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Molecular sequencing of collagen-binding adhesin genes in *Streptococcus mutans* isolated from patients with severe dental caries in Iraq

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Abstract

Objective

One of the most important bacteria causing dental caries is *Streptococcus mutans*. This agent is known as the main etiological agent of dental caries. The pathogenicity of this bacterium is closely related to collagen-binding adhesins and cell surface-associated proteins. This bacterium also has a high ability to adhere to dental surfaces, form resistant biofilms, produce acid through carbohydrate fermentation, and survive in acidic conditions. This increases the pathogenicity of this bacterium. Investigating and identifying the genetic sequences of these agents is very important. Because valuable information can be obtained about conserved regions, evolutionary diversity, and pathogenesis mechanisms. The aim of this study was to investigate and identify the sequences of WapA, SpaP, Cnm, and Cbm genes in *Streptococcus mutans* Iraqi isolates, compare these sequences with international reference strains, and evaluate the phylogenetic relationships between them.

Materials and methods

A total of 138 oral swab samples were collected from patients with severe dental caries, including those with and without type 1 diabetes mellitus. 32 control samples were also prepared from healthy individuals without dental caries. Sampling was performed under strict sterile conditions at the Health Education Center and Specialized Dental Center in Babylon Province, Iraq, between October 2024 and February 2025. Oral swabs were collected from patients with severe dental caries in two subgroups, those with and without type 1 diabetes, and from healthy individuals. Streptococcus mutans isolates were then isolated and genomic DNA was extracted. The genes of interest were amplified by PCR and sequenced. Reference sequences in the GenBank database

were used to perform sequence alignment and BLAST analyses. Phylogenetic trees were drawn and analyzed to assess the evolutionary relationships between isolates and reference strains.

Results

Sequencing results showed that for all four genes studied, there was 99-100% similarity between local isolates and global reference strains. A limited number of substitution mutations were identified in the WapA and SpaP genes. However, no sequence changes were observed in the Cnm and Cbm genes. This indicates that the Cnm and Cbm genes are completely conserved. The results of phylogenetic analyses showed that the cluster of Iraqi isolates is close to the clusters of international pathogenic strains. This could indicate high genetic similarity and close evolutionary relationships between them.

Conclusion

These findings indicate high genetic conservation of adhesion genes in *Streptococcus mutans* among different populations and at the same time indicate the existence of microevolutionary variations that could contribute to strain-specific pathogenicity. Gene sequencing is recognized as an important tool in monitoring the evolution of pathogenicity and can play an effective role in guiding preventive strategies for oral health and controlling dental caries.

Keywords: collagen-binding proteins, dental caries, gene sequencing, phylogenetics, Streptococcus mutans

Paper Type: Research Paper.

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Introduction

Dental caries is one of the most common chronic infectious diseases worldwide. Approximately 2.3 billion people worldwide are affected by this disease. It is considered the most common non-communicable disease (Peres et al., 2019; Li et al., 2025). Its prevalence is higher in developing countries. This is because access to preventive dental services is limited in these countries and dietary patterns are changing towards higher sugar consumption (Rajasekaran et al.,

2024; Marin et al., 2025). Among the oral microbiota, Streptococcus mutans is known as the main etiological agent of dental caries. The pathogenicity of this bacterium is closely related to collagen-binding adhesins and cell surface-associated proteins. This bacterium also has a high ability to adhere to dental surfaces, form resistant biofilms, produce acid through carbohydrate fermentation, and survive in acidic conditions. This increases the pathogenicity of this bacterium (Mukasa et al., 1973; Shehab et al., 2024). SpaP (antigen I/II), Cnm, Cbm and WapA are among the most important surface adhesin and collagen-binding proteins (Nomura et al., 2020; Wahlenmayer et al., 2023). These molecules play a key role in bacterial colonization and persistence, as they mediate bacterial attachment to host tissues and extracellular matrix proteins. In addition to dental caries, these adhesin also play a role in the development of systemic complications such as infective endocarditis and atherosclerosis (Nomura et al., 2013; Lukomski et al., 2017; Yin et al., 2025). Investigating and identifying the genetic sequences of these agents is very important. Because valuable information can be obtained about conserved regions, evolutionary diversity, and pathogenesis mechanisms (Duncan, 2003). These data can provide valuable insights into the adaptation and evolution of microorganisms. Therefore, they can be used to design targeted preventive and therapeutic strategies (Lemos et al., 2019). Studies conducted in different regions of Asia, Europe and North America have shown that the sequence of S. mutans adhesion genes varies across regions. Genetic diversity is likely to be one of the reasons for the observed differences in dental caries prevalence among populations (Peixoto et al., 2017; Fitri et al., 2025). Moreover, biochemical tests are incapable of distinguishing different types of microorganisms, such as bacteria, viruses, and parasites. Genomic techniques such as PCR and sequencing are the most modern practical technology in diagnosing infectious diseases and compared with classical techniques, it has been shown to be more rapid, with results obtained in a few hours, and also more reliable. Genomic techniques allow a faster identification directly from clinical samples. Unfortunately, molecular data on S. mutans in the Middle East, especially in Iraq, are very limited (Al-Charrakh et al., 2014). In addition, the Iraqi population has a high prevalence of dental caries. Little information is available on the genetic composition of S. mutans isolates in this country (Merritt et al., 2012; Hmood et al., 2019). Therefore, the aim of this study was to investigate and identify the sequences of WapA, SpaP, Cnm, and Cbm genes in *Streptococcus mutans* Iraqi isolates, compare these sequences with international reference strains, and evaluate the phylogenetic relationships between them.

Materials and methods

Sample collection: A total of 138 oral swab samples were collected from patients with severe dental caries, including those with and without type 1 diabetes mellitus. 32 control samples were also prepared from healthy individuals without dental caries. Sampling was performed under strict sterile conditions at the Health Education Center and Specialized Dental Center in Babylon Province, Iraq, between October 2024 and February 2025. Oral swabs were collected from

patients with severe dental caries in two subgroups, those with and without type 1 diabetes, and from healthy individuals.

