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ISOLATION AND IDENTIFICATION OF THE *STREPTOCOCCUS PYOGENES* CAUSING ACUTE TONSILLITIS AND DETECTING FOR SOME VIRULENCE FACTOR BY PCR

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By Mohammed Nsaif Jasim JASIM

February 2023

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

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Master of Science in Biology

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In this study, 100 samples were collected from tonsillitis patients of both genders aged 1-40 years who applied to Al-Harith National Hospital in Salah al-Din Governorate of Iraq between 9/10/2021-30/01/2022. The isolates were diagnosed by bastracin susceptibility as well as microscopic and culture characteristics and biochemical tests, and the diagnosis was confirmed by the Vitek 2 system. The isolation rate was recorded as 10%, ie 10 isolates belonged to *Streptococcus pyogenes*. The results showed that the highest isolation rate was 76% in tonsil samples and the highest isolation rate was in the 1-10 age group. Some of the virulence factors of these bacteria were also examined and the results showed that all isolates have bacterial encapsulation and the ability to produce DNase and hemolysin enzymes in addition to their biofilm forming abilities. Drug susceptibility testing was performed for all isolates of 10 antibiotics. All isolated bacteria were highly sensitive to Amoxicillin clavulanic, Imipenem, Meropenem and Levofloxacin and highly resistant to Amoxicillin and Ampicillin. Some virulence factors were studied molecularly and using thermal polymerase PCR technique. Agarose gel electrophoresis results revealed that all isolates contained Emm, ScpA, Spe1 and GyrB genes.

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Keywords: Acute tonsillitis, PCR, *S. pyogenes,* Virulence factor

ÖZET

AKUT TONSİLİTE NEDEN OLAN *STREPTOKOK PİYOJENLER*'İN İZOLASYONU VE TEŞHİSİ VE PCR İLE BAZI VİRÜLANS FAKTÖRLERİNİN TESPİTİ

Mohammed Nsaif Jasim JASIM
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Şubat 2023

Bu çalışmada, 9/10/2021-30/01/2022 tarihleri arasında Irak'ın Salah al-Din Valiliği'ndeki Al-Harith Ulusal Hastanesine başvuran her iki cinsiyetten 1-40 yaş arası bademcik iltihabı hastalarından 100 örnek toplanmıştır. İzolatların teşhisi bastracin duyarlılığının yanı sıra mikroskobik ve kültür özellikleri ve biyokimyasal testler ile teşhis edilmiş ve teşhis Vitek 2 sistemi ile doğrulanmıştır. İzolasyon oranı %10 olarak kaydedildi, yani 10 izolat Streptococcus pyogenes'e aitti. Sonuçlar bademcik örneklerinde en yüksek izolasyon oranının %76 olduğunu ve en yüksek izolasyon oranının 1-10 yaş grubunda olduğunu göstermiştir. Bu bakterilerin virülans faktörlerinden bazıları da incelendi ve sonuçlar, tüm izolatların bakteri kapsülüne sahip olduğunu ve biyofilm oluşturma yeteneklerine ek olarak DNaz ve hemolizin enzimleri üretme kabiliyetine sahip olduğunu göstermiştir. 10 antibiyotiğin tüm izolatları için ilaç duyarlılık testi yapılmıştır. İzole edilen tüm bakteriler, Amoxicillin clavulanic, Imipenem, Meropenem ve Levofloxacin'e karşı oldukça duyarlı ve Amoxicillin ve Ampicillin'e karşı oldukça dirençlidir. Bazı virülans faktörleri moleküler olarak ve termal polimeraz PCR tekniği kullanılarak incelenmiştir. Agaroz jel elektroforez sonuçları tüm izolatların Emm, ScpA, Spe1 ve GyrB genlerini içerdiğini ortaya çıkarmıştır.

2023, 69 sayfa

Anahtar Kelimeler: Akut tonsillit, PCR, *S. piyojen*, Virülans faktörü

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Mohammed Nsaif Jasim JASIM
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CONTENTS

LIST OF SYMBOLS

%	Percent
mL	Milileter
µm	Micrometer
BP	Base pair
CFU	Colony forming unit
GR	Gram
KDA	Kilodalton
L	Liter
N	Normal
ºC	Celsius degrees
PPM	One in a million
VER	Version
β	Beta

LIST OF ABBREVIATIONS

A-CHO	A-carbohydrate
CLSI	Clinical and laboratory standards ınstitute
CT	Computed tomography
D.W	Distilled water
HCL	Hydrochloric acid
ICU	Intensive care unit
KOH	Potassium hydroxide
MDR	Multiple drug resistance
PCR	Polymerase chain reaction
SLO	Streptolysin O
SLS	Streptolysin S
WHO	World health organization

