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Molecular Detection of Tetracycline-Resistant *Streptococcus viridans* Bacteria Using the rpoA Gene

Aseel Jalil Ibrahim Al-Karawi[1], Abdulhameed Salim Hameed[2], Sally Talib Da'aj[3], Hadeel J. Ibrahim[4]

[1]*University of Bilad Alrafidain, Diyala, Iraq*
[2]*Ministry of Health, Diyala, Iraq*
[3]*University of Bilad Alrafidain, Diyala, Iraq*
[4]*General Directorate of Education, Diyala, Iraq*

Abstract

Objective: The aim of this study was to established diagnostic approaches to dental caries using polymerase chain reaction technology.

Methods: PCR series. 60 samples of oral bacteria were collected between 8/6/2023 and 12/1/2023, where 27 teeth showed a growth rate for bacterial culture (45%). The bacterial isolates under study were characterized. The sensitivity of the bacterial isolates of *Streptococcus viridans* under study to eight antibiotics was tested. The results of the current study showed that the resistance rates were as follows: 71.4% for tetracycline, 57% for augmentin, 57% for ciprofloxacin, 28% for clindamycin, 57% for erythromycin, 57% for doxycycline, and 28% for doxycycline for clarithromycin.

Results: The minimum inhibitory concentration (MIC) for the bacterial isolates under study was determined for the tetracycline antibiotic. *S. viridans* isolates showed resistance to this antibiotic through a sensitivity test using the disk method, as the MIC value. for the isolates ranged from (1024-8) micrograms/m l.

The percentage of S. *viridans* isolates producing virulence factors was as follows: protease enzyme 42%, and membrane protease. Bioactive 71%, bacteriocin 14%, hemolycin 57%, DNAase 71%, capsule 28%, and lipase 28%.

Conclusion: The total DNA of all bacterial isolates under study was extracted, after which a polymerase chain reaction (PCR) was performed for the S. viridans resistant to tetracycline and with an MIC value higher than 64 µg/ml using specialized primers targeting the specific sequence of the tetM gene with a size of 1,862bp. When the amplified products were migrated on an agarose gel, one band appeared in all tracks in the gel at the same level for all samples. The results showed that the presence of the tetM gene was 100%.

Keywords: *Streptococcus* viridans, rpoA, tetM, Sequencing, PCR

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Email: aseeljaleel68@gmail.com

Introduction

Streptococcus is a gram-positive coccus that includes several species, as they share common characteristics, as they are facultative anaerobic and spherical in shape [1]. Some of them are obligate anaerobic and some are facultative anaerobic that ferment carbohydrates and grow with the availability of CO2 and are negative to produce oxidase and catalase enzymes. The growth requirements of this species are complex as they require culture media rich in blood, such as blood agar [2].

The pathogenicity of the Streptococcus genus is linked to its ability to produce several virulence factors, which include the production of hemolytic toxins such as hemolysin, in addition to its production of intestinal toxins that cause food poisoning. It also possesses the ability to produce many extracellular enzymes such as lipase, proteinase, and streptokinase, which help the bacteria in tissue invasion and spread of infections. Most of its types are surrounded by a capsule and grow at a temperature of 37°C,

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which is the optimum temperature for their growth, and growth is weak in ordinary solid or liquid media [3].

S. viridans are gram positive, facultative anaerobic, and negative for oxidase and catalase tests. Their growth is inhibited by optochin and their colonies do not dissolve in bile salts. Unlike pneumococci, some Streptococci viridans, such as S. mutans, accumulate large polysaccharides, such as dextran or levans of sucrose, and contribute to the development of tooth decay [4].

Some types of Streptomyces may enter the bloodstream by tooth brushing or during dental work [5]. When these organisms enter the bloodstream, they will form colonies and form biofilms, causing chronic endocarditis.

Resistance to tetracycline and its other derivatives is widespread in spherical gram-positive bacteria, and the mechanisms of resistance against the tetracycline family are either by changing the target site for the action of the antibiotic or reducing the permeability of the antigen through the outer membrane, or through genetic expression of the transposon gene containing it [6].

