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Phenotypic and Molecular Identification of *R. dentocariosa* Bacteria from Samples Isolated from the Oral Cavity.

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ABSTRACT

Background

Rothia dentocariosa is a part of the normal flora found in the oral cavity of many humans, which can become opportunistic pathogens in cases of oral infections, diseases, and viral infections such as coronavirus, and other causes that may lead to immunodeficiency in the host.

Material and methods

A total of 120 samples (swabs) were collected from the oral cavity of individuals suffering from gum and dental problems. Phenotypic, biochemical, and molecular tests were conducted on the collected samples to obtain the necessary results.

Results

After culturing and biochemical testing, 25 samples containing the target bacteria were identified. Following the application of PCR technique on the isolates using a specific primer for *R. dentocariosa* and conducting gel electrophoresis, the results showed 11 positive samples out of 25 isolates, with genetic bands at a single molecular level, and a molecular weight of (551 bp).

Conclusions

The DNA sequencing of the positive isolates was conducted, and a phylogenetic tree was constructed to determine their genetic similarity with isolates registered in the NCBI-GenBank.

Keywords: *Rothia; R. dentocariosa*; Phylogenetic tree; PCR technique; NCBI-GenBank.

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INTRODUCTION

The human oral cavity contains various environments suitable for a multitude of microorganisms, including the teeth, gums, tongue, tonsils, and extending to the respiratory tract. The oral cavity ranks second after the digestive system in terms of the diversity and variety of microorganisms present, including bacteria, fungi, archaea, and viruses [1]. The microorganisms that colonize the oral cavity are called the oral microflora, oral microbiota, or oral microbiome [2].

R. *dentocariosa* is a Gram-positive, non-acid-fast, non-spore-forming, non-pigmented, non-hemolytic, and non-motile bacterium. Chemically, it is characterized by a guanine to cytosine (G+C) content in its DNA ranging from 47-53 mol%. The main cellular fatty acids are C15:0 ai, C17:0 ai, C16:0 i, and C16:0. Its peptidoglycan is of type A3x, meaning D-alanine is present at position 4, L-lysine at position 3, and the peptide bridge consists of L-alanine. The polar lipids include diphosphatidylglycerol and monophosphatidylglycerol, with menaquinone containing seven isoprene units. The cell wall sugars are galactose, fructose, glucose, and ribose. This bacterium ferments sugar, and the end products of carbohydrate metabolism are lactic and acetic acids [3,4].

R. dentocariosa is found in the mouth, pharynx, respiratory passages, and often in the duodenum of humans. It can also be found in the air, soil, and some healthy mammals [5,6]. As part of the normal flora, its presence in blood cultures is often considered contamination rather than infection. It is frequently associated with dental caries, gum diseases, and respiratory tract infections [7]. It has also been discovered to cause systemic infections, primarily in hosts with weakened immune systems [8] and those with predisposing conditions such as infections of natural and prosthetic heart valves and intravenous drug users [9].

From a taxonomic perspective, the genus Rothia spp., to which *R. dentocariosa* belongs, was initially classified within the family Actinomycetaceae based on morphological characteristics [10]. Later, based on phylogenetic analysis, it was reclassified into the family Micrococcaceae, which belongs to the suborder Micrococcineae, order Actinomycetales, subclass Actinobacteridae, within the phylum Actinobacteria. This family includes several genera in addition to Rothia, such as Micrococcus, Kocuria, and Arthrobacter, all of which exhibit distinctive nucleotides in their 16S rRNA gene sequences [11,12]. The classification of this species has undergone several changes; it was initially named *Micrococcus dentocariosa.* In 1982, it was reclassified by Bergey and Goker as a species under the proposed genus Stomatococcus. Later, in 2000, during the discovery of *R. nasimurium*, Collins and colleagues proposed reclassifying this species under the genus Rothia based on 16S rRNA gene sequences and cellular protein profile [13] [14].

In the late 1970s, studies began to emerge regarding human pathological cases caused by *R. dentocariosa*, most of which involved patients suffering from endocarditis [15,16]. Despite Kronvall and his research group describing *R. dentocariosa* genomovar II [17], R. dentocariosa remained the only species within the

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genus Rothia for a long time since its discovery by Brown & Georg [18], despite the phenotypic heterogeneity within the species. It was not until Collins and his research group discovered *R. nasimurium* and reclassified *S. dentocariosa* to *R. dentocariosa*[13].

