Iraqi Journal of Biotechnology, 2025, Vol. 24, No. 1, 283-294

Molecular Identification and Phylogenetic Analysis of S*treptococcus Pyogenes* Clinical Isolates from Human Throat Swab

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Received: May 2, 2024 / Accepted: July 4, 2024 / Published: March 5, 2025

Abstract: *Streptococcus pyogenes* is gram-positive, versatile human pathogen produced many virulence factors and toxins that target various component of immune response and caused multitude of local and systemic infection. The aim of the study was to use conventional PCR to identify *Streptococcus pyogenes* based on *16S rRNA*, *speB*, and *smeZ* genes. During the period from January to May 2022, fifty throat swabs were collected from humans suffering respiratory signs. They detected the infection with standard biochemical tests such as gram stain and sensitivity to bacitracin disc, then confirmed by PCR. The synthesized DNA was further amplified through conventional PCR targeting the *16S rRNA*, *speB,* and *smeZ* genes. These amplified products were submitted to Macrogen, South Korea, for sequencing. The result of the sequences analysis revealed multiple points of mutation within *16S rRNA* gene G/A, showing transition mutation at 433 and 911 locations, while T/A and G/C had trans version mutation at 554 and 693 respectively. The phylogenetic analysis indicated 99% similarity of the 16sRNAgene of the isolated bacteria with a known strain of Egypt and India strains. Overall, the result concluded that standard molecular technique shows *16S rRNA*, *speB,* and *smeZ* genes found with long lengths 1250bp, 978bp and 246bp, respectively when detection by conventional PCR as essential virulence genes which have crucial role in the confirmation of *S. pyogenes* infection with establishes that the circulating strain exhibits high genetic resemblance to Egypt and India strains.

Keywords: *Streptococcus pyogenes*, *16S rRNA*, *speB*, *smeZ,* genes, phylogenetic.

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Introduction

Streptococcus pyogenes is an pathogenic gram positive bacterium (1), known as group A streptococcus (GAS), which cause both local and systemic infection in animal and humans by production multiple virulence factors like M protein and superantigenes (SAgs), M protein an important analyzed type of *S.pyogenes* virulence factor which are located in the region of fibronectin-collagen-T antigen (FCT) and encoded by *emm* gene and classified bacteria to many serotypes based on vibration of amino acid sequences for that used the genotyping as an effective method to observe the bacterial strain, sequence of *emm* gene and their correlation with diseases caused by group A *streptococcus pyogenes*, also M protein have essential role in limiting the phagocytosis by bind to high molecular weight glycoprotein of the extracellular matrix fibronectin (2), also helping in microcolony formation and invasion of

epithelial cells, binding to complement regulatory proteins contributes to the bacterial resistance by limiting the deposition C3b opsonin on their surface and enhance pathogenesis by enable promoting adhesive, facilitate colonization, and invasive bacterial product to deep tissues then degrade proteins to resist immunity and overstimulation of the immune inflammatory cascade that fated by a toxic shock syndrome (3).

The SAgs are highly mitogenic exotoxin synthesized by ribosomes and have an important role in the pathogenesis of group A *streptococcus pyogenes,* which have classical signal peptides, cleaved to excrete stronger T cell activators as mature toxins called SAg toxins, which have many terms such as streptococcal pyrogenic exotoxins (spe), streptococcal mitogenic exotoxin Z (*smeZ*), and distribution of Sag genes used as procedures to detect the heterogeneity of the genome, binding between the content of genes and determining their clinical appearance (4).

SpeB is a cysteine protease exotoxins associated with invasive infection of *S.pyogenes* and degrading several substrates on the host side like cytokines, chemokines, complement components, and IgGs, and inhibiting the serum protease and replacement for bacterial chromosome; the recent identification of mucosal-associated invariant T-cells (MAIT cells) as primary contributors to the cytokine storm as highly activated in infected host with streptococcus toxic shock syndrome(STSS) (5).