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Streptococcus pyogenes is a Gram-positive, spherical, non-motile, paired or chained, non-motile bacteria capable of fully hydrolyzing blood cells (Shulman *et al*. 2012). It is part of the normal flora in the respiratory system, and it plays an important role in causing many diseases. These diseases range from mild superficial skin infections to life-threatening systemic diseases such as joint infection (arthritis) and blood poisoning in newborns, childbirth, meningitis, lung infection, and glomerulonephritis (Eraso *et al*. 2020). It affects the pharynx and causes infections such as pharyngitis, necrosis of the fascia, and toxic shock syndrome. Its pathogenesis is linked to its possession of many virulence factors that enable these bacteria to colonize, grow, and exist between cells, the host as a capstone, and form biofilms (Alamiri *et al*. 2020). *S. pyogenes* bacteria carry a number of genes encoding multiple virulence factors, and their presence is often associated with disease severity; these factors are either in the form of exotoxins such as *SpeA*, *SpeC*, and *SpeB* or in the form of membrane-bound toxins such as protein M *emm* and peptidase enzyme C5a *scpA* (Babbar *et al*. 2018).

These bacteria are very sensitive to penicillin antibiotics, but the irregular and indiscriminate use of these antibiotics, as well as the use of other types of antibiotics, has caused the emergence of strains resistant to these antibiotics, and therefore these antibiotics are inefficient to treat diseases caused by *S. pyogenes*. Furthermore, bacteria have several mechanisms that aid in antibiotic resistance, such as changing the barrier to permeability, changing the location of the target, or producing enzymes that degrade antibiotics, such as enzymes that break down the beta-lactam ring and render it ineffective molecules (Sun *et al.* 2012).

Aim of study: 1. Isolation and identification of *S. pyogenes* from patients with tonsil infections, different age groups and both genders. 2. Investigation of virulence factors genetically and phenotypically. 3. Studying the pattern of resistance to antibiotics and determining the dominant pattern of resistance among local isolates.

2. LITERATURE REVIEW

2.1 *Streptococcus* Spp.

The term *Streptococcus* consists of two syllables, and its root is strepto, which means twisted or coiled, and coccus, which means spherical in shape. The term "*Streptococcus*" was used for the first time by Billroth in 1847 to describe bacteria, which form chains of spherical shapes that he saw in wounds and abscesses in the bodies of animals. Ogston was able to isolate rosarias from highly inflammatory wounds in 1881 and distinguished between them and *Staphylococci,* describing their pathogenicity (Mpala *et al.* 2019).

Crocodilians need to grow in rich nutrient media, most of which are anaerobic. Croshaids on blood sugar medium exhibit varying degrees of hemolysis in the medium, and this trait, along with the phenotypic shape of the colonies and serospecification Lancefield classification, is considered diagnostic, early, and important in determining the type of clinical isolates, which is of the alpha type a-hemolysis for some types of *Streptococcus*, such as *Streptococcus pneumonia*, and the apical group of *Streptococcus*, which (Almasaudi *et al.* 2017).

Streptococcus that completely degrades red blood cells of type B hemolysis can be divided into groups from A to V with the exception of I and J. The rosarias are part of the natural flora of the skin, vagina, and respiratory system (Todar 2002). It is also the cause of many diseases for humans of different ages, and the diseases it causes range from moderate severity, such as pharyngeal infection and tissue infection of the skin, to severe, such as life-threatening systemic diseases such as lung infection, meningitis, endocarditis, septicemia, and glomerulonephritis (Mpala *et al*. 2019).

2.2 Classification of *Streptococci*

Scientists have adopted many bases in classifying *Streptococcus* bacteria, including colony shape, external appearance and color, as well as the location of the bacteria and their presence in the host's body, in addition to biochemical tests and the extent of their tolerance and resistance to physical factors. Beta-Hemolytic *Streptococci*, these include the following: *S.Pyogenes, S.agalactiae, S.dysgalactiae, S.canis, S.porcinus, S.iniae.* Non-Beta-Hemolytic *Stococci,S.Pneumoniae, S.infantarius, S.lutetiensis* (Tweten 2005).

2.3 Complete Beta-Hemolytic *Streptococci*

The types of bacteria in this group are characterized by their ability to completely decompose red blood cells on the medium of the blood, and there is a clear area around the colonies indicating the decomposition of red blood cells by the enzyme hemolycin (Brook *et al*. 2014). Brown was the first researcher to describe the interactions of *Streptococci* on a medium of blood in 1919, and the hemolytic *Streptococci* were classified as causing complete lysis according to the Latsfield classification. Lancefield classified germs into 20 groups based on the antigenic characteristics of the carbohydrate group present in the cell wall of the germs in each group, and each group was assigned an English letter symbol from A to V, omitting the I and J, including *S.pyogenes* (Golińska *et al*. 2016).