MATERIALS AND METHODS

Preparation of Culture Media

Preparation of Commercially Available Culture Media

The commercially prepared culture media were prepared according to the manufacturer's instructions. They were then sterilized in an autoclave at 121°C and 15 psi for 15 minutes. After sterilization, the media were poured into Petri dishes or tubes as required for the experiment. The media were incubated at 37°C for 24 hours to ensure no contamination. Finally, they were stored at 4°C in a refrigerator until use [19].

Preparation of Synthetic Culture Media

The synthetic culture media were prepared according to the method described by Tille [19] as follows:

A- Blood Agar Medium

The Blood Agar base was prepared according to the manufacturer's instructions by dissolving 40 grams of the medium in 1 liter of distilled water. It was then sterilized in an autoclave, allowed to cool to 45-50°C, and then 5% blood was added. The mixture was well stirred and poured into plates, allowed to solidify at room temperature, and incubated at 37°C for 24 hours to ensure it was contamination-free. The plates were then stored at 4°C in a refrigerator until use. This medium is used to detect the ability of bacterial isolates to produce the enzyme hemolysin [20].

B- Urea Agar Medium

The Urea Agar medium was prepared by dissolving 2.4 grams of Urea Base in 95 ml of distilled water, sterilized in an autoclave, then cooled to 45°C. Afterward, 5 ml of 40% solution (sterilized using filter paper) was added. The mixture was poured into sterile test tubes and allowed to solidify in a slanted position. This medium was used to detect the ability of bacteria to produce the enzyme urease [20].

C- Motility Test

It was prepared by dissolving 0.8 grams of nutrient broth and 10 grams of agar in 90 ml of distilled water, adjusting the pH to 7.0, and then completing the volume to 100 ml. The mixture was sterilized in an autoclave at 121°C for 15 minutes at 15 psi. After cooling, it was placed in sterile tubes and allowed to solidify in a slanted position. This medium was used to determine motile and non-motile bacteria [20].

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Preparation of the Reagents Used

A- Oxidase Enzyme Reagent

This reagent was prepared as described in Forbes and his fellow researchers by dissolving 0.1 g of Tetramethyl-p-phenylene diamine dihydrochloride in 9 ml of distilled water and completing the volume to 10 ml, achieving a final concentration of 1%. The solution was then stored in a sterile and opaque bottle until use [21].

B- Catalase Enzyme Reagent

Prepared by diluting 3% of concentrated hydrogen peroxide (30%) using sterile distilled water to detect the ability of bacterial isolates to produce the enzyme catalase [22].

Isolation Identification

The initial identification of the isolates was based on phenotypic and microscopic characteristics, including the shape, color, and consistency of the colony growing on the selective medium used in the study. Biochemical tests were also employed to identify the bacteria under study and determine their ability to produce oxidase and catalase enzymes. Additionally, the ability of the bacteria to hemolyze blood on Blood Agar medium and grow on the differential Chromogenic Agar medium specific for the genus Rothia spp. was assessed.

Gram Stain Method

Preparation of a Slide Smear

A Loop was used to transfer a small amount of bacterial colony onto a slide with a drop of distilled water. The bacteria were spread with the drop on the slide, then air-dried or quickly passed over a heat source. This procedure helps the sample adhere to the glass slide and prevents its loss during washing[23].

Gram Staining

Crystal violet stain is added to the slide prepared in the previous step. After one minute, iodine solution is added to cover the smear, followed by rinsing the slide with running water. A few drops of de-colorizer, usually a mixture of ethanol and acetone, are then added. The slide is rinsed with water within 5 seconds. Basic function solution is applied for 40-60 seconds, followed by rinsing the slide and air-drying [23].

Microscopic Examination

The slide is examined under the microscope, initially using a 40x magnification lens to ensure that the smear is correctly distributed. Then, the examination proceeds using a 100x lens after adding oil [24].

Biochemical Tests

The biochemical tests were conducted according to MacFaddin [22]:

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A- Catalase Test

This test was conducted by placing a bacterial colony aged 18-24 hours with a drop of 3% hydrogen peroxide (H2O2) on a clean glass slide. The appearance of air bubbles indicates a positive test, showing the production of the catalase enzyme, which breaks down H2O2 into water and oxygen gas.

B- Oxidase Test

A single bacterial colony aged 18-24 hours was transferred using a wooden stick to a filter paper moistened with the oxidase reagent. The appearance of dark purple colonies within 5-10 minutes indicates a positive result.

C- Coagulase Test

It was prepared using the slide coagulase method, a differential test where a bacterial suspension is made from bacterial colonies with a drop of sterile distilled water on a glass slide, ensuring that the suspension is well-mixed with the drop of water on the slide. Then, 0.5 ml of rabbit plasma is added to the suspension and mixed well. The result is considered positive if clumping occurs within 10 seconds .

D- Methyl Red Test

The peptone water medium was used in this test. The purpose was to determine if the bacteria could ferment glucose and produce organic acid, which would lower the pH of the medium to below 4.5. The tubes containing the medium were inoculated with a part of the bacterial colony to be tested and incubated at 37°C for 48 hours. After the incubation period, 5 drops of methyl red reagent were added. The medium turning red indicates complete glucose breakdown and acid production, which is a positive result, while the yellow color indicates a negative result [25].

E- Urease Test

The tubes containing the solid slanted urea medium were inoculated with the bacteria under study and incubated at 37°C for 24 hours. A color change in the medium from yellow to pink indicates a positive test. This test is used to detect the ability of bacteria to produce the enzyme urease, which breaks down urea in the medium, producing ammonia, carbon dioxide, and water.

2.3: Preservation of Isolates

The bacterial isolates used in the study were preserved according to Atlas [26]. Pure bacterial colonies were taken from the Chromogenic Agar medium and inoculated into tubes containing Brain Heart Broth to activate the isolates. The tubes were incubated at 37°C for 24 hours. The isolates were then transferred using an inoculating loop to Brain Heart Agar medium and incubated again at 37°C for 24 hours. The isolates were stored in the refrigerator until use, with periodic renewal and maintenance every 3-4 weeks.

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Molecular Detection of *R.dentocariosa*

The bacterial DNA was extracted from the bacteria under study using a Genomic DNA extraction kit provided by the American company Geneaid, and the extraction was performed according to the company's instructions.

DNA Extracted Purity Examination

The purity and concentration of the extracted DNA were determined using a Nano-drop spectrophotometer. The DNA is detected by measuring its concentration (ng/µl) and its purity by reading the absorbance at wavelengths ranging from 280 nm to 260 nm. The extracted DNA is considered pure when the absorbance ratio is less than or equal to 1.8.

The extraction was carried out according to the company's instructions as follows:

1. Transferring 1 ml of the bacterial suspension from each isolate grown on Brain Heart Broth medium aged 24 hours into sterile 1.5 ml Eppendorf tubes. The tubes were then centrifuged at 15,000 rpm for 3 minutes to collect bacterial cells, followed by discarding the supernatant.
2. Adding 20 µl of Proteinase K solution (25 mg/ml) and mixing the mixture using a vortex mixer for 5 seconds.
3. Incubating the mixture at room temperature for 10 minutes, during which the tubes were inverted to ensure complete cell lysis.
4. Adding 200 µl of GB Buffer prepared from the extraction kit to the lysed cell mixture and mixing well using the vortex mixer for 5 seconds.
5. Incubating the mixture at 60°C for 10 minutes using a water bath.
6. Adding 200 µl of absolute ethanol to the lysed mixture and mixing with the vortex mixer for 10 seconds.
7. Transferring the mixture from the Eppendorf tube to 2 ml Collection tubes containing GD filter columns equipped with the extraction kit for DNA purification.
8. Placing the collection tubes with columns in a centrifuge, spinning at 15,000 rpm for 1 minute to remove lysate debris.
9. Discarding the filtrate and transferring the GD column containing DNA to a new collection tube.
10. Adding 400 µl of W1 Buffer from the kit to the column for DNA washing, centrifuging at 15,000 rpm for 30 seconds.
11. Discarding the filtrate, adding 600 µl of Wash Buffer containing absolute ethanol to the column to remove lipids, centrifuging at 15,000 rpm for 30 seconds.
12. Discarding the filtrate and centrifuging the columns again at 15,000 rpm for 3 minutes to dry the columns.