Streptococcal mitogenic exotoxin Z(*sme*Z) its chromosomally encoded genes released from different isolates of *S.pyogenes*, frequently present in strains carrying *spe*A and *ssa*, together with *spe*K, and *sme*Z have apathogenic mechanism by passing the generating process of typical antigen presentation and overstimulation of the immune inflammatory cascade (6). Thus, this study aimed to detect of *Streptococcus pyogenes* isolated humans and then identified by molecular assay by using 16sRNA, *speB,* and *smeZ* genes.

Materials and Methods

Sample collection

In this study, throat swabs conducted on a total of fifty swabs from humans were collected under sterilized conditions by using sterile swabs with media and labeled randomly; the collection occured during the period from January to May 2022, then transported to the pathology laboratory, Collage of Veterinary Medicine/University of Baghdad.

Bacterial culture media

The culture media that are used blood agar, both brain heart infusion agar, and broth (Himedia). All media were prepared according to manufacturing instructions. The pH was adjusted to 7.3 (± 0.2) and sterilized in an autoclave at 121 ^{o}C for 15 minutes at 15 pounds per square inch. After that, they were distributed into Petridishes and test tubes and then were incubated at 37°C for 24 hr. according to Hiba *et al*. 2024(7).

Isolation of *S. pyogenes*

The clinical isolate of *S.pyogenes* isolated from humans. The swabs were inoculated in an autoclaved and sterilized universal containing 10 ml of brain heart infusion broth**,** then incubated overnight in a candle jar at 37˚C under (5–10)% CO_2 condition (8, 9).

After that, a loopful from the broth culture were used for streaking on the selective media agar to give quickly and

direct identification then the isolated colonies were detected.

Identification of *S. pyogenes*

The suspected bacterial colonies were confirmed as *S.pyogenes* by using standard microbiological techniques such as *B*-hemolytic on blood agar plates, catalase test, morphological appearance of gram-positive cocci (Streptococci) under light microscope (10), susceptibility of bacteria to bacitracin disc, and further confirmation was done by conventional PCR and analysis of DNA sequencing. The protocol of gene detection was used according to Tamura *et al*., 2013 (11).

Extraction of genomic DNA

The extraction of chromosomal DNA and detection of some *S.pyogenes* virulence genes by polymerase chain reaction (PCR): DNA was extracted from the fresh overnight bacterial isolate culture grown in the blood agar at 37˚C by using a specific kit for bacterial isolation manufactured by Macrogen, Korea, according to the protocol of the Favor Prep Blood/ Cultured Cells Genomic. DNA Extraction Intron Mini Kit, Korea.

Primers and PCR reaction

The virulence genes (*speB* and *smeZ*) primers that were used in the current study, in addition to the 16sRNA gene for detection of *S.pyogenes* were amplified using forward and reverse primer respectively.

The amplified of 16SrRNA was:
5'- AGAGTTTGATCCTGGCTCAG- 3'
5'- GGTTACCTTGTTACGACTT- 3’
respectively, while *speB* gene amplified 5'-GGAGAACTTTCTGGCTCTAA- 3',
5'-GATGCCTACAACAGCACTTT-3’
respectively, and *smeZ* amplified was
5'- TTTCTCGTCCTGTGTTTGG- 3',
5'TTCCAATCAAATGGGACGGAGAAC-3’ respectively, (Table 1).

They were prepared by lyophilizing followed by dissolving in free ddH2O to reach 100 pmol/µl and kept as stock solution at -20 to prepare 10 pmol/µl concentration as suspended work primer. After that they must dissolve the stock solution (10 µl) in 90 µl of the free ddH2O water to get (100 µl) as the final required volume(12).

Table (1): Sequence of primer used in the study.

