above, it can be seen the category of smoking behavior degree (Table 2) as researched by Zami and Shofi [7] explained the Brinkman Index by classifying the degree of smoking behavior into three categories, namely light, moderate and heavy smokers based on the duration of smoking in years multiplied by the number of cigarettes smoked per day.

Table 1. Respondent characteristics

No	Characteristic		Total (person)	Percentage (%)
1	Age	25-30 tahun	15	37.5
		35-40 tahun	25	62.5
2	Duration of smoking	5 – 10 tahun	10	25.0
		> 10 tahun	30	75.0
3	Cigarettes consumed/day	5 – 10 batang	25	62.5
		>10 batang	15	37.5
4	Frequency of brushing teeth	Once a day	28	70.0
		≥ twice a day	12	30.0

Table 2. Degree of Smoking Behavior

Degree of Smoking Behavior	Number (person)	Percentage (%)
Light smoker	8	20
Moderate smoker	14	35
Heavy smoker	18	45
Total	40	100

B. Identification of Microbes

The oral microbiome, oral microbiota, and oral microflora are microorganisms in the human oral cavity, including bacteria, fungi, eukaryotes, and viruses [2]. The oral microbiome can change rapidly in composition and activity both spatially and temporally, and is dynamic as the host evolves [1]. Smoking is more vulnerable to infectious diseases, decreased arterial perfusion, and neuropathy; moreover, a suppressed immune response intensifies the frequency and severity of infectious diseases [4].

The results of microbial identification in 40 samples showed that 31 samples were positive for pathogenic microbes; forms of bacteria, fungi, or protozoa (Table 3). The other nine samples were overgrown with other microorganisms not included in *Staphylococcus, Candida*, and *Entamoeba gingivalis*. Sample code S is Saliva, and OC is Oral Cavity swab.

Table 3. Microbial Identification Results

No.	Sample Code	Types of Microbes		
		***Staphylococcus* sp.**	***Candida* sp.**	***Entamoeba ginggivalis***
1	S01	+	+	-
2	S03	-	+	-
3	S04	+	+	-
4	S06	-	+	-
5	S09	+	+	-
6	S13	+	-	+
7	S14	+	+	-
8	S16	+	+	-
9	S17	-	-	+
10	S18	+	-	-
11	S19	-	-	-
12	S20	-	+	-
13	S22	-	-	+
14	S29	+	-	-
15	S36	-	-	-
16	S39	-	-	-
17	OC01	-	+	+
18	OC03	-	-	-
19	OC04	-	-	-
20	OC05	-	+	-
21	OC09	+	-	-
22	OC10	-	-	+
23	OC12	-	-	-
24	OC18	+	+	+
25	OC19	-	-	-
26	OC22	+	-	+
27	OC25	-	-	-
28	OC29	-	-	-
29	OC30	+	+	-
30	OC33	-	+	-
31	OC36	+	-	-
Total samples positive		13	13	7
Percentage (%)		39.4	39.4	21.5

Saliva was used for this research because it is an easily accessible and patient-friendly method for diagnosing pathogenic microorganisms. This can be used as a screening tool for potential pathogenic changes before more serious and financially expensive detection methods are applied [10]. Samples from saliva have positive results for *Staphylococcus sp., Candida sp.,* and *Entamoeba gingivalis*. The protozoan *Entamoeba gingivalis* was found less than bacteria and yeast. It is because the protozoan *Entamoeba gingivalis* is not a normal flora. *Entamoeba gingivalis* is a pathogen. Whenever these parasites are found, it is a sign of unhealthy teeth and poor oral care. This parasite is included in the infectious parasites and causes infections.

Diabetes, hypertension, smoking, and heart disease are among other reasons that increase the possibility of periodontitis and gingivitis [11].

C. Identification of Microbes: *Staphylococcus* sp.

Sample positive *Staphylococcus* sp. as many as 13 samples (39,4%) are S01, S04, S09, S13, S14, S16, S18, S29, OC09, OC18, OC22, OC30, OC36. In this study, identification was limited to microscopic and macroscopic observations, so the results could only refer to the genus, not the species. The positive samples have specific microscopic and macroscopic characteristics (Table 4). The results of this study are also in line with Zami and Shofi [7] for macroscopic and microscopic characteristics.

Table 4. Macroscopic and Microscopic Identification of *Staphylococcus* sp.

Type	Shape	Coccus
Macroscopis	Color	White
	Edge	Flat
	Surface	Convex
	Shape	Coccus
Microscopis	Arrangement	In a group
	Color	Violet
	Gram	Positive
	Koagulase	Negative

The presence of *Staphylococcus* sp. is related to the composition of cigarettes and the cleanliness of the oral cavity and teeth. Tar in cigarettes can accumulate and form plaque. The plaque is a place for bacteria to grow [12]. So it can be concluded that the longer you smoke, the more plaque is formed, which can cause the growth of various types of bacteria

D. Identification of *Candida* sp.

Sample positive *Candida* sp. as many as 13 samples (39,4%) are S01, S03, S04, S06, S09, S14, S16, S20, OC01, OC05, OC18, OC30, OC33. The positive samples have specific microscopic and macroscopic characteristics (Fig. 2 and Fig. 3).

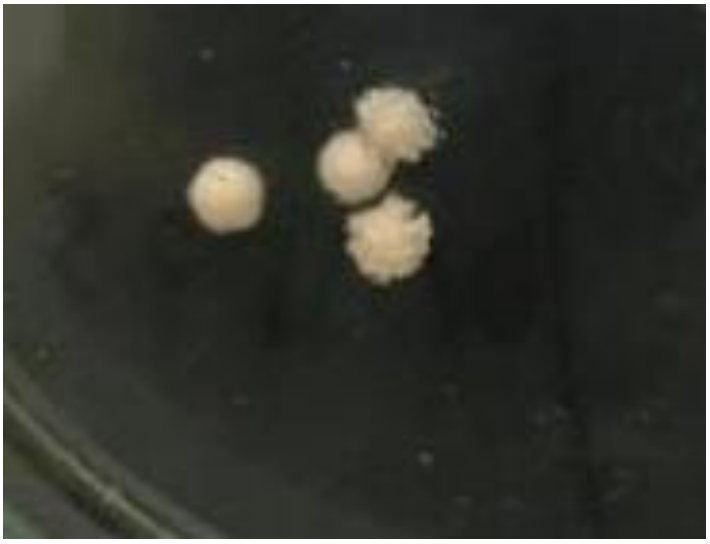


Fig. 2. Colony *Candida* sp. in PDA

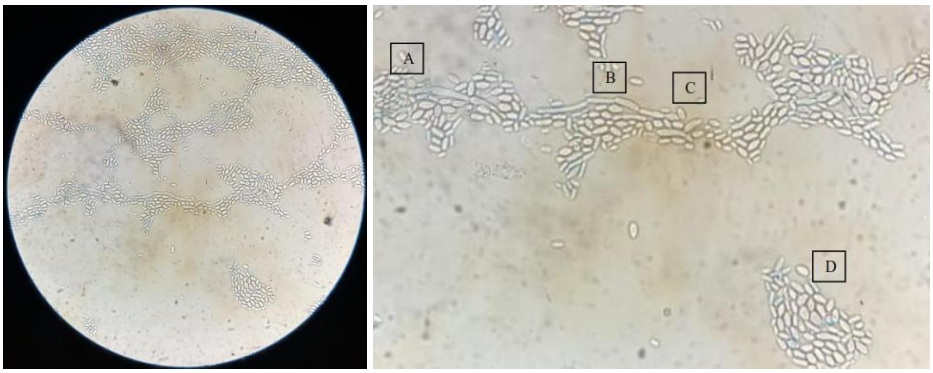


Fig. 3. Microscopic of *Candida* sp. (a) Candida sp. Zooming out showed the colony grows very densely. (b) Parts of *Candida* sp. Cells: A: chlamydospores, B: pseudohyphae, C: blastosphere, D: yeast

Candida sp. and *Staphylococcus* sp. were detected in similar numbers (13 samples positive) because both are opportunistic pathogenic. *Candida* sp. grows well at 39 °C, which allows differentiation between commensal and pathogenic strains. The pathogenic isolates show optimal growth at temperatures above 37 °C due to their ability to adapt and form biofilms [13], [14]. In this study, *Candida* sp. colonies were macroscopically round colonies, slightly raised from the surface, smooth, yellowish white, and smelled like yeast. Microscopically, it has yeast cells, blastospores, and chlamydospores.

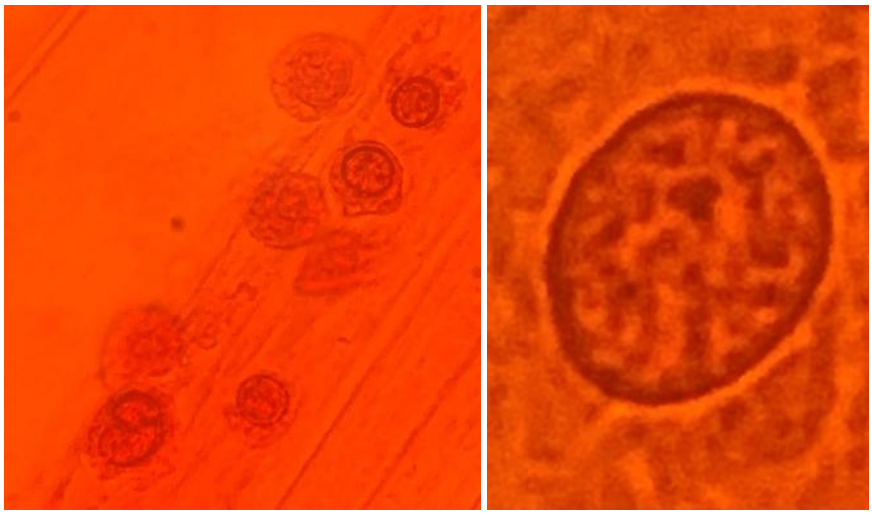
At a temperature of 39 °C, the cell walls and hyphae of *Candida* sp. pathogens are thicker, so this can cause disease in the host. The adaptation of *Candida* sp. pathogens at a temperature of 39°C can also cause *Candida* sp. to adapt to high temperatures in its host [15]. Factors that influence the presence of *Candida* sp. in the mouths of active smokers are that cigarette materials contain toxins that can cause changes in the condition of the oral cavity, such as thickening of the epithelial cell layer, decreased salivary flow rate, and decreased salivary pH. Brushing teeth once a day can show a higher percentage of positive *Candida* sp. According to Gani et al [16], one of the factors that increases the number of *Candida* sp. colonies in the oral cavity is influenced by the duration of smoking. Long-term smoking can cause salivary gland dysfunction, so that the rate of saliva flow can decrease.

A decrease in the saliva flow rate because of smoking can decrease cleaning stage activity, so poor oral hygiene allows *Candida* sp. to increase. In addition, changes in heat due to smoking can cause changes in vascularization and secretion of the salivary glands [4]. Thinning of the mucosal epithelium and decreased salivary secretion cause *Candida* sp. to easily multiply and invade the oral cavity and cause oral candidiasis [17].

Sample positive *Entamoeba gingivalis*: As many as seven samples (21,5%) are S13, S17, S22, OC01, OC10, OC18, and OC22. The positive samples have specific microscopic characteristics. Furthermore, these findings suggest a possible association between smoking-related oral environment changes and the increased presence of opportunistic protozoan and fungal organisms.

E. Identification of Protozoa

Identification of *E. gingivalis* was dependent on its shape, the growth and formation of the pseudopodia, and the presence of vacuoles [11]. In this research, the trophozoites of Entamoeba gingivalis can be seen in Fig. 4, which has the following characteristics: round shape and a nucleus with a karyosome in the middle and a small endosome.



(A) (B)

Fig. 4. *Entamoeba ginggivalis* (400x magnification). (A) Tropozoid phase (B) Tropozoid phase has a karyosome in the middle and a small endosome. The protozoan *Entamoeba gingivalis* was found in the trophozoite phase. The trophozoite phase in protozoa is unique, so by observing the trophozoite alone, we can determine the species' name [18].

The risk of decreased salivary function increases with the duration of smoking and the number of cigarettes smoked per day. In people who smoke and are routinely exposed to cigarette smoke every day, this certainly has an impact on decreasing antibodies in saliva, thereby decreasing the oral cavity defense system, causing the mouth area to be susceptible to infection, especially by anaerobic bacteria [19].

Conclusion

Based on the results, it can be concluded that 31 positive samples were found for *Staphylococcus* sp., *Candida* sp., and *Entamoeba gingivalis* from both saliva samples and oral swabs. The highest percentage of results was for *Staphylococcus* sp. and *Candida* sp., each with 13 positive samples. At the same time, seven samples were positive for *Entamoeba gingivalis*. Saliva samples provide better results than oral swabs because saliva can represent the overall condition of the oral cavity. Smoking habits and oral hygiene greatly influence the results.

Limitations

This study has several limitations. The sample size was relatively small (n = 40) and all participants were recruited from a single geographic area (East Sak-Sak). Therefore, the

findings should be interpreted as preliminary and may not be generalizable to broader populations. Larger, multi-site studies are needed to confirm these results.

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Conflict of Interest

The authors declare no conflict of interest

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