2.4 *Streptococcus Pyogenes*

2.4.1 General characters of *S.pyogenes*

They are spherical in shape, positive for gram stain, unable to move, and they are not spores; they are also called flesh-eating anaerobes. They need rich growth media because they are very sensitive, but in general, they grow on the medium of blood traces, through which decomposition can be detected. Whole blood cells are Pinna type, with a diameter ranging from 0.5 to 1.0 m (Shulman *et al*. 2012), which can be in the form of long chains

in liquid media and sometimes short chains or in pairs in clinical samples. Friedrich Fehleisen has been able to It was cultivated for the first time in the year 1883, as he was the first to be able to do so, and it is the main cause of many diseases that afflict people of all ages and are widespread infections and diseases (Brook *et al*. 2014).

In 1928, Rebecca Lancefield published the method used to determine serotypes based on the M protein and was able to describe the antigen seroclassification by detecting four out of twenty T antigens (Almasaudi *et al.* 2017). Estimates indicate that 5–15% of normal individuals can have *S. pyogenes* in their respiratory system and have it occur without symptoms as a normal home for them (Chang *et al.* 2011). It also kills 500,000 people each year, making it the world leader (Cho and Caparon 2008). It has many virulence factors, including M protein, streptokinase, teichoic acid, stretolysin, peptidoglycan, and hyaluronidase, in addition to secreting toxins out of the cell to attack immune cells (Walker *et al*. 2005).

They possess *streptococcal* pyrogenic exotoxins, as well as superantigen properties that lead to rapid activation of second lymphocytes, proliferation and release of large amounts of proinflammatory and cytokines, which leads to the formation of toxic shock (Golińska *et al*. 2016). Bacteria use their virulence factors in order to be able to enter the body, as well as to weaken the defense mechanisms of the immune system, as well as to evade the immune system (Ogawa *et al*. 2013). It can weaken the mechanism of swallowing by cytolysin, which helps in the decomposition of phagocytic cells and also works on programmed cell death (apoptosis) (Kwinn and Nizet 2007).

2.4.2 Genome of *S. pyogenes*

The chromosome length of *S. Pyogenes* ranges between 1.67 and 1.98 million base pairs and is estimated to encode between 1495 and 1976 genes, most of which are not functionally classified (Brook *et al*. 2014). Distinguishing features of the *S. pyogenes* genome include the presence of 5–6 16 tRNAs and rRNAs (67–57), 0–10 protective elements, and 0–1 plasmids, and the core genomes of other crop species are estimated to be smaller than this (Davies *et al*. 2019).

DNA gyrase is a major enzyme that facilitates the lysing and segregation of strands and supercoiling, which depends on ATP, and it consists of two units (B and A) necessary for DNA replication, transcription, recombination, and repair (Davies *et al*. 2019). Other topological isomers of dsDNA loops, such as catenins and complex loops, can also be converted by this enzyme in an ATP-dependent manner (Vekemans *et al*. 2019)**.** Gyrase belongs to a class of enzymes known as topoisomerases that are involved in controlling the topological transformations of DNA, making DNA gyrase an intracellular target for a number of antibacterial agents (Davies *et al*. 2019).

2.5 Virulence Factors of *S. Pyogenes*

S. pyogenes also possesses *S. pyogenes* has numerous other virulence factors that allow it to bind to host tissues, evade the immune response, penetrate into the tissue layers of the host skin, and spread (Bisno *et al.* 2003). For swimming pools, especially *S. pyogenes,* there *are many* and varied factors that help in pathogenesis and increase their virulence, and some of them are part of the antigenic structure of bacteria, which includes each of the antigens of the group A carbohydrate (A-CHO), the capsule, peptidoglycan, M LTA lipoteichoic acid, and another type of virulence factors that are extracellular products such as toxins (Walker *et al.* 2005).

2.5.1 Hemolysin

It is a type of blood-breaking protein in red blood cells, which has a molecular structure that varies according to the type of bacteria. *S. pyogenes* secretes two types of main and basic toxisthatide the cell called *Streptococca*l cytolysins, streptolysin O (SLO) and streptolysin S (SLS), which can infect a variety of eukaryotic organisms, have varying effects, and cause complete hemolysis (Feil *et al*. 2014).

A 69-kDa cholesterol-dependent altered oxygen and cytolysin capable of forming large pores in the host cell's membrane to induce apoptosis is a relay-dependent pore-formed for entry and transport of NAD + -glycohydrolase, contributing to NADase's

pathogenicity. It is delivered to the cytoplasm of host cells and expressed in association with SLO via a procedure called cytolysin-mediated translocation (Farrand *et al*. 2015). SLO-mediated NADase delivery interferes with cellular repair mechanisms and prevents phagocytosis, thus preventing bacterial destruction and death. NADase plays an important role in promoting intracellular survival and gas proliferation (Mozola and Caparon 2015).

