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Molecular diagnosis of nitrate reducing bacteria in healthy oral cavities

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ABSTRACT
Background: The oral cavity harbors a highly diverse microbial community, within which nitrate-reducing bacteria (NRB) constitute a functional core group contributing to microbial balance and systemic health through nitric oxide (NO) production. Objectives: The present study aimed to detect and characterize NRB from the oral cavities of healthy individuals using a selective molecular approach. Methods: A total of ten oral samples were collected and cultured on Brain Heart Infusion (BHI) agar prior to DNA extraction. A species-specific primer targeting Rothia mucilaginosa, a well-documented oral NRB, was designed and applied in PCR assays. Results: The analysis revealed the presence of Rothia mucilaginosa in one positive sample (10%). Although the prevalence was limited compared to previous studies reporting broader NRB diversity, the detection of Rothia mucilaginosa underscores its ecological relevance in nitrate reduction within the oral environment. Conclusion: Collectively, these findings demonstrated the feasibility of selective molecular detection, highlight Rothia mucilaginosa as a potential biomarker for oral and systemic health, and emphasize the need to expand primer panels in future research to capture the full diversity of oral nitrate-reducing bacteria.
Keywords: ***Nitrate-reducing bacteria, Oral microbiota, Molecular identification***

1. INTRODUCTION

Recent developments in high-throughput sequencing technology, along with an increase in microbiological research, have led to in an increasing number of studies investigating the role of microbiota in systemic health and the underlying mechanisms of action [1]. Within this context, the oral microbiome has been recognized as a tremendously various community comprising up to one thousand microbial species, consisting of bacteria, fungi, viruses, archaea, and protozoa that colonize the oral cavity [2]. The concept of the core oral microbiome has largely been limited to bacteria because of their numerical predominance [3]. Consequently, maximum investigations have primarily centered on the bacterial issue, while fungi, archaea, protozoa, and viruses have acquired comparatively much less attention. In terms of health, the oral microbiome represents balanced and dynamic surroundings that contributes to retaining oral and systemic homeostasis [4]. However, disturbances in this stability had been associated with the onset of each oral and systemic diseases, such as diabetes and cardiovascular situations [5-8]. More than 700 species of bacteria have been identified in the oral cavity, meaning it has the second largest and most diverse microbiota after the gut [9]. The oral microbial community has been thoroughly described by earlier research [10-12]. Oral nitrate-reducing bacteria (NRB) are of special importance among these. The majority of NRB are facultative anaerobes [12]. They are necessary to the nitric oxide (NO) production procedure through the nitrate–nitrite–NO pathway that helps maintain the metabolic homeostasis of the oral environment and systemic health [1], [13], [14]. *Rothia, Veillonella, Corynebacterium, Haemophilus*, and are among the genera that are commonly found in healthy people [10], [11], [15]. which consistently demonstrated that NRB is a crucial functional group

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in preserving microbial equilibrium and promoting host health. The diagnostic and therapeutic potential of these methods for upcoming clinical applications is highlighted by this integrative framework, which also improves the accuracy of microbial community profiling. Therefore, the current study uses species-specific primers for molecular validation to characterize oral nitrate-reducing bacteria in samples from healthy volunteers. To provide a precise diagnostic tool and expand our knowledge of the function of NRB in oral and systemic health, this methodology aims to directly connect functional activity and genetic identification.

2. MATERIALS AND METHODS

1.1.Sample collection:

Oral samples were taken in the morning from 10 healthy youths (5 females and 5 males) using sterile cotton swabs, and they were then soaked in sterile PBS.

1.2. Isolation of nitrate reducing bacteria:

A 10 samples suspended in PBS was streaked onto BHI agar plates to isolate a unique colony. Plates were incubated at 37°C for 24-48 hours in a situation that was aerobic. The colonies were then moved to new BHI agar plates and were put again within the incubator for more purity. Colonies have been installed in 5 mL of liquid BHI and stored in the identical conditions. After incubation, 1 mL of the lysate was blended with sterile glycerol (25%) to create long-time-period inventory cultures of each isolate for the next molecular characterization.

1.3. Extraction of bacterial genomic DNA:

Colonies selected for DNA extraction were first Gram-stained to confirm purity and Gram reaction. Based on the Gram result (positive or negative), samples were processed following the manufacturer's instructions of Genomic DNA Mini Kit according to FAVORGEN company. The DNA quality was examined using a normal 1% (W/V) agarose and 95 V at 35 min and photographed under UV light.

1.4.Primer design:

The primer of 16S rRNA was designed using NCBI-Genbank and primer3 software (http://www.embnet.sk/cgi-bin/primer3_www.cgi) and synthesized commercially http://dna.macrogen.com/main.do. as in Table (1).

Table (1): The PCR primer of 16S rRNA gene of *Rothia mucilaginosa*.

Bacterial strain	Accession number	Primers: Forward/Reverse	Length	Tm C°	GC %	Amplicon length
Rothia mucilaginosa	X87758.1	GCAGGGAAGAAGAGAGATTGAC	22	62	50%	371 bp
		ACCTAGTGCCCAACGTTTAC	20	62	50%	

1.5. Polymerase chain reaction master mix preparation:

The master mix (Bioneer, Korea) was used in which, each PCR tube had a final volume of 25µl for the amplification reaction. This included 12.5µl of the master mix, 5µl of

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the DNA template, 4µl of all the primers (2µl of the forward primer and 2µl of the reverse primer), and 3.5µl of nuclease-free water as shown in Table: (2).