There is an interest in Diyala Governorate on methods to detect resistance of Streptococcus viridans bacteria to tetracycline antibiotics among hospitalized patients. The aim of this study was to isolate S. viridans and characterize them phenotypically, biochemically and by molecular methods using 16SrRNA and rpoA.

Material and Methods

Isolation and diagnosis of the bacteria under study

Oral samples were cultured on blood agar and on mitis salivarius agar medium and incubated in an anaerobic growth cylinder candle jar for 48 hours at a temperature of 37°C, and then the isolates were morphologically characterized based on the shape of the colony [7], and microscopically, based on the shape, color, cell clusters, and type of pigmentation [8]. In addition, biochemical characterization, based on the ability of the bacteria to produce enzymes such as oxidase [9] and catalase [10] was performed. Finally, we tested the solubility in bile salts [11] and tested sensitivity to optogen [12].

Sample collection

60 oral samples were collected from patients at the Specialized Dental Center in Baquba, Diyala, Iraq and from outpatient dental clinics, under the supervision of specialized dentists, during the period between August and December 2017. The samples were taken using sterile cotton swabs inside a closed carrier medium for the purpose of avoiding external contamination as well as to prevent contamination of samples during their transport to the laboratory.

Detection of virulence factors

Various methods were utilized for determination of virulent factor: the ability of bacterial isolates to produce hemolysin [13] lipase [11], protease [14], DNase [15] enzymes, biofilm production [16], composition of the portfolio [17], and the ability to produce bacteriocin [18].

Antibiotic susceptibility testing

The sensitivity of the S. viridans isolates under study to eight antibiotics was tested using the disk diffusion method on Mueller-Hinton agar medium [19,20]. Antibiotics used in this study were doxycycline (30 µg), erythromycin (15 µg), clindamycin (15 µg), clarithromycin (15 µg), ciprofloxacin (5 µg), bacitracin (0.04 micrograms), tetracycline (30 micrograms), and augmentin (30 micrograms).

Extraction of total DNA and PCR amplification

Total DNA extraction and PCR amplification of the tetracycline-resistant bacterial isolates under study was done using Geneaid. Genomic DNA was purified and the rpoA and tetM genes (445 base pairs) were amplified using specific primers: rpoA F- (CAC AGT TCC AGG TGT TCG) and R- (TGC TGA

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AAG CCC TAA AGCAT) and tetM F-(AGTTTTAGCTCATGTTGATG) and

R- (TCCGACTATTTGGGACGACGG), respectively. For the PCR master mix, Geneaid, Accupower®Profi Taq PCR Premix was placed in stander Accupower®Profi Taq PCR Premix) that contained all other components. To execute the PCR reaction, Taq DNA polymerase, dNTP, Tris-HCl, pH 9, KCl MgCl2 stabilizer, and tacking dye were used (Table 1).

Table 1. Components for the polymerase chain reaction (PCR).

Components	Volume
Master Mix PCR solution	5
DNA template	3
Forward primer	2
Revers primer	2
PCR water	13
Total volume	25

PCR tubes were prepared according to manufacture instructions. They were transferred into a spin vortex centrifuge for 3 minutes at 3000 rpm and then placed in the PCR thermocycler. PCR amplification conditions for rpoA were denaturation at 950C for 5 minutes followed by 30 cycles of annealing at 500C for 30 seconds, extension at 72°C for 30 seconds, and a final extension cycle at 72°C for 5 minutes. For the tetM gene, the reaction conditions were denaturation at 950C for 5 minutes, followed by 30 cycles, annealing at 640C for 30 seconds, extension at 72°C for 30 seconds, and a final extension cycle at 72°C for 5 minutes. PCR products were resolved in 1% agarose gel and visualized by UV gel documentation and the image was captured by digital camera (Canon, USA) [21].

RESULTS AND DISCUSSION

Isolation and identification of Streptococcus spp

The results showed that 27 samples, representing 45% of the total 60, had Streptococcus.

Isolation rate of S. viridans bacteria in the current study was 25.9%. These results were inconsistent with previous work [22,23], which showed the isolation rate of these bacteria as 11% and 5%, respectively, and more like others [24-26] that found percentages of 43%, 19.6%, or 24.5%. The reason for the difference in the percentage of isolation of Hemolytic Streptococcus in the current study in comparison with previous studies is possibly due to differences in the source of isolation, geographical location, ages, or nutritional and health habits.