However, SLO induces an immune response during infection, and citrateolicin antibodies are still used to confirm streptococcal infection (Chiarot *et al*. 2013). In addition to SLO, *S. pyogenes* produces a second hemolysin known as SLS, so named because its activity is almost stationary in relation to oxygen and is encoded by nine genes (SAG and *operm*, SLS is secreted by many Christian strains, and is responsible for hemolysis of the b-hemolytic type surrounding the colonies in the middle of the blood agar. Similar to SLO, SLS possesses a broad spectrum of cytotoxicity that targets red blood cells, platelets, and white blood cells under unfavorable conditions (Molloy *et al*. 2011). And that through the action of pores in the membrane, SLS is hydrophilic and disrupts the osmotic balance, which leads to cell lysis, however, recent studies of SLS indicate a more complex role in increasing the pathogenicity of *Streptococcus* bacteria (Sumitomo *et al*. 2013).

2.5.2 Lipoteichoic acid (LTA)

Linoteichoic acid (LTA) is an important hydrophobic component on the cell surface of Gram-positive bacteria, contributing to adhesion and biofilm formation (Russell and Herwald 2005). In addition, it can improve many physiological properties, such as, B regulation of autolysase, which may also play a role in the removal of heavy metals (Percy and Gründling 2014). This acid consists of lipid membranes or glycolipids, covalently bound to polymers; the first reported LTA receptor was fibronectin, and it was activated by glycerol phosphate or diphenol phosphate, with a variety of sugars and amino acids acting as substitutes. The resulting surface profile of LTA is a fatty acid moiety that complexes LTA with other *Streptococcal* surface proteins such as M protein and other as yet unidentified trypsin-sensitive proteins (Harry *et al*. 2009).

2.5.3 Multiple gene activator (mga)

Streptococcus polygenic activator. pyogenes are very large DNA-binding proteins containing 500 amino acids with a molecular size of approximately 62 kDa (Vahling and McIver 2006). Mga is known as an integrative virulence regulator because it regulates a wide range of genes involved in the pathogenesis of streptococcal disease. purulent (Hondorp and McIver 2007).

Mga is a ubiquitous virulence modulator whose products are essential and play a role in immune evasion, such as M protein, Ca peptidase, and serum turbidity factor (Limbago *et al.* 2001, Hondorp and McIver 2007). MGA controls the expression of genes that code for proteins that allow bacteria to colonize particular host tissues, such as the upper respiratory tract, deep soft tissue infections, and *E. coli* colonization. In the early stages of infection, immune responses to stress and carbohydrate metabolism are prevented (Ribardo and McIver 2006).

Mga was the first apparent regulatory network in Streptococcus. The pyogenes and themselves are important virulence regulators that enable the pathogen to adapt and thrive in a host environment conducive to growth (Hondorp and McIver 2007). Mga is also regulated by many other streptococcal proteins, such as temperature, pH, carbon dioxide levels, and iron concentration (Finn *et al.* 2006). pyogenic bacteria such as B. streptococcus complement inhibitor and bicistronic FBP (sof-sbfX) (Cunningham 2000, Geyer and Schmidt 2000).

2.5.4 Capsule

It is considered the first line of defense for bacteria as it consists of hyaluronic acid and works to protect bacteria from the process of phagocytosis as it has to do with adherence and invasion (Wessels 2019), because it contains the majority of *S. pyogenes* strains and pathogenic strains from the Christian C and G serogroups on the portfolio, which is one of the most important factors in its virulence (Stevens and Kaplan 2000). The capsule is

formed in the early stages of bacterial growth in the medium, as well as when it spreads in the blood and tissues. It contributes with M protein to resisting and preventing the process of phagocytosis, and in the process of persistence, the bacteria are lacking the capsule (Hancz *et al.* 2007). The capsule is characterized by being sensitive to the enzyme hyaluronidase and also not stimulating immunity because it is chemically similar to the available Hyaluronate, the naturally occurring material in the host's binding tissues, works to mask bacterial antigens and prevent the host immune system from recognizing them (Todar 2002).

2.5.5 Sags protein

SAgs are proteins with prominent cleavage peptides and a molecular weight of 22–28 kDa (Mccormick *et al*. 2006). It is a member of a wide family of toxins that also contains structurally related *Streptococcal* SAgs and shares an amino acid sequence between 17% and 48% (Proft *et al*. 2003). In *S. pyogenes*, about 13 toxins have been identified so far: *SpeA, SpeB, SpeM, Spel, SpeK, Spel, Spel, Spel, SpeH, SpeG, SpeF, SpeC*, and *streptococcal* mitogenