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Morphological Identification and Colony Count of Bacteria in Residual Root Pulp for Supporting Human DNA Examination

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Abstract

Necrotic root canal residual pulp provides a confined environment with limited oxygen availability that favors bacterial colonization. The presence of bacteria, particularly anaerobic and Gram-negative species, has the potential to reduce the quality of human DNA through enzymatic degradation or co-extraction of bacterial DNA during the isolation process. Therefore, information regarding bacterial characteristics within residual pulp tissue may serve as important supporting data in human DNA examination. This study aimed to identify bacterial morphology and quantify bacterial colony numbers in root canal residual pulp as supplementary information for human DNA analysis. This analytical observational study employed a cross-sectional design involving sixteen root remnant samples. Bacterial morphology was examined using Gram staining, while colony enumeration was conducted using the spread plate method on Mueller Hinton agar. Colony forming units per milliliter were calculated using a colony counter, followed by descriptive analysis. All samples demonstrated the presence of Gram-positive bacteria, whereas Gram negative bacteria were detected only in several samples. Rod-shaped bacteria were predominant, alongside cocci and mixed bacterial forms. The mean colony count was 108.94 CFU, indicating a consistent and substantial degree of bacterial colonization within necrotic root canal residual pulp. These findings suggest that necrotic residual pulp predominantly harbors Gram positive rod-shaped bacteria, and the bacterial load observed may have implications for the integrity and reliability of human DNA examination.

Keywords: Root canal residual pulp; Bacterial morphology; Gram staining; Colony count; CFU; Human DNA analysis

1. Introduction

Human identification in forensic science plays a crucial role, particularly in disaster victims, fire incidents, mutilation, and advanced decomposition cases where soft tissues can no longer be used as biological evidence. Deoxyribonucleic acid (DNA) analysis is considered one of the most reliable identification methods because each individual possesses unique DNA characteristics. However, under extreme conditions such as high temperatures or severe decomposition, DNA from soft tissues often degrades, making it unsuitable for examination; therefore, a more stable DNA source is required [1]. Teeth represent the hardest tissue in the human body and are chemically resistant to degradation and high temperatures, making them highly valuable in identifying burned or fragmented remains [1,6]. Their high hydroxyapatite content provides superior environmental resistance compared to bone, resulting in more stable DNA preservation [16]. Among dental components, the pulp contains the highest DNA concentration and is protected by enamel and dentin, making it a promising forensic DNA source [3,10].

This condition forms a complex anaerobic microbial ecosystem that may directly interact with DNA [5]. Microorganisms may co-extract with human DNA during isolation, generating complex mixtures and potentially producing artifacts that interfere with genetic profiling, including Short Tandem Repeat (STR) interpretation [3]. Additionally, bacteria can

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produce enzymes that damage DNA and degrade organic tissue [7]. Studies have also reported that most bacteria in necrotic root canals are anaerobes [15,8].

Given the high prevalence of severe untreated dental disease in Indonesia, with caries prevalence reaching 82.8% [17], identifying bacterial morphology and colony counts in residual root pulp is important to understand their potential influence on the success of forensic human DNA identification.

2. Material and methods

2.1. Sample Collection

Samples in this study were obtained from extracted residual tooth roots at the Dental and Oral Hospital, in accordance with medical indications for tooth extraction. Prior to extraction, patients underwent periapical radiographic examination to evaluate the presence of root resorption and to detect possible periapical lesions [12]. After extraction, the residual tooth roots were immediately wrapped in sterile gauze, placed into tubes containing 10 ml of thioglycolate transport medium, and transported to the Research Center, Faculty of Dentistry, Universitas Airlangga under controlled conditions at 4 °C using a cooler box for subsequent bacterial isolation [9].

2.2. Bacterial Isolation from Residual Root Teeth

In teeth presenting as residual roots, the initial procedure involved inserting a sterile paper point size No. 15 into the root canal for approximately 60 seconds to absorb fluids and microorganisms present within the canal [15]. In certain cases where the root canal exhibited attrition or narrowing (atretic), making paper point insertion difficult, a No. 10 K-file was used to create an access pathway to facilitate optimal penetration. After sampling, the paper point was immediately placed into a sterile tube containing 1 mL of thioglycolate transport medium to maintain bacterial viability. The tube was then incubated for 24 hours at 37 °C to allow subsequent culture and microbiological analysis [14].

2.3. Bacterial Morphology Identification

For bacterial morphology identification, samples were first introduced into Brain Heart Infusion (BHI) medium by immersing them into tubes containing the medium. The tubes were then incubated at 37°C for 24 hours [14].

The next stage involved inoculation onto Mueller Hinton (MH) agar using the spread plate technique with a spreader, followed by incubation for 24 hours at 37°C [11]. After incubation, a bacterial colony from the MH agar was taken using an inoculating loop, placed onto a glass slide, evenly spread, air-dried, and heat-fixed.

Gram staining was subsequently performed sequentially, beginning with the application of crystal violet as the primary stain for one minute, followed by rinsing with water. Iodine solution was then added as a mordant for one minute and rinsed again. The slide was then treated with 95% alcohol as a decolorizing agent for one minute, rinsed, and counterstained with safranin for one minute before a final rinse. After drying, immersion oil was applied, and the preparation was observed under a microscope at 100× magnification. Gram-positive bacteria appeared purple due to their thick cell walls retaining the crystal violet–iodine complex, whereas Gram-negative bacteria appeared red because their thinner cell walls were unable to retain the primary stain and instead absorbed the safranin counterstain [10].

2.4. Bacterial Colony Count

Before colony enumeration on Petri dishes, bacterial samples were first diluted using a four-step serial dilution technique. This was performed by gradually mixing portions of the sample with sterile diluent so that the bacterial concentration decreased at each dilution stage [17]. Following dilution, samples from each dilution level were inoculated onto Mueller Hinton (MH) agar using the spread plate technique with a spreader to ensure even distribution of colonies across the agar surface. The plates were then incubated for 24 hours at 37°C. After incubation, the grown colonies were counted using a colony counter. The ideal number of colonies for accurate enumeration ranges between 30 and 300 colony-forming units (CFU) per plate to ensure valid and representative results [12].

3. Results and discussion

3.1. Results of Bacterial Morphology Identification in Residual Root Pulp Using Gram Staining

In this study, an analysis of bacterial morphology in residual root pulp was conducted based on Gram staining results. Descriptive analysis was performed by differentiating bacterial morphology according to staining color and structural form. The microscopic observation findings of bacterial morphology are presented in the following table.

Table 1 Results of Bacterial Morphology Identification Based on Gram Staining

Sample	Gram Positive	Morphology Form	Gram Negative	Morphology Form
PP 1	+	Cocci	-	-
PP 2	+	Cocci	-	-
PP 3	+	Rods, Cocci	-	-
PP 4	+	Rods	+	Rods
PP 5	+	Rods	-	-
PP 6	+	Rods	-	-
PP 7	+	Rods	-	-
PP 8	+	Cocci	+	Rods
PP 9	+	Rods	-	-
PP 10	+	Cocci	-	-
PP 11	+	Rods	-	-
PP 12	+	Rods	-	-
PP 13	+	Rods	+	Rods
PP 14	+	Rods	-	-
PP 15	+	Rods	-	-
PP 16	+	Cocci	-	-

Based on the identification results of sixteen residual root pulp samples, variations in bacteria were observed according to Gram staining characteristics and morphological forms. All samples demonstrated the presence of Gram-positive bacteria, as seen in PP1 to PP16, although Gram-negative bacteria were also identified in several samples, namely PP4, PP8, and PP13. These findings indicate that Gram-positive bacteria were present in all samples, while Gram-negative bacteria were only detected in three samples.

In terms of morphology, the samples showed variations in bacterial forms, including cocci, bacilli, and combinations of both. Bacilli were the most frequently observed morphology, found in PP3, PP4, PP5, PP6, PP7, PP9, PP11, PP12, PP13, PP14, and PP15. Cocci were identified in PP1, PP2, PP3, PP8, PP10, and PP16. In addition, some samples demonstrated more than one morphological form, with both bacilli and cocci observed in PP3.

3.2. Results of Bacterial Colony Count in Residual Root Pulp

Descriptive statistical analysis was carried out to provide an overview of the distribution and characteristics of bacterial colony counts in each residual root pulp sample. The calculation included the minimum, maximum, mean, median, range, and standard deviation values to determine data variation. The results of the descriptive statistical analysis are presented in the following table.

Table 2 Results of Bacterial Colony Count

Statistic	Value
Sample Size	16
Mean	108.94
Standar Deviation	13.02
Minimum	85
Median	108.5
Maximum	136

Based on the table, it can be seen that the bacterial colony count data show a reasonable variation with a minimum value of 85 and a maximum value of 136. The mean value of 108.94 and the median of 108.5 indicate that the data distribution is symmetrical, while the standard deviation of 13.02 demonstrates a moderate level of variation among samples. Overall, this descriptive analysis shows that the bacterial colony counts in residual root pulp samples have a relatively uniform distribution pattern and do not indicate any substantial differences between samples.

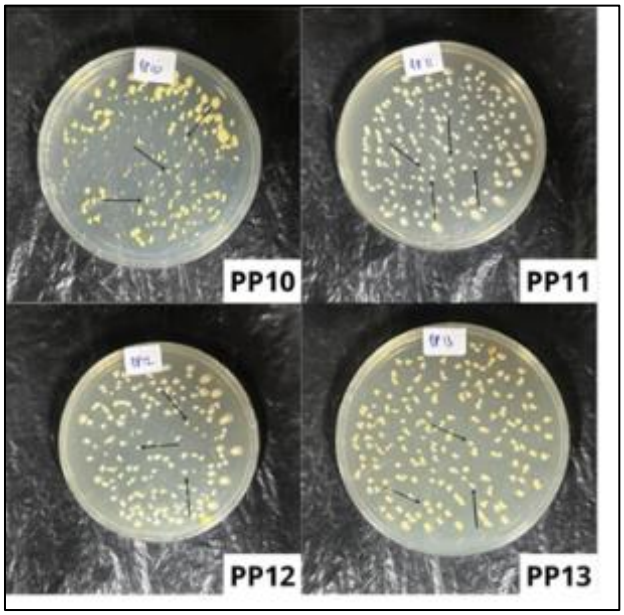

Figure 1 Results of the bacterial colony count in several root remnant pulp samples

This study was an observational investigation aimed at identifying the morphology and colony counts of bacteria in residual root pulp. The results showed a dominance of anaerobic Gram-positive bacteria, while anaerobic Gram-negative bacteria were detected only in a few samples, likely influenced by the isolation methods and culture conditions that did not fully support anaerobic growth. The dominance of Gram-positive bacteria is associated with their ability to adapt to the root canal environment characterized by limited nutrients, low oxygen, acidic conditions, and a thick peptidoglycan cell wall, whereas Gram-negative bacteria require optimal anaerobic conditions to thrive [4].

In terms of morphology, the identified bacteria included rods, cocci, or a combination of both within a single sample. Rod-shaped bacteria were the most prevalent, followed by cocci, reflecting a polymicrobial infection capable of forming biofilms and surviving unfavorable environmental conditions. Species such as *Fusobacterium nucleatum*, *Prevotella intermedia, Porphyromonas gingivalis, Lactobacillus spp., Streptococcus mutans, Streptococcus anginosus, and Enterococcus faecalis* demonstrated anaerobic adaptability, proteolytic activity, and contributed to periapical inflammation and tissue degradation [1].

The mean colony count of 108.94×10^4 CFU reflects normal colonization in necrotic pulp and a bacterial community that has stably adapted. Variations in colony counts among samples indicate heterogeneity in the microenvironment,

influenced by local hypoxia, mature biofilm formation, and host immune responses. Although bacterial degradation activity had not reached maximal levels, these findings indicate that human DNA in residual pulp remains potentially recoverable with adequate integrity. Overall, the results highlight that residual root pulp harbors a complex microbiological ecosystem with Gram-positive dominance, diverse morphology, and heterogeneous colony distribution [12].

4. Conclusion

This study showed that the residual root pulp contained an average bacterial colony count of 108.94×10^4 CFU, with bacterial morphology predominantly consisting of cocci and bacilli. All samples demonstrated Gram-positive bacteria, while Gram-negative bacteria were observed in only a portion of the samples. Therefore, these findings indicate that residual root pulp can be used as supporting data for human DNA examination.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Statement of ethical approval

This study was reviewed and approved by the Health Research Ethical Commission, Faculty of Dental Medicine, Universitas Airlangga. All research procedures were conducted in accordance with applicable ethical standards, and the use of extracted residual tooth root samples was performed based on medical indications. The confidentiality of patient data was strictly maintained throughout the study.

Statement of informed consent

Informed consent was obtained from all participants prior to sample collection. Participants were provided with a clear explanation regarding the purpose and procedures of the study, and agreed to the use of their extracted residual tooth root samples for research purposes. All participants' identities were kept confidential, and no personal identifying information was disclosed.

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