13. Transferring the columns containing DNA to sterile Eppendorf tubes, adding 50 µl of Elution Buffer from the kit to the center of the column, and incubating for 5 minutes. Finally, centrifuging at 15,000 rpm for 30 seconds to elute the DNA and storing it at -20°C until PCR analysis.**

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) test was conducted to identify R. dentocariosa under study using the 16S rRNA gene with a species-specific primer designed for its identification. The reaction mixture was prepared using a kit from the company Abm, according to the company's instructions. After the preparation of the reaction mixture, the tubes were sealed and carefully mixed using a vortex mixer for 10 seconds, then transferred to the PCR Thermocycler for DNA amplification.

Gel Electrophoresis

This technique was used to separate DNA molecules based on their sizes [27] [28]. A DNA ladder of 3000 bp was used to determine the size of the amplified DNA fragments. After the electrophoresis, the gel containing the PCR products was examined using a UV Transilluminator at a wavelength of 336 nm to identify the bands and measure the molecular weights by comparing them to the standard DNA values. The gel was then photographed using a digital camera.

DNA Sequencing

To detect the genetic sequences of the two genes, the products of these genes were excised from the agarose gel, and the genetic material was extracted using the extraction kit from ELK Biotechnology according to the manufacturer's instructions. Finally, the tubes containing the genetic material of the two genes, comprising four isolates, were sent to Macrogen in South Korea for DNA sequencing using the AB DNA sequencing system. The results were then analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI (National Center for Biotechnology Information) website to determine the number and sequences of the nucleotide bases of the target genes. Subsequently, a phylogenetic tree analysis was conducted using the MEGA11 program to identify the genetic variations between Rothia spp. isolates and the standard isolate. Finally, the isolates were registered in the NCBI-GenBank.

STATISTICAL ANALYSIS

The study data obtained were statistically analyzed after being electronically entered using the statistical program SPSS version 29. The statistical tests used included the Chi-Square test (X2) for comparing percentages and the T-test for comparing means of the studied indicators and for comparing between affected and unaffected individuals. Significant differences were determined at a probability level of 5% [29].

RESULTS AND DISCUSSION

After collecting 120 isolates from oral infections of individuals visiting the first and second specialized dental centers in Al-Diwaniyah Governorate between December 2023 and February 2024, they were cultured on Brain Heart Infusion (BHI) medium at 37°C for 18-24 hours. As a result, bacterial growth appeared in 107 isolates

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(89.16%) out of 120, while 13 isolates (10.83%) showed no growth and were excluded.

After adding part of the bacterial culture to Blood Agar, phenotypic characteristics of the growing colonies (shape, color, size, edge, height) were observed and recorded. Additionally, biochemical tests were conducted. As a result, 25 isolates (23.36%) were identified, while 82 isolates (76.64%) belonged to other bacterial species. This is higher than the percentage reported by Odeberg and his fellow researchers in their study on Rothia infections and contamination, which was 10.74% with 26 isolates out of 242 showing bacterial growth [30]. This increase in prevalence might be attributed to different environmental conditions, varying infectious causes, and incorrect health practices like arbitrary antibiotic use, leading to higher colonization rates of this opportunistic bacterium. Ramanan found the development of oxacillin resistance in hospitalized patients with conditions such as cancer and catheter operations [31]. They also reported a higher prevalence compared to the total isolates studied, with a presence rate of 37.31%, with 25 positive cases out of 67 positive samples, confirming its opportunistic nature that becomes more virulent in immune-compromised conditions.

3.1: Phenotypic Characteristics and Biochemical Tests

The growth on blood agar showed white or milky colonies, mucous and sticky, and their adherence to the agar surface increased over the days. They were convex, shiny, smooth, and non-hemolytic. The bacteria were non-motile, oxidase-negative, catalase-positive, non-urease-producing, and coagulase-negative. Table (3-1).

These results were compared with the characteristics of *R. dentocariosa* bacteria, which had been previously documented by scientists and researchers. Whitman his research team found that it does not hemolyze blood on blood agar [32]. It is non-motile [33], and Bergan & Kocur found it ferments glucose, is coagulase-negative, urease-negative, and oxidase-negative [34]. However, their results contradict the finding that it is catalase-negative, while the catalase test result agrees with Getzenberg and his research group [35].