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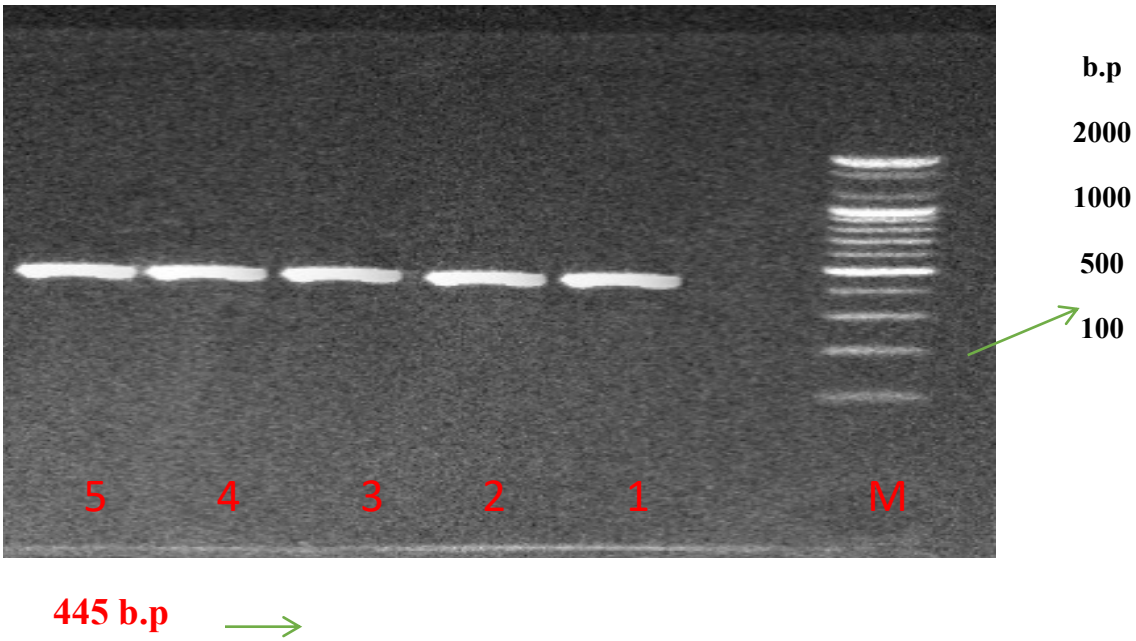

Figure 1. Electrophoresis of amplification products of *rpo*A (445 base pairs) in an agarose gel.

Detection of Streptococcus viridans using the diagnostic rpoA gene

rpoA was detected in all samples (Figure 1).

Antibiotic susceptibility testing for Streptococcus viridans

The sensitivity of Streptococcus viridans isolates to eight antibiotics (doxycycline, tetracycline, augmentin, ciprofloxacin, clindamycin, bacitracin, clarithromycin, and erythromycin) was tested. The results of the current study showed that the rate of resistance of viridans to the tetracycline antibiotic was 71.4%, which is inconsistent with previous results [27,28], that showed the rate of resistance of S. viridans bacteria to this antibiotic was 2.7% or 15.8%. The reason for this resistance is suggested as the inability of the antibiotic to reach inhibitory concentrations into the bacterial cell because of the presence of plasmids that encode efflux pumps, which are responsible for either increasing the transfer of the antigen outside the bacterial cell or reducing its permeability [4]. The tetracycline antagonist inhibits protein synthesis by binding to the 30S ribosomal unit. As a result of this inhibition, it prevents the binding of amionacyl-tRNA to the special site on the mRNA.

The resistance rate of ciprofloxacin for S. viridans isolates was 57%, which is like results of the resistance of S. mutans defined as 75% [29]. The antibiotic effect of ciprofloxacin is bactericidal by inhibiting the DNA synthesis process by stopping the action of the enzyme DNA gyrase [30]. As for the antibiotic erythromycin, the resistance rate of the isolates under study was 57%. The reason for the resistance of bacterial isolates to the anti-erythromycin is likely due either to the presence of plasmids that carry genes responsible for this resistance or to a change in the target site, or to add a methyl group to adenine to rRNA [31,32].