Gene sequencing: Genomic DNA was extracted from confirmed *S. mutans* isolates. The *WapA, SpaP, Cnm,* and *Cbm* genes were amplified and sequenced using Sanger sequencing. Sequences were analyzed with BLASTn against GenBank references (M37842.1, MF959099.1, AB102689.1, AB610592.1). Phylogenetic analysis was performed using MEGA X software with neighbor-joining methods and bootstrap validation.

Ethical statement: This study was submitted to the Research Ethics Committee of the Faculty of Medicine, University of Babol and then reviewed and approved. The approval number issued for this study was BMC/ETH/2024/118. Before sampling, an oral informed consent was provided to all participants. Only those who confirmed this consent were sampled.

Statistical analysis: SPSS v25 was used to perform statistical analysis. Gene frequencies were expressed as percentages. Sequence similarity values were presented as mean ± SD. Bootstrap analysis with 1,000 replicates was used to assess the phylogenetic clustering. Statistical significance was set at $p < 0.05$.

Results and discussion

Sequencing of the four virulence genes (*WapA, SpaP, Cnm,* and *Cbm*) revealed a high degree of similarity between Iraqi isolates and global reference strains. The *WapA* gene sequences demonstrated 99% similarity to GenBank reference M37842.1, with minor single nucleotide substitutions detected in two isolates. Similarly, *SpaP* sequences showed 99% identity to MF959099.1, and minor mutations were identified in the alignment. In contrast, *Cnm* and *Cbm* sequences were fully conserved, showing 100% similarity with their respective references (AB102689.1 and AB610592.1). These findings confirm the evolutionary stability of adhesive genes. However, limited local variation in them must be considered. The genomic locations and amplicons sizes of the genes studied, along with their GenBank accession numbers, are shown in Table 1. These results confirm the presence of the expected fragments in all isolates. Figure 1 shows the genomic mapping of the amplified regions. With this information, sequence variations can be more accurately assessed. In addition, these results confirm the accuracy of primer design and targeting. Multiple alignment of the Iraqi isolates' sequences and their comparison with the reference strains are shown in Figure 2. In this figure, the conserved regions and the locations of the nucleotide substitutions observed in the WapA and SpaP genes are highlighted. Sequencing chromatograms of selected isolates are presented in Figure 3. These results confirm the accuracy of the nucleotide peaks and the presence of the identified mutations. Figure 4 shows the phylogenetic analysis of the isolates. The results of this analysis showed that Iraqi isolates clustered closely with international virulent strains, indicating close genetic relationship and evolutionary conservation of these isolates.

Table 1. Genomic positions, accession numbers, and amplicon sizes of *Streptococcus mutans* virulence genes (*WapA, SpaP, Cnm,* and *Cbm*) obtained from Iraqi isolates and compared with reference sequences in GenBank

Amplicon	Reference locus sequences (5′ - 3′)	Length
A) *Wapa* gene	GACTTTGACTGATGTTGTCGGAGAAGATCAAGAAATCGTAAAAG ATTCCTT GGTTGCTGCACGCTTGCAGTACATTGCTGGTGATGATGTTGACAG TTTAGATGAAGCTGCTTCGGAAAAATCCTCAGCATAAGGTCGC	139 bp
B) *Spap* gene	AACGACCGCTCTTCAGCAGATACCATTCAAAAAGGATTTTACTAT GTAGATGATTATCCAGAAGAAGCGCTTGAATTGCGTCAGGATTT AGTGAAGATTACAGATGCTAATGGTAATGAAGTTACTGGTGTTA GTGTGGATAATTATACTAGTCTTGAAGCAGCCCCTAGAAAGAAC ATCTCTAATTTCTTG	192 bp
C) *Cnm* gene	GACAAAGAAATGAAAGATGTGTTATAATAGATTTGTAATATTCTT GTTACAAGAAAGGACTAAAAATATGAAAAGAAAAGGTTTACGAA GACTATTAAAGTTTTTTGGGTATTGTTTTGTCACTTCT CGGTCTTGCAACTGTCTCAGTGACT GGTCTAGTTTACCGTAAATATCATA GCTGATATTAATTAAAAACAGGATG AAAGAGGCAGGGACAAGAGTCTTTGCCTCTTTGCAAAGACTCTTG TCCCTGC	1728 bp
D) *Cbm* gene	GACAAACTAATGAAATCTAATTATACTATATTAGTAATAGTTTTG TTATAGAAAGGACTAAAAAATGAAACAGTGACTGGTCTAGTTTAC CGTAAATATCATAGCTGATATTAATTACGAAGAAAGCGGCACTGT TTTGTCACTTCTTGGTCTTGCAGCTGTCTCAGTGATTGGTTTATTCT ATTATCGTAAATATCATAGCTGATGTTGATTGAAAACAGGATGAA AGGCAAAGACTCTTGTCCCTGC	1814bp

The results of this study showed that Streptococcus mutans isolates isolated from Iraqi patients carry adhesion genes associated with pathogenicity. In addition, these isolates have high genetic conservation and are closely related to global reference strains. The 99-100% sequence similarity observed in them indicates that these genes have high evolutionary stability. Therefore, they play a fundamental and vital role in the processes of bacterial attachment, colonization and survival (Lévesque et al., 2005; Rued et al., 2021). Limited substitution mutations were identified in the WapA and SpaP genes. This indicates the occurrence of continuous microevolutionary changes in S. mutans populations. Such mutations have also been reported in studies conducted in East Asia and Europe (Masuda et al., 1979; Guo et al., 2015; Choudhari et al., 2023). These studies have shown that sequence variations in the adhesive regions influence the strength of attachment and the capacity to form biofilms. These subtle genetic differences reported in the present study and other studies could explain the observed variability in susceptibility and severity of dental caries among different populations. In phylogenetic clustering, Iraqi isolates were placed alongside global pathogenic strains. This could indicate that these bacteria have a pathogenic potential comparable to their international counterparts.