On Chromogenic agar, the colonies appeared green, circular or oval, and shiny, distinguishing them from other types of bacteria found in the oral and upper respiratory tract, such as Staphylococcus, which stains golden yellow on this medium, and Streptococcus, which usually appears blue or blue-green. The green color in the culture of *R. dentocariosa* may be attributed to the bacteria's consumption of the sugars present in the medium, resulting in a color reaction that appears green on the surface of the medium (Figure 3-1).

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Table (3-1): Biochemical Tests for *R. dentocariosa* Isolated from the Oral Cavity of Patients

	النتيجة
Gram Stain	+
Production of Oxidase Enzyme	-
Production of Catalase Enzyme	+
Production of Urease	-
Fermentation of Glucose	+
Motility Test	-
Coagulase Test	-
Hemolysis Test	-

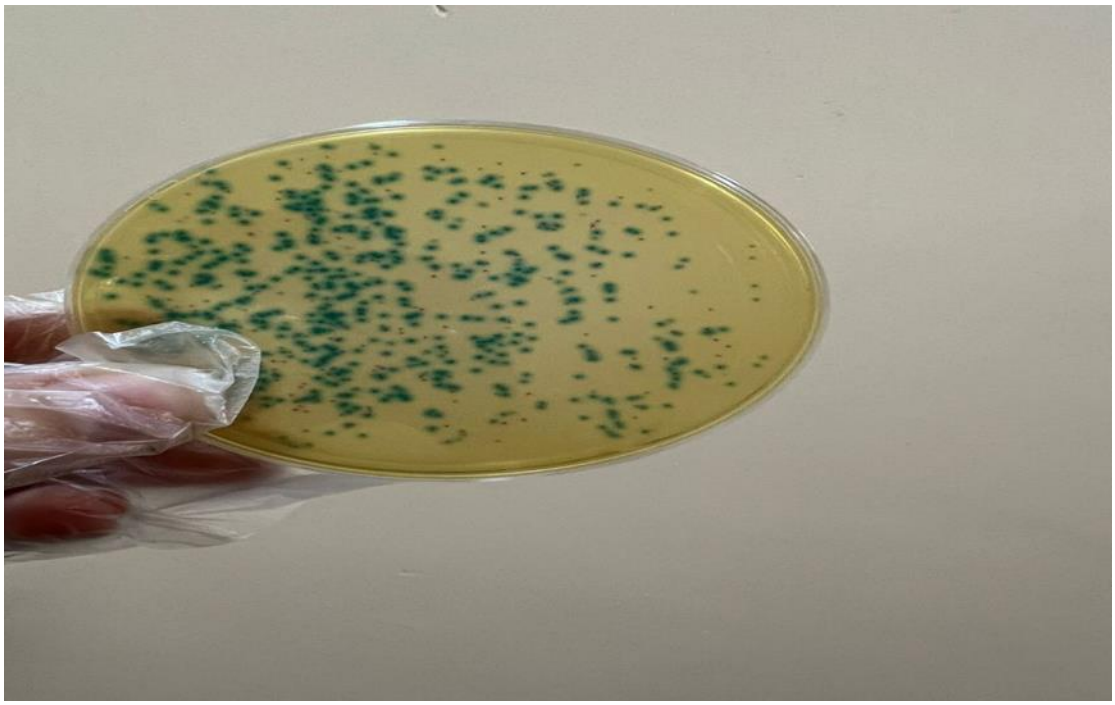

Figure (3-1): Colonies of R. dentocariosa on Chromogenic Agar, appearing green.

3.2: Molecular Identification of *R. dentocariosa*

The molecular detection of the bacteria was performed using a species-specific primer for the bacteria under study. The results were positive for 11 isolates (44%) compared to 14 samples (56%) were negative out of a total of 25 samples, subjected to PCR and gel electrophoresis, showing genetic bands at a single level and a molecular size of 551 bp, as shown in figure (3-1). Subsequently, DNA sequencing was conducted on 4 of the positive isolates using the AB DNA sequencing system.

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The results were analyzed using the BLAST program on the NCBI website to determine the number and sequence of the nucleotide bases of the target genes. A phylogenetic tree was then constructed, as shown in figure (3-2), to show the genetic variations between the *R. dentocariosa* isolates under study and the standard isolates, determining the genetic similarity percentage, as shown in table (3-2). Finally, the isolates were registered in the NCBI gene bank, as listed in table (3-3).