Resistance of the bacterial isolates of S. viridans to the antibiotic clindamycin was 28%. Previous data showed resistance of S. pyogenes bacteria to this antibiotic was 1.6% [33]. Clindamycin is a macrolide, and bacteria become resistant to macrolides by acquiring the mef (A) gene. This gene encodes the

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synthesis of efflux pump protein synthesis, which works on pumping 4-15 rings of the macrolide group [34].).

As for the resistance of bacterial isolates to the antibiotic augmentin, it reached 57% for S. viridans, which is different than previous work that showed a resistance rate of S. pyogenes of 6.6% [22]. The resistance of S. viridans isolates to doxicyclin was 57%, which was different to S. mutans isolates in previous work that were sensitive at 100% level [29]. For clarithromycin, the resistance of S. viridans was 71%. These results were close to the results reached earlier for S. viridans [35]. The increase in resistance resulting from misuse of antibiotics leads to the transfer of this resistance through genetic factors such as transposons or plasmids, or because of a change in the permeability of the bacterial cell wall [36]. The results of the study showed that all isolates were 100% resistant to bacitracin. These results were close to results reached earlier, where the resistance of S. mutans bacteria to this antibiotic was 100% [37].

Detection of the tetM gene using PCR technology

Five bacterial isolates under study, belonging to the S. viridans bacterium, were tested [38]. The results of the current study showed that all isolates had the tetM gene that encodes resistance to tetracycline in S. viridans. The results of our study were inconsistent with previous work, which indicated S. viridans resistance of 43% [39].

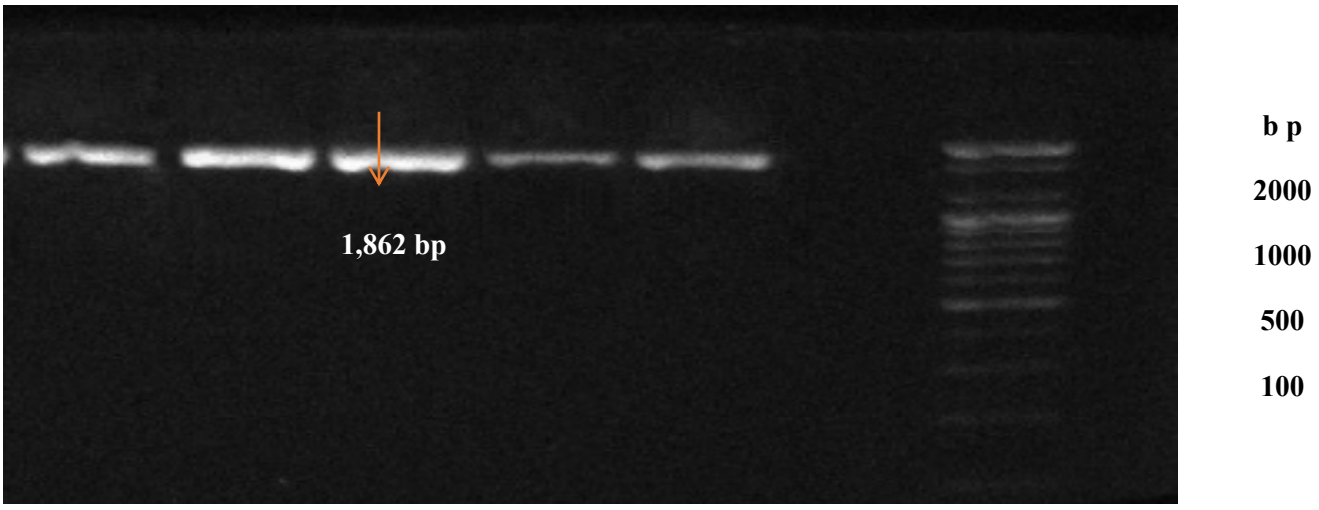

Figure 2. Electrophoresis of the amplification products of the tetM gene in an agarose gel at a concentration of 1% and a voltage of 100 V for an hour and using a DNA Ladder (100bp-2000bp), as this appears in the M path and starts from 100 base pairs, and the gene packages are 1,862 base pairs long in S. viridans bacteria.

Conflicts of interest

The authors declare no competing interest.

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