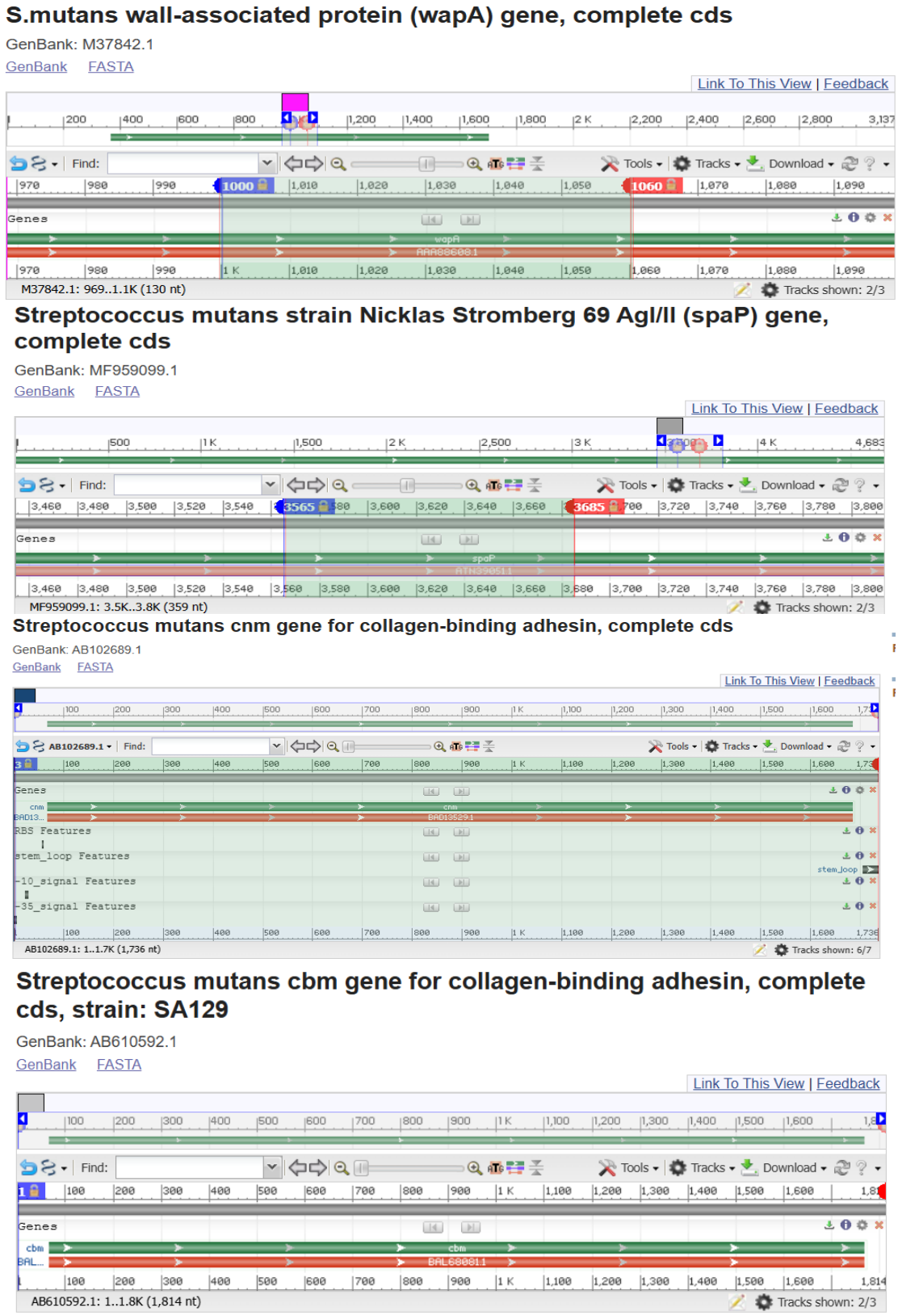
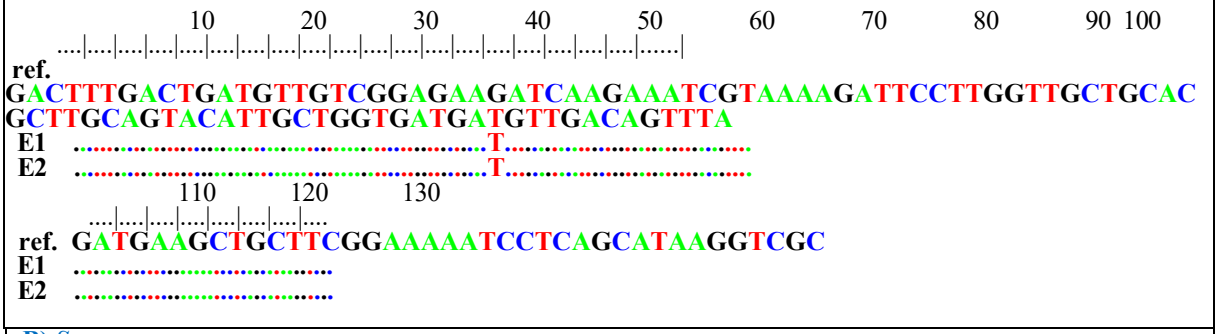
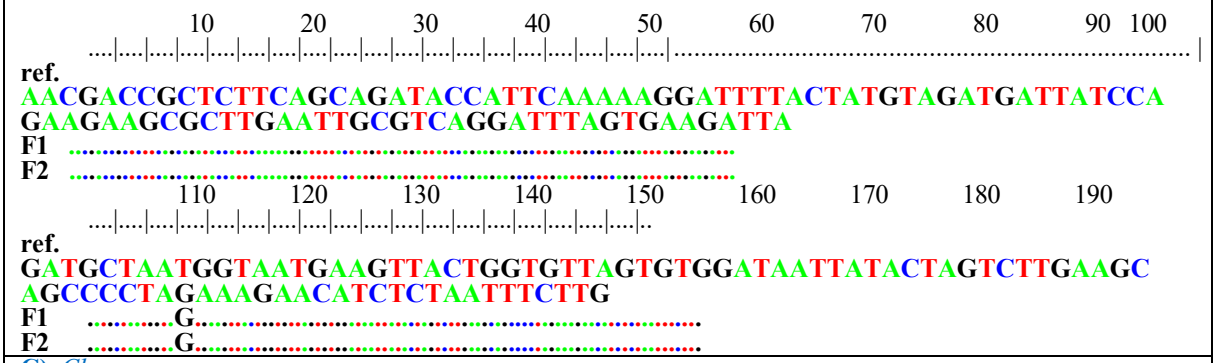


Figure 1. Schematic representation of amplified genomic regions of *WapA, SpaP, Cnm,* and *Cbm* genes in *Streptococcus mutans* isolates, showing positions of primers relative to reference sequences

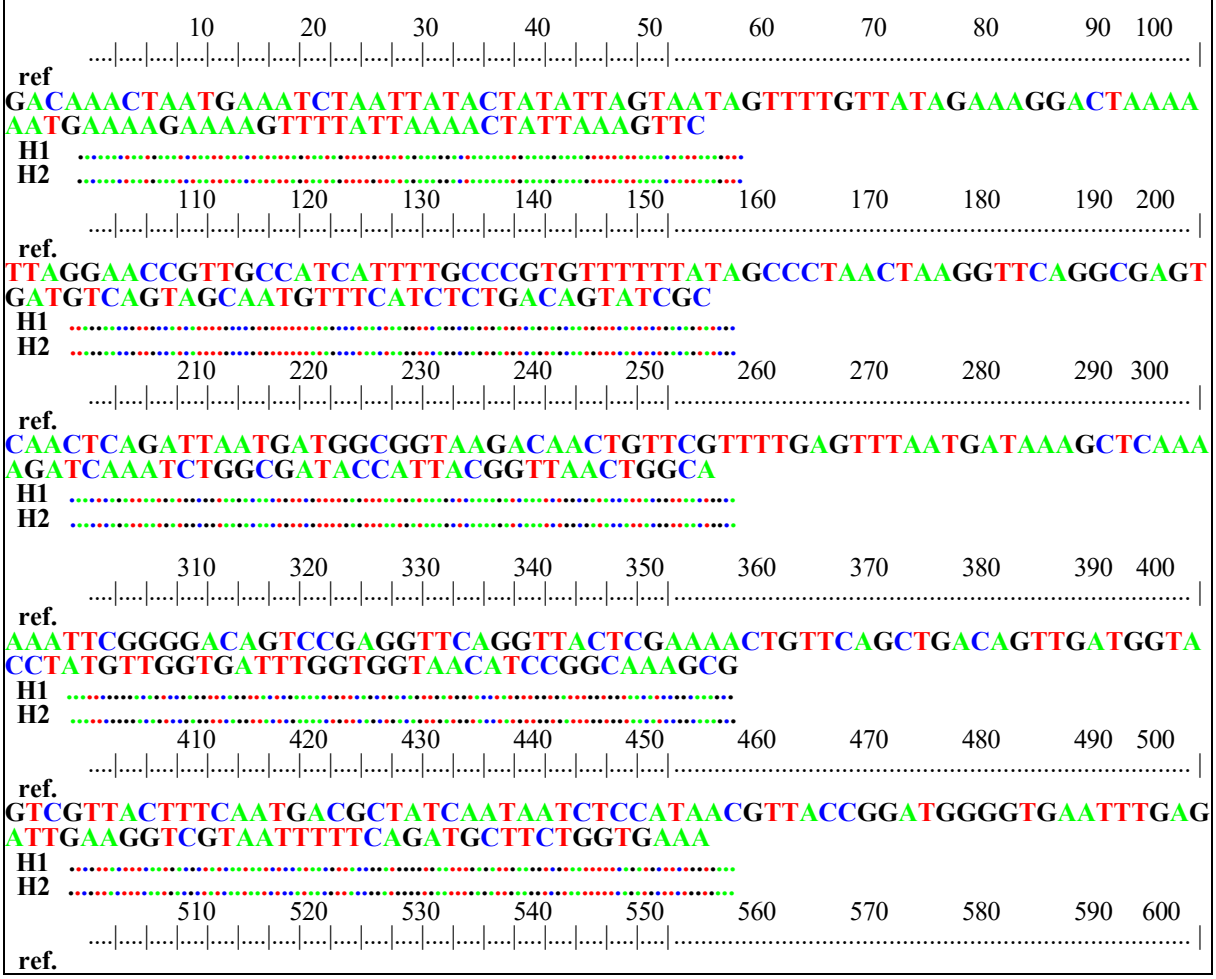
A) *Wapa* gene

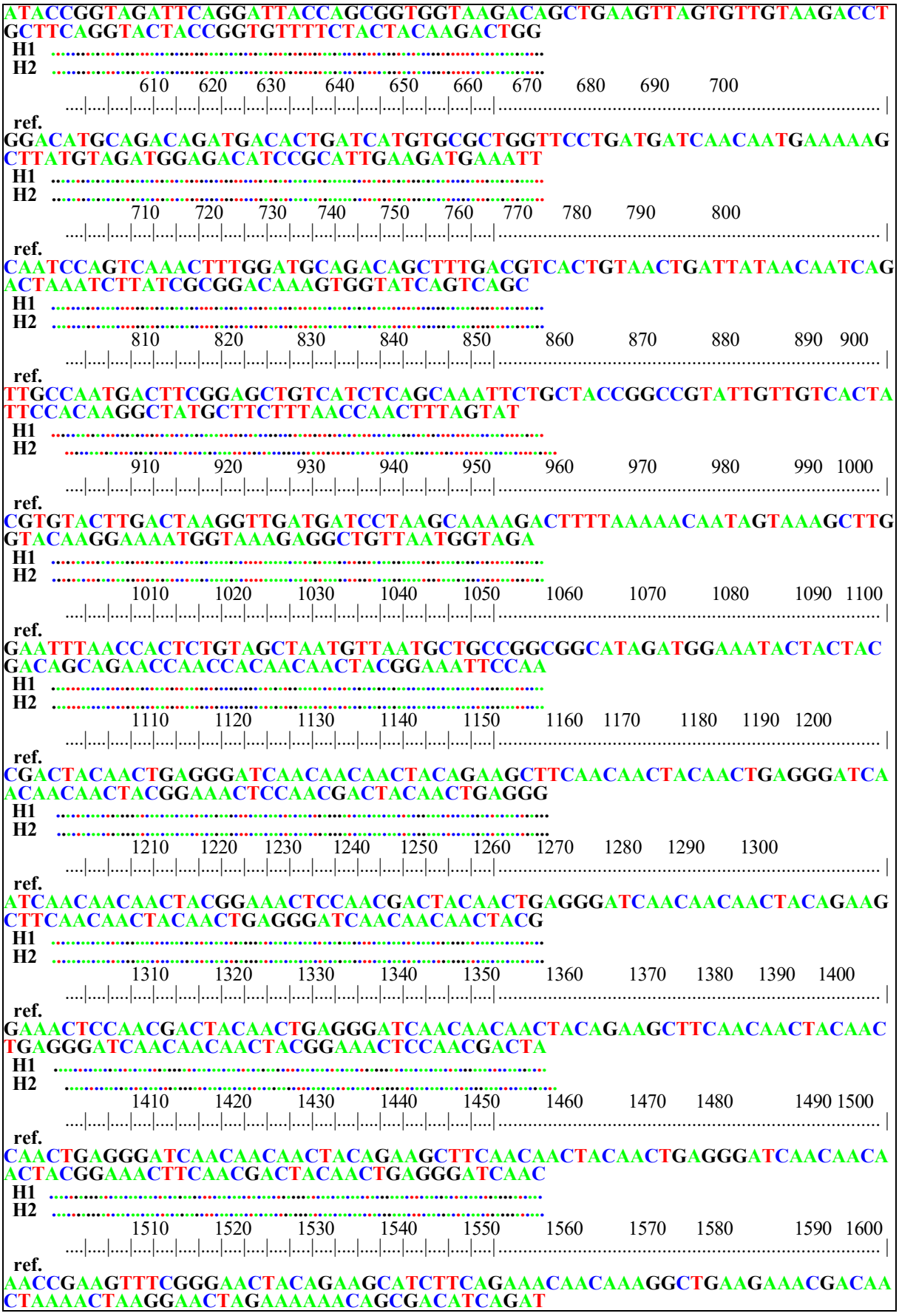


B) *Spap* gene



C) *Cbm* gene

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H1
H2
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ref.
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H1
H2
710 720 730 740 750 760 770 780 790 800
ref.
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H1
H2
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ref.
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H1
H2
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ref.
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H2
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H2
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ref.
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H1
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H2
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