Table (2):The PCR master mix methodology

component	25 µl reaction volume
Template DNA	5µl
Forward primer 10 pmol/ µl	2µl
Reverse primer 10 pmol/ µl	2µl
Deionized water	3.5µl
Master mix	12.5µl
Total volume	25µl

1.6. Polymerase chain reaction conditions:

PCR amplification was performed with the following program: Initial denaturation: 94 °C for 5 min; 35 cycles of denaturation at 92 °C for 30 s; annealing at 57 °C for 30 s (optimized by gradient PCR); extension at 72°C for 30 s and final extension at 72°C for 5 min. This program's reliability for species-specific detection was confirmed by the consistent production of distinct amplification bands with minimal non-specific products as in Table (3). After 45 minutes of electrophoresis at 95V through a 2.0% agarose gel containing ethidium bromide (1.2 µl), the products were observed and photographed under UV light.

Table (3): Program of PCR thermocycling conditions of 16S rRNA gene.

No.	Steps	Temp.(°C)	Time	No. of cycle
1.	Pre-denaturation	94	5 min.	1 cycle
2.	Denaturation	92	30 sec.	35 cycles
3.	Annealing	57	30 sec.	
4.	Extention	72	30 sec.	
5.	Final extention	72	5 min	1 cycle

3. RESULTS AND DISCUSSION

Ten oral samples were obtained from healthy volunteers and analyzed for molecular characterization utilizing species-specific primers aimed at *Rothia mucilaginosa*. PCR

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amplification detected *Rothia mucilaginosa* in one of the ten samples (10%) showed in Figure (1). Even though the detection rate was low, this finding is in line with earlier studies that showed Rothia to be one of the most common nitrate-reducing taxa in the mouth.The identification of *Rothia mucilaginosa* in this study underscores its ecological significance as a representative oral nitrate reducer and corroborates its functional role in preserving microbial equilibrium and facilitating nitric oxide metabolism. One specific identification of *Rothia mucilaginosa* indicates the specificity of the primer design and the limited sample size. *Rothia* has been consistently identified as a predominant nitrate reducer in the oral microbiome; however, other genera, including *Veillonella* and *Corynebacterium*, have also been emphasized in prior research. The lack of these taxa in the current results does not contradict previous findings; instead, it highlights the selective nature of the molecular approach employed here. Subsequent investigations involving larger cohorts and augmented primer sets are essential for a more thorough comprehension of oral nitrate-reducing communities.

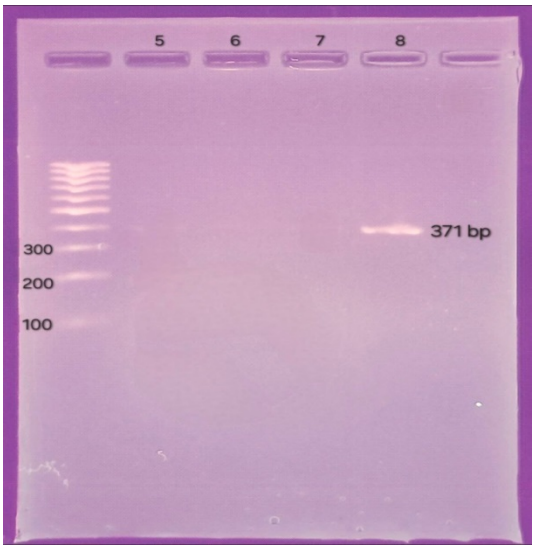

Figure (1): The agarose gel electrophoresis shows that the PCR successfully detected specific primers for *Rothia mucilaginosa* (371 bp), M: DNA ladder (a 100 bp).

The primary objective of this study was to employ molecular techniques to identify and describe nitrate-reducing bacteria in the oral cavity of healthy persons. This approach was selected because molecular methods provide enhanced precision in species identification, avoiding the limitations of culture-based methods influenced by variable growth conditions. Our study samples contained nitrate-reducing bacteria, which shows how crucial they are for preserving the microbial balance in the oral cavity and enhancing human health. The identification of taxa such as *Rothia mucilaginosa* in 10% of samples applying specific primers demonstrated the precision of bacterial isolate identification by PCR, which is consistent with the findings of [10], [11], who emphasized the importance of these species in the conversion of nitrate to nitrite, a vital process in nitric oxide (NO) synthesis. It is generally established what chemicals have antibacterial, vasodilatory, and signaling effects. The nitrate-reducing community offers protective benefits against systemic disorders, especially cardiovascular diseases, while simultaneously promoting ecological stability within the oral microbiome, as demonstrated by [4], [11]. supported this by demonstrating that the disruption of the nitrate-reducing community is associated with hypertension and weakened immune system responses. The presence or absence of certain nitrate-reducing taxa in individuals illustrates the dynamic

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characteristics of the oral microbiome. The lack of some taxa in some healthy people should not be taken as a symptom of poor health but rather as a usual inter-individual variation within the microbial community. This study shows that nitrate-reducing bacteria are a crucial part of the oral microbiome. A molecular-based approach can precisely identify these microorganisms, thereby improving our understanding of their ecological adaptation and clinical significance. These results underscore the beneficial effects of employing nitrate-reducing bacteria as biological mediators to enhance oral and systemic health through nitric oxide pathways.

4. CONCLUSION

This study suggests that the microbiomes of healthy individuals' oral cavities include various microorganisms capable of reducing nitrate. Additionally, this study employs species-specific PCR as a reliable and accurate technique for molecularly identifying these beneficial microorganisms. The results demonstrate the importance of nitrate-reducing microorganisms in preserving both oral and overall health, highlighting that humans can possess unique species of bacteria. To enhance cardiovascular and metabolic fitness, future research should build on these findings by investigating larger populations and utilizing effective enzymatic activity assays to explore the potential of nitrate-lowering microorganisms as organic agents

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Author's Contributions

Both authors contributed equally to achieve this project

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