Target gene	Sequence	Primer sequence 5'- 3' From (Microgen Korea)	Tm (°C)	GC%	Product Size (bp)	Reference
16s RNA	F	5'-AGAGTTTGATCCTGGCTCAG-3'	54.30	50.00	1250bp	Srinivasan *et al.* (13)
	R	5'-GGTTACCTTGTTACGACTT-3’	49.40	42.10		
speB	F	5'-GGAGAACTTTCTGGCTCTAA-3'	54.39	45.00	978bp	Dong *et al.* (14)
	R	5'-GATGCCTACAACAGCACTTT-3’	56.33	45.00		
smeZ	F	5'- TTTCTCGTCCTGTGTTTGG- 3'	57.01	45.00	246bp	Borek *et al.* (15)
	R	5'-TTCCAATCAAATGGGACGGAGAAC-3’	62.74	44.00		

Preparation of PCR

The PCR reaction components were prepared by a mixture (10 µl) Maxter Mix, (0.5 µl) of each primer pair, (5 µl) DNA, and up to (20 µl) RNase-free water. Then, the PCR amplification program was done through the use of Applied Biosystem of PCR as follows: initial denaturation: 5 minutes at 95°C; denaturation: 45 sec. at 95°C; annealing as an important optimal condition: 45 sec. at 58°C, extension-1: 1 minute at 72°C, and extension-2: 5 minutes at 72°C, all (35) cycles except initial denaturation and extension-2(1) cycle (Table 2).

Table (2): The optimal detection for thermocycling.

No.	Phase or Steps	Tm (°C)	Time	No. of cycle
1	Initial Denaturation(Hold)	95°C	5 min	1 cycle
2	Denaturation -2	95°C	45 sec	35 cycle
3	Annealing	58°C	45 sec	
4	Extension-1	72°C	1 min	
5	Extension -2	72°C	5 min.	1 cycle

The PCR product bands, when the bacterial amplification occurred partially by using 16 sRNA gene, were subjected to electrophoresis on 1.5% agarose gel at 100 volts for 1.30 hours. Then, the UVtransilluminator was used to visualize DNA bands, and the photos were captured using a special camera.

Sequencing of DNA and Phylogenetic Tree

The product of PCR for one sample already amplified by PCR targeting the 16sRNA as well as the forward primer was submitted to the sequencing service (Macrogen, Korea) to perform Sanger sequencing. Then, the sequences were analyzed using BLAST (Basic Local Alignment Search Tool) at NCBI to investigate a similar sequence that had previously been registered in this genomic bank, and the phylogenetic tree was organized using the maximum composite likelihood and minimum evolution method by Molecular Evolutionary Genetics Analysis (MEGA) version 6.0. Software that eliminates the gaps and missing data in all positions (11, 16).

Results and Discussion

Isolation and Identification

Streptococcus pyogenes clinically isolates from humans suffering respiratory signs such as nasal discharge, sneezing, anorexia, restlessness, depression and fever and identified by morphological evidence as gram-positive cocci, arranged in single or pairs, long or short chains shown in (Figure 1), with notice clear zone of inhibition when used bacitracin disc shown in (Figure 2), then confirmed by molecular study.

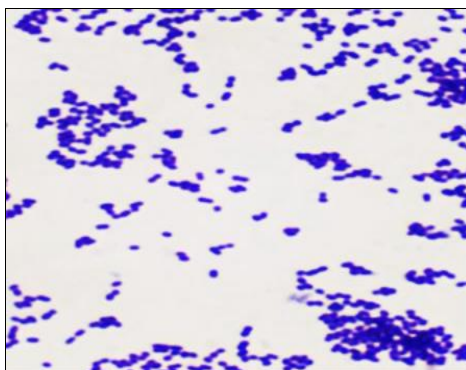

Figure (1): ***Streptococcus pyogenes*** **cocci. (Gram stain X100).**

Figure (2): Inhibition zone around the bacitracin disc.

Molecular analysis of 16S rRNA genes

S. pyogenes isolates from humans were further analyzed for the presence of the 16sRNAgene according to the response state reported in (Table 2). PCR results of the 16S rRNA gene demonstrated the presence of 16S rRNA gene; which is display like a bundle on agarose gel with a size of 1250 bp, shown in (Figure 3).

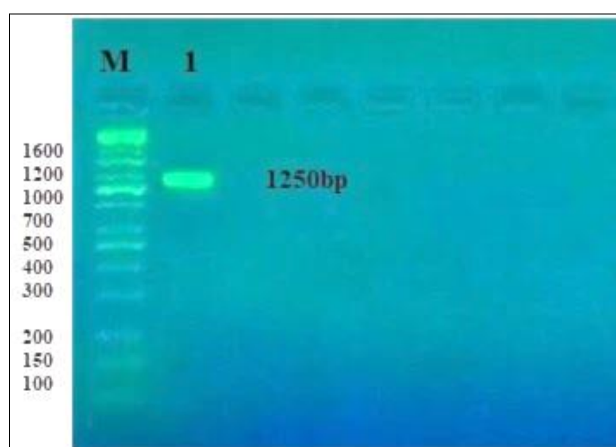

Figure (3): PCR Product the band size. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2 . 1%TBE buffer for 1:3 hours. M: DNA ladder(100).

Detection and sequencing of genes

The DNA fragments successfully amplified with a length of 1250 bp were evaluated as positive for 16sRNA and 978 bp for *speB* gene, while the band of approximately 246 bp represented *smeZ* gene shown in (Table 1) (Figure 4).

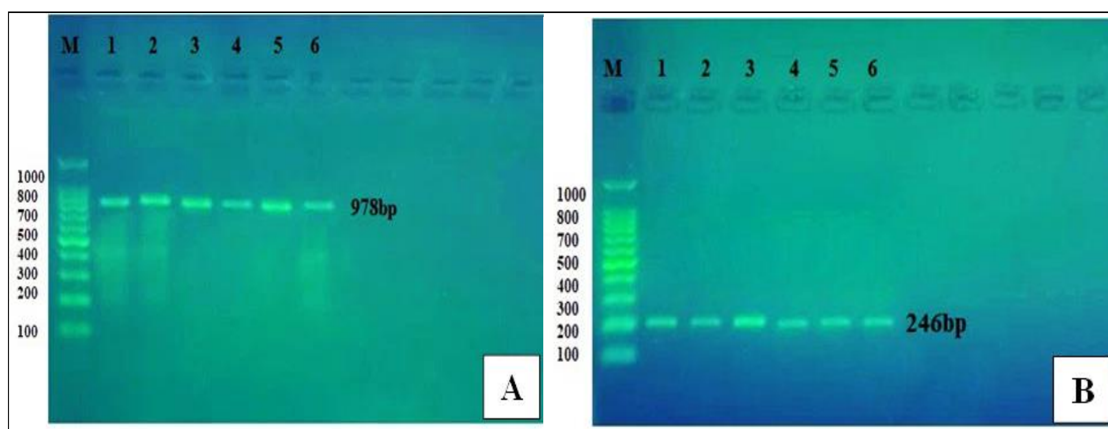

Figure (4): PCR reaction showing *Streptococcus pyogenes* positive for SpeB(A) and smeZ(B) genes in band size (978bp and 246bp), respectively.

S. pyogenes 16S rRNA gene sequencing isolated from humans was analyzed in the NCBI GenBank database and compared with isolated sequences retrieved from the GenBank databases. The sequenced DNA showed a match of 99% of the *S. pyogenes* strain found in NCBI has the accession numbers (ID: ON680896.1); these matches could be distinguished by three different nucleic acid substitutions as in (Table 3) illustrated that the nearest national strain possessed the Sequence ID: MT256086.1 has compatibility of 99% with three variants including; G\A showed transition mutation at 433 and 911 location, while T\A and G\C had transversion mutations at 554 and 693 respectively. Iraqi *S.pyogenes* was registered at NCBI and established as a global reference with the accession number ON680896.1; These nucleocapsid changes may result in a high degree of antibacterial resistance and/or serve a supporting role in the formation of high levels of pathogenicity in bacteria or vice versa.