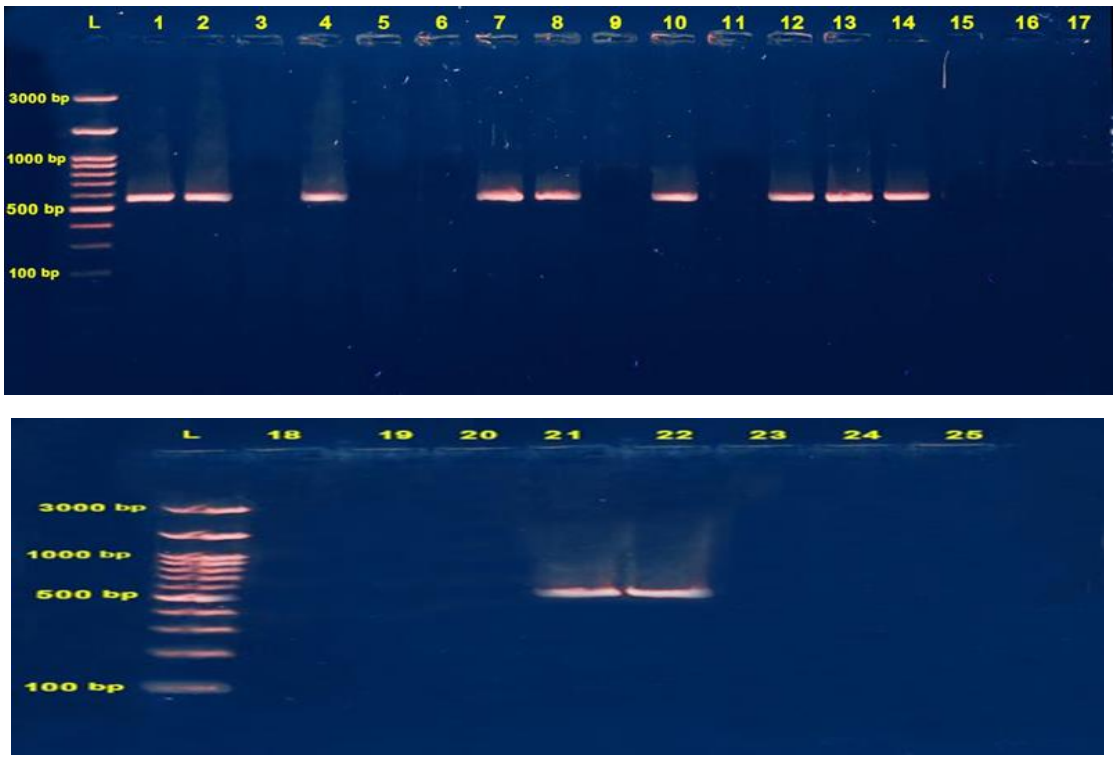

Figure (3-1): Result of agarose gel electrophoresis from PCR reaction using a specific primer for the 16S rRNA gene of *R. dentocariosa.* Lanes 1-25 represent the 16S rRNA gene product with a size of 551 bp, while (L) represents the DNA Ladder with a size of 3000 bp.