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H1
H2
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
ref.
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H1
H2
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ref.
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GCTGATGTTGATTGAAAACAGGATGAAAGAGGCAAAA
H1
H2
1810
ref. ACTGTTGTCCCTGC
H1
H2
D) Cnm gene
10 20 30 40 50 60 70 80 90 100
ref.
GACAAAGAAATGAAAGATGTTATAATAGATTTGTAATATTCTTGTTACAAGAAAGGACTAAAAAT
ATGAAAAGAAAAGGTTTACGAAGACTATTAAAGTT
G1
G2
110 120 130 140 150 160 170 180 190 200
ref.
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G1
G2
210 220 230 240 250 260 270 280 290 300
ref.
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G1
G2
310 320 330 340 350 360 370 380 390 400
ref.
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G1
G2
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ref.
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G1
G2
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ref.
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G1
G2
610 620 630 640 650 660 670 680 690 700
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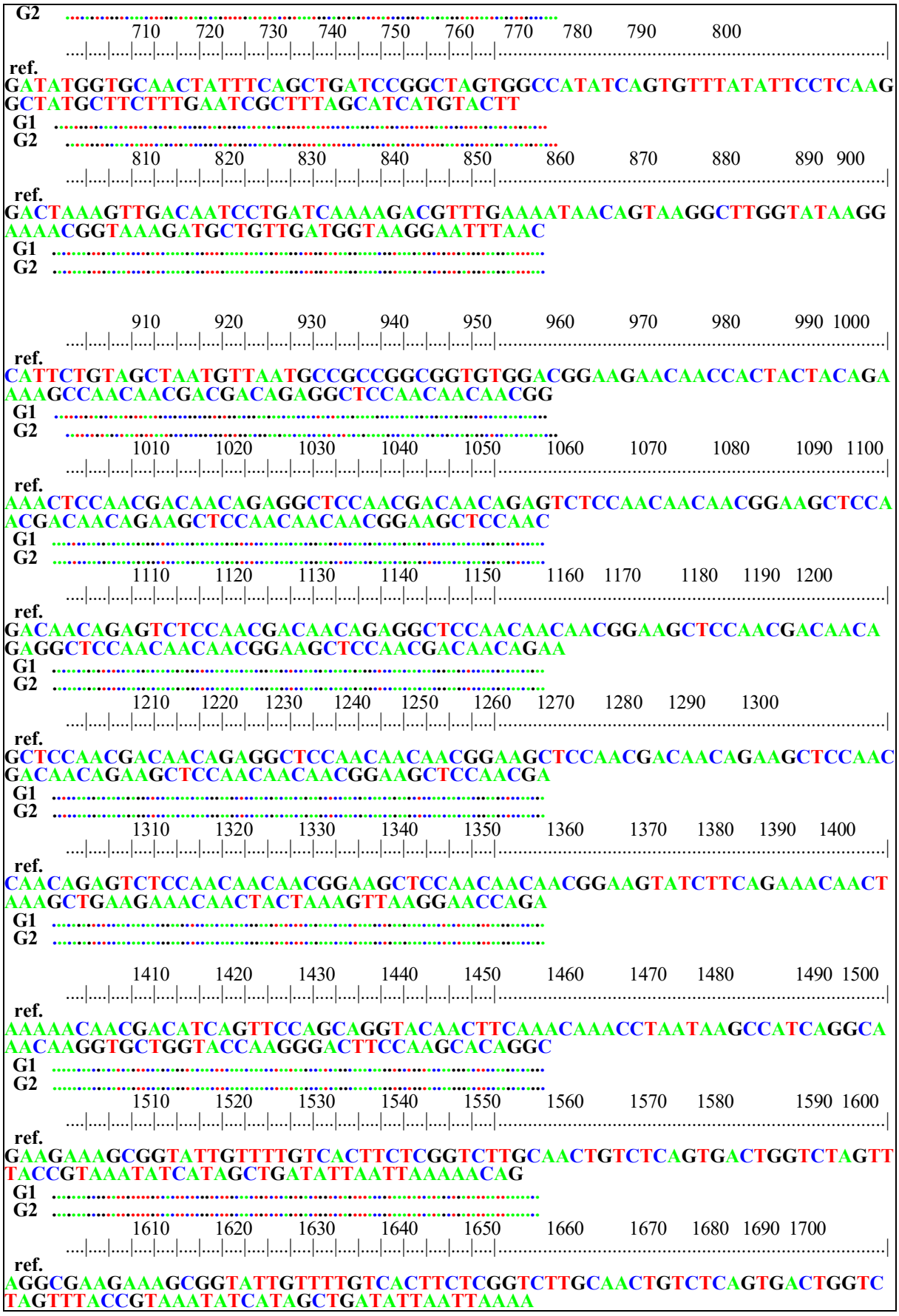
G1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G2
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G2
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G1
G2
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
ref.
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GAGGCTCCAACAACAACGGAAGCTCCAACGACAACAGAA
G1
G2
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GACAACAGAAGCTCCAACAACAACGGAAGCTCCAACGA
G1
G2
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G2
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ref.
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G2
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
ref.
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G1
G2
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
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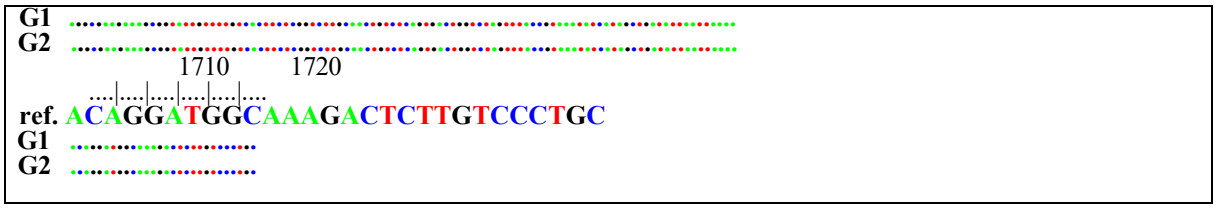

Figure 2. Multiple sequence alignment of Iraqi isolates with corresponding GenBank reference sequences, highlighting conserved regions and nucleotide substitutions in *WapA* and *SpaP*

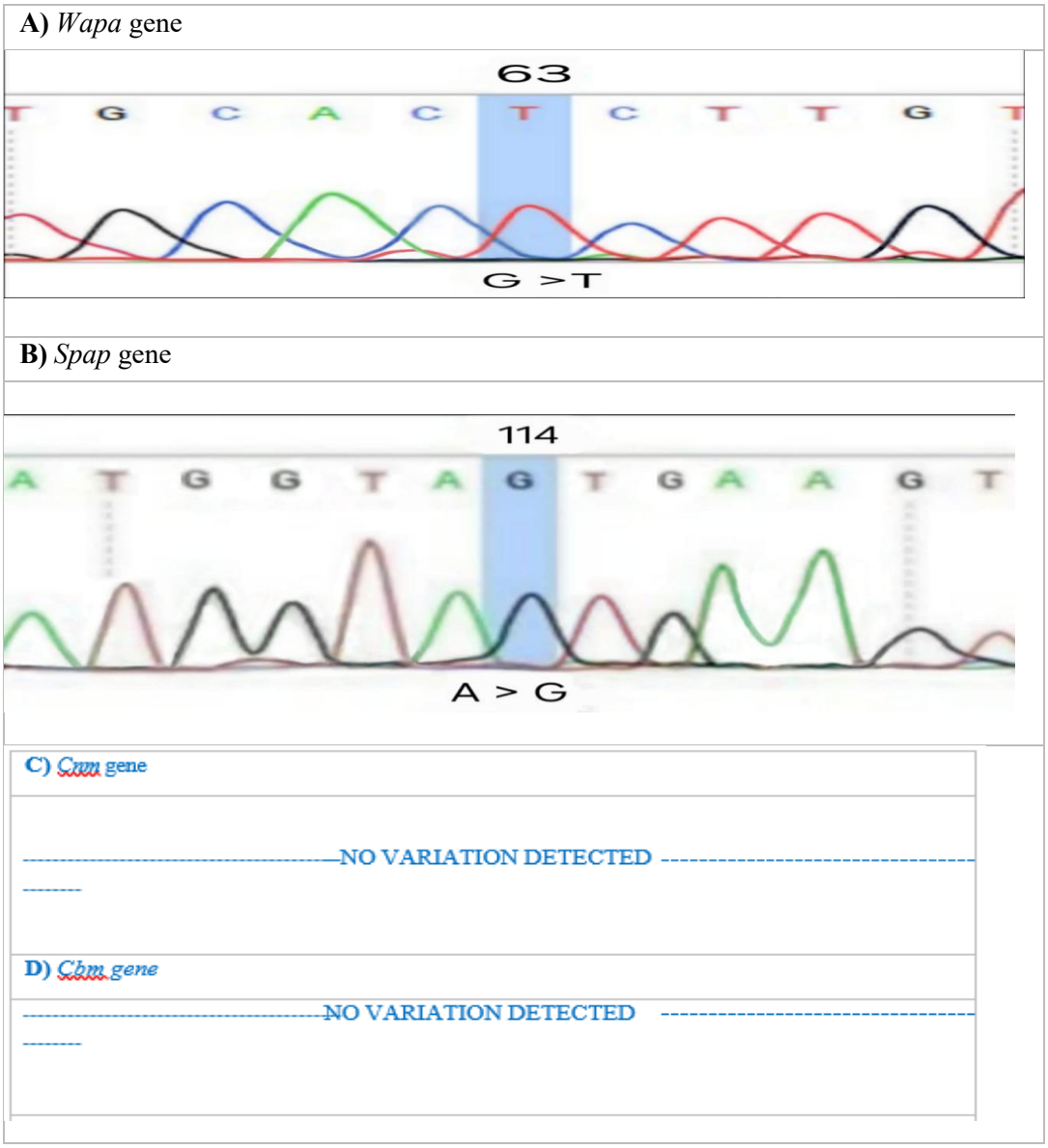

Figure 3. Chromatograms of sequenced isolates illustrating nucleotide peaks for representative *WapA* and *SpaP* genes, confirming the accuracy of base calling and identification of single nucleotide variations

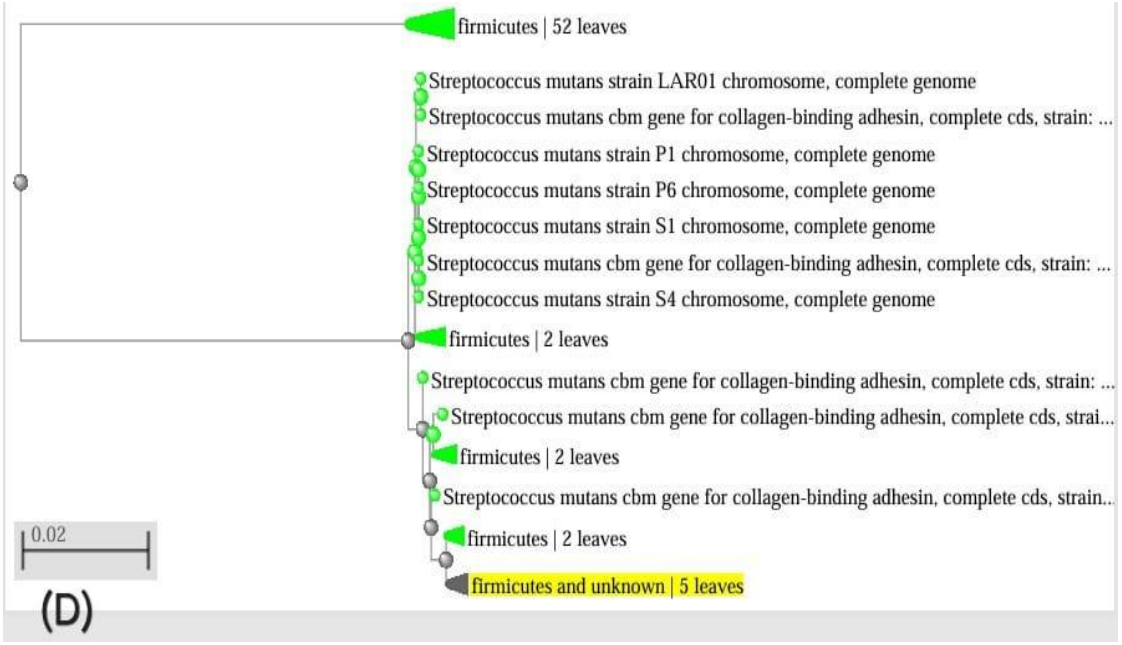

Figure 4. Phylogenetic trees of *WapA, SpaP, Cnm,* and *Cbm* genes constructed by the neighbor-joining method with 1,000 bootstrap replicates, showing the clustering of Iraqi isolates with global pathogenic strains

This finding is also clinically important and indicates that there is a potential risk of systemic infections associated with S. mutans beyond dental caries, especially in patients with underlying diseases such as diabetes mellitus or cardiovascular disease (Carletto-Körber et al., 2015; Rasheed et al., 2025). From a regional perspective, the results of this study fill an important gap in the molecular epidemiology of S. mutans in Iraq. Previous studies in Iraq have focused mainly on bacterial culture and PCR-based identification of these isolates, with limited attention to genetic sequencing (Murray et al., 2024). However, this study used sequencing. The sequencing data presented in this study provide more detailed information about the degree of genetic conservation and diversity. Therefore, its results can be used in designing preventive oral health strategies and effective therapeutic interventions. The present findings are also in line with global trends, as they emphasize the integration of molecular microbiology in oral health surveillance. Gene sequencing can be used as a biomarker tool to monitor the evolution of pathogenicity and assess the effect of interventions such as vaccines, probiotics, or targeted antimicrobial agents against S. mutans (Gomez et al., 2017; Chandra Nayak et al., 2025). As with most studies, limitations should be noted. Some of these limitations include the limited number of isolates sequenced and the small number of pathogenic genes studied. Therefore, it is recommended to use whole genome sequencing, as its results can provide a more comprehensive view of the genetic landscape and adaptation mechanisms of S. mutans. In addition, functional studies should be performed to investigate the association of specific sequence changes with phenotypic traits.