Table(3): Identical level of *Streptococcus pyogenes* isolated with nearest national strain.

16S ribosomal RNA gene						
Type of substitution	**Location**	**Nucleotide**	**Sequence ID with compare**	**Sequence ID with submission**	**Source**	**Identities**
Transition	433	G\A	ID: MT256086.1	ID:ON680896.1	*Streptococcus pyogenes*	99%
Trans vertion	554	T\A				
Trans vertion	693	G\C				
Transition	911	G\A				

Streptococcus pyogenes strain St Py6 16S ribosomal RNA gene, partial sequence Sequence ID: MT256086.1Length: 1411 Number of Matches: 1

Range 1: 160 to 1069GenBankGraphics

Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1624 bits(1800)	0.0	906/910(99%)	0/910(0%)	Plus/Plus

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Query 1    TTGCTCCACTATGAGATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACC 60
Sbjct 160  ............................................................ 219
Query 61   AAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG 120
Sbjct 220  ............................................................ 279
Query 121  CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCG 180
Sbjct 280  ............................................................ 339
Query 181  AGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGAA 240
Sbjct 340  ............................................................ 399
Query 241  TGATGGTGGGAGTGGAAAATCCACCAAGTGACGATAACTAACCAGAAAGGGACGGCTAAC 300
Sbjct 400  ................................G........................... 459
Query 301  TACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGT 360
Sbjct 460  ............................................................ 519
Query 361  AAAGCGAGCGCAGGCGGTTTTTTAAGTCTGAAGTAAAAGGCATTGGCTCAACCAATGTAC 420
Sbjct 520  .................................T.......................... 579
Query 421  GCTTTGGAAACTGGAGAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGT 480
Sbjct 580  ............................................................ 639
Query 481  GAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTCTAACTG 540
Sbjct 640  .....................................................G...... 699
Query 541  ACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG 600
Sbjct 700  ............................................................ 759
Query 601  TAAACGATGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGGAGCTAACGCATT 660
Sbjct 760  ............................................................ 819
Query 661  AAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCC 720
Sbjct 820  ............................................................ 879
Query 721  CGCACAAGCGGTGGAGCATGTGGTTTAATTCAAAGCAACGCGAAGAACCTTACCAGGTCT 780
Sbjct 880  ..............................G............................. 939
Query 781  TGACATCCCGATGCCCGCTCTAGAGATAGAGTTTTACTTCGGTACATCGGTGACAGGTGG 840
Sbjct 940  ............................................................ 999
Query 841  TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA 900
Sbjct 1000 ............................................................ 1059
Query 901  CCCCTATTGT 910
Sbjct 1060 .......... 1069
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Phylogenic tree of the *16sRNA* gene from *Streptococcus pyogenes*

The Iraqi isolates recorded in NCBI were in concordance of 99% with isolates from different sources including (Brazil ID: CP041615.1,Nigeria ID: MZ914644.1, Poland ID: MZ951159.1, Saudi Arabia ID: CP094944.1, New Zealand ID: CP035455.1, United Kingdom ID: LR590483.1, Japan ID: AP019548.2, China ID: MK341735.1, Netherlands ID: MK330587.1, USA ID: CP044093.1, Australia ID: CP045930.1, France ID: CP036531.1,Croatia ID: MN923021.1, India ID: CP049800.1 and Egypt ID: MT256086.1) shown in (Figure 5) and (Table 4).

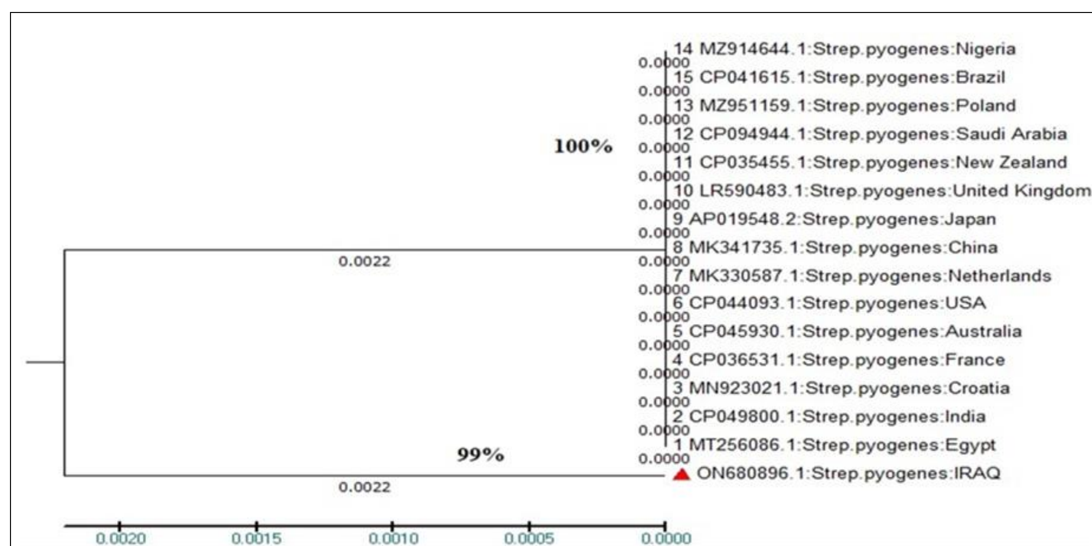

Figure (5): Phylogenetic tree of *Streptococcus pyogenes.* The isolate used in the current study is labled with red color. MEGA software version 6.0 was used to create the analysis using the neighbor-joining method.

Table (4): Comparative analysis of the 16S rRNA gene for *Streptococcus pyogenes*.

	Accession	Country	Source	Host	Compatibility
1.	ID: MT256086.1	Egypt	Streptococcus pyogenes	Homo sapiens	99%
2.	ID: CP049800.1	India	Streptococcus pyogenes	Homo sapiens	99%
3.	ID: MN923021.1	Croatia	Streptococcus pyogenes	Homo sapiens	99%
4.	ID: CP036531.1	France	Streptococcus pyogenes	Homo sapiens	99%
5.	ID: CP045930.1	Australia	Streptococcus pyogenes	Homo sapiens	99%
6.	ID: CP044093.1	USA	Streptococcus pyogenes	Homo sapiens	99%
7.	ID: MK330587.1	Netherlands	----------------	---------------	---------
8.	ID: MK341735.1	China	----------------	---------------	---------
9.	ID: AP019548.2	Japan	----------------	---------------	---------
10.	ID: LR590483.1	United Kingdom	----------------	---------------	---------
11.	ID: CP035455.1	New Zealand	Streptococcus pyogenes	Homo sapiens	99%
12.	ID: CP094944.1	Saudi Arabia	Streptococcus pyogenes	Homo sapiens	99%
13.	ID: MZ951159.1	Poland	Streptococcus pyogenes	Homo sapiens	99%
14.	ID: MZ914644.1	Nigeria	----------------	---------------	---------
15.	ID: CP041615.1	Brazil	Streptococcus pyogenes	Homo sapiens	99%

Streptococcus pyogenes is a *B*-hemolytic type of streptococcus that produces many virulence factors helping to colonize and adhere to the mucosal surface of host epithelial cells with a changeful rate of *S.pyogenes* infection in both humans and animals dramatically as a result of genotyping wide diversity in genetic in addition to lteration of host susceptibility. As the first step toward the biochemical identification of these types of gram-positive bacteria which appeared dark blue cocci when stained by gram stain because their peptidoglycan cell wall absorbed crystal violet while (17) indicated staining of microcolonies by blue-violet color revealed investigative of biofilms in specimens that give apositive result for the present of *streptococcus pyogenes*, however, in the current study used bacitracin disc to

presumptive distinguish of *S.pyogenes* from other streptococcus types by formation zone of inhibition around a bacitracin disc indicate that the organism is sensitive to these antibiotics and these observation like with (18,1) who notice large inhibited zone around the bacitracin disc because this type of antibiotic is a polypeptide produced by Bacillus subtilis and inhibits the synthesis of bacterial cell wall and disrupts their cell membrane.