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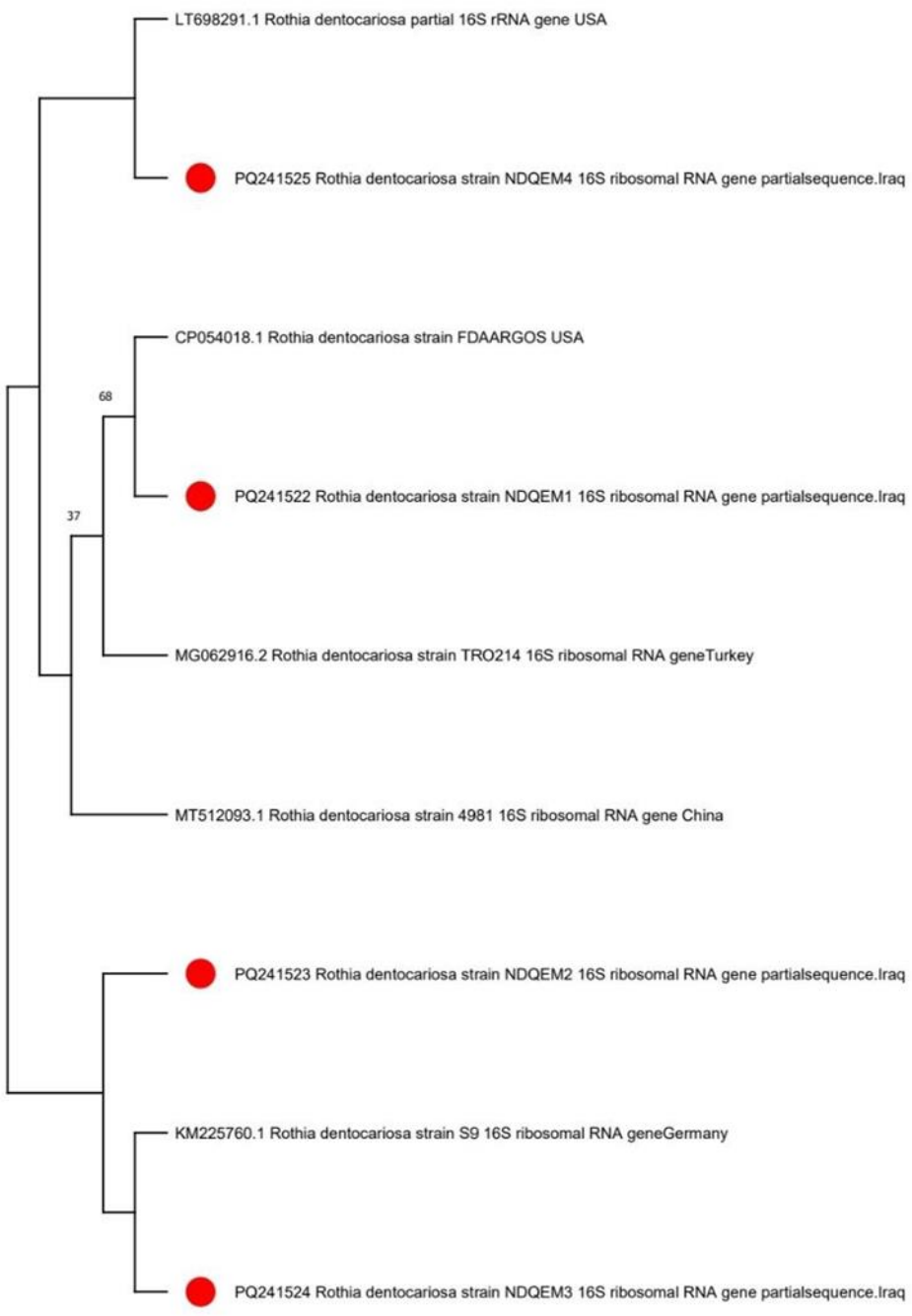

Figure (3-2): Phylogenetic tree analysis of local *R. dentocariosa* strains based on the genetic sequence of the 16S rRNA gene, namely NDQEM4, NDQEM1, NDQEM2, and NDQEM3. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in MEGA version 6.0.

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Table (3-2): The Percentage of Genetic Similarity Between Local *R. dentocariosa* Isolates (IQD-NO.1—IQD-No.4) and Genetically Similar R. dentocariosa Isolates in NCBI-BLAST

Local Isolated gene	Genbank accession number	NCBI-Sequence	Country	NCBI-Identity
R.dentocariosa isolate No.1	PQ241523	LT698291.1	USA	% 99
R.dentocariosa isolate No.2	PQ241525	CP054018.1	USA	% 97
R.dentocariosa isolate No.3	PQ241524	KM225760.1	Germany	% 99
R.dentocariosa isolate No.4	PQ241522	KM225760.1	Germany	% 98

Table (3-3): Links to the NCBI-GenBank Registration of Local *R. dentocariosa* Isolates

Rothia dentocariosa strain NDQEM1 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI
Rothia dentocariosa strain NDQEM2 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI
Rothia dentocariosa strain NDQEM3 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI
Rothia dentocariosa strain NDQEM4 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI

CONCLUSIONS

Cultivation of Rothia dentocariosa on Chromogenic agar resulted in green-colored colonies. The specific s16 rRNA gene was effective for diagnosing and characterizing oral cavity isolates. Sequence analysis of the s16 rRNA gene revealed high genetic similarity between local R. dentocariosa strains and those in the global gene bank.

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Conflict of interests.

There are non-conflicts of interest

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