Conclusion: Sequencing of WapA, SpaP, Cnm and Cbm genes in Iraqi isolates of Streptococcus mutans showed that these isolates have strong genetic conservation compared to international reference strains. Although limited nucleotide changes were observed. These findings indicate high genetic conservation of adhesion genes in *Streptococcus mutans* among different populations and at the same time indicate the existence of microevolutionary variations that could contribute to strain-specific pathogenicity. Gene sequencing is recognized as an important tool in monitoring the evolution of pathogenicity and can play an effective role in guiding preventive strategies for oral health and controlling dental caries.

Author Contributions

Sajjad Haider Jasim: Conceptualization, methodology, software, validation, formal analysis, investigation, data curation, resources, and writing-original and subsequent draft. Zainab Hamoud Muhaisen: Visualization, and supervision. All authors have read and agreed to the published version of the manuscript

Data Availability Statement

Data are available from the authors upon reasonable request.

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The author thanks all the subjects who participated in this research.

Ethical Considerations

The study was carried out with integrity, with no fabrication, falsification, plagiarism, or scientific misconduct.

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Conflict of Interest

There is no conflict of interest.

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توالی‌یابی مولکولی ژن‌های چسبنده متصل‌شونده به کلاژن در *Streptococcus mutans* جداشده از بیماران مبتلا به پوسیدگی شدید دندانی در عراق

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چکیده

هدف: *Streptococcus mutans* یکی از مهم‌ترین باکتری‌های عامل پوسیدگی دندان به شمار می‌رود و به‌عنوان عامل اصلی اتیولوژیک این بیماری شناخته می‌شود. بیماری‌زایی این باکتری ارتباط نزدیکی با چسبنده‌های متصل‌شونده به کلاژن و پروتئین‌های وابسته به سطح سلولی دارد. این باکتری از توانایی بالایی در اتصال به سطوح دندانی، تشکیل بیوفیلم‌های مقاوم، تولید اسید از طریق تخمیر کربوهیدرات‌ها و بقا در شرایط اسیدی برخوردار است که همگی موجب افزایش پاتوژنیسیته آن می‌شوند. بررسی و شناسایی توالی‌های ژنتیکی این عوامل از اهمیت بالایی برخوردار است، زیرا می‌تواند اطلاعات ارزشمندی درباره نواحی حفاظت‌شده، تنوع تکاملی و سازوکارهای بیماری‌زایی فراهم کند. هدف از این مطالعه، بررسی و شناسایی توالی ژن‌های WapA، SpaP، Cnm و Cbmدر ایزوله‌های عراقی *Streptococcus mutans*، مقایسه آن‌ها با سویه‌های مرجع بین‌المللی و ارزیابی روابط فیلوژنتیکی میان آن‌ها بود.

مواد و روش‌ها: در مجموع، ۱۳۸ نمونه سواب دهانی از بیماران مبتلا به پوسیدگی شدید دندانی، شامل افراد مبتلا و غیرمبتلا به دیابت ملیتوس نوع ۱، جمع‌آوری شد. همچنین ۳۲ نمونه شاهد از افراد سالم و فاقد پوسیدگی دندانی تهیه گردید. نمونه‌برداری با رعایت کامل شرایط استریل در مرکز آموزش بهداشت و مرکز تخصصی دندان‌پزشکی استان بابل، عراق، طی بازه زمانی اکتبر ۲۰۲۴ تا فوریه ۲۰۲۵ انجام شد. پس از جداسازی ایزوله‌های *Streptococcus mutans*، DNA ژنومی استخراج گردید و ژن‌های مورد نظر با استفاده از واکنش زنجیره‌ای پلیمراز (PCR) تکثیر و سپس توالی‌یابی شدند. هم‌ترازی توالی‌ها و تحلیل‌های BLAST با

استفاده از توالی‌های مرجع موجود در پایگاه داده GenBank انجام شد. در نهایت، درخت‌های فیلوژنتیکی به‌منظور ارزیابی روابط تکاملی میان ایزوله‌ها و سویه‌های مرجع ترسیم و تحلیل گردید.

نتایج: نتایج توالی‌یابی نشان داد که برای هر چهار ژن مورد بررسی، شباهت ۹۹ تا ۱۰۰ درصدی میان ایزوله‌های محلی و سویه‌های مرجع جهانی وجود دارد. تعداد محدودی جهش جانشینی در ژن‌های WapA و SpaP شناسایی شد، در حالی که در ژن‌های Cnmو Cbm هیچ‌گونه تغییر توالی مشاهده نشد که بیانگر حفاظت کامل این دو ژن است. تحلیل‌های فیلوژنتیکی نشان داد که ایزوله‌های عراقی در خوشه‌هایی نزدیک به سویه‌های پاتوژن بین‌المللی قرار می‌گیرند که حاکی از شباهت ژنتیکی بالا و روابط تکاملی نزدیک میان آن‌ها است.

نتیجه‌گیری: یافته‌های این مطالعه نشان‌دهنده حفاظت ژنتیکی بالای ژن‌های چسبنده در *Streptococcus mutans* در میان جمعیت‌های مختلف است و در عین حال وجود تغییرات ریزتکاملی را تأیید می‌کند که می‌توانند در بیماری‌زایی اختصاصی سویه‌ها نقش داشته باشند. توالی‌یابی ژن‌ها به‌عنوان ابزاری مهم در پایش تکامل بیماری‌زایی شناخته می‌شود و می‌تواند نقش مؤثری در هدایت راهبردهای پیشگیرانه سلامت دهان و دندان و کنترل پوسیدگی‌های دندانی ایفا کند.

کلمات کلیدی: پروتئین‌های متصل‌شونده به کلاژن، پوسیدگی دندان، توالی‌یابی ژن، فیلوژنتیک، *Streptococcus mutans*

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