The data of our study estimate that the *S.pyogenes* isolation percentage was (20%) and this result was recorded after the identification of isolated bacteria by previous biochemical tests and PCR; this percentage was supported by (19, 20) who identified tonsillitis and pharyngitis caused by *Streptococcuspyogenes* and *Staphylo coccus aureus* reach to 20% while the frequency of *S.pyogenes* near to these results was documented with a percentage of 20.2 % and consider higher comparative with 11.3% which reported in Ethiopia (21) and 12% in Turkey (22) and lower than other percentages that noted by (23) 25.5% , (24) 24.1%, (25) 30%, and (26) 46% This result harmonized with another study by (27), who indicated that human was the primary source of infection by *Streptococcus pyogenes*; after that, maybe transmission by Asymptomatic carriers to domestic animals such as domestic cats, dogs, and rabbits when consuming food-contaminated by *S. pyogenes*.

In order to detect the presence of 16 sRNA in the isolated sample (28, 23) reported that 16S ribosomal RNA as central structural component and primer that is widely used for genetic evolutionary studies and identification of numerous bacterial types (29) like *Streptococcus pyogenes* because their fixative ability for that required longer time to change, in addition, the data base of16srRNA sequence were available at GenBank and the discovery of novel species this interpretation was supported by (30, 31) who indicate that 16S rRNA gene has more than 20 million deposited sequences for over 90,000 of 16S rRNA gene comparative the sequences of unknown isolated bacteria with many previously known deposited sequences, It used extensively in bacterial phylogenetic for that consider dependable markers for the phylogenetic analysis (32).

Severity and pathogenic properties of *S. pyogenes* infection associated with production of multiple virulence factors that can predictor in bacterial adapted and invasive in infected host and to indicate present these pathogenic bacteria and their virulence genes used 16S ribosomal RNA, *speB* and *smeZ* in current work which product at band size 1250 bp, 978 bp and246 bp respectively as marker because *SpeB* specific and major targeting genes of *S.pyogenes* recorded as cysteine protease by (3) modern study and recorded substantial role in promoting the spreading and demeaning of bacteria also this gene existing in all strain of group A streptococcus but not all strains produce it while (33) explain the super antigenic activity of *SpeB* gene while (34) explain their role in increasing the immunity resistance, another studies by (35) explain role of *SpeB* in pathogenic side by regulating the proteolytic cleavage of infected host and streptococcal in addition their role as pro-inflammatory mechanism by activate precursors of IL-10-alpha and IL 36-gamma.

S.pyogenes produced several movable elements that help in the

transferred of *smeZ*, *speA*, *speC*, *speH*, *speJ,* and *ssa* genes through chromosomal DNA due to correlation between these several elements and chromosomal DNA(26) for that used the superantigen streptococcal mitogenic exotoxin (*smeZ*) gene in current work as primer to the identification of *S.pyogenes* infection because it has imperative role and coded as highly distribution mitogenic proteins and T cells forceful activators (34), in addition, their crucial role in prophages (integrated of the bacteriophages by the bacterial chromosome).

The crucial technique used for genomic comparison is molecular phylogenetics, in which categories, metagenomic sequences, and genetic identification, the phylogenetic trees for different species of Streptococcus explained in many investigations (36, 37).

In our study the result of 16S rRNAgene sequencing and phylogenetic data analysis evidence the similarity of *S.pyogenes* throat swabs sample with national strains such as India and Egypt, this consoled to the entry of tourists from these countries to Iraq (38).

Conclusion

Conventional PCR is the best specific technique with high accuracy used for confirmed *S.pyogenes* infection by using 16S ribosomal RNA, speB, and smeZ virulence genes, in addition to phylogenetic tree that indicated high genetic resemblance of current isolate to Egypt and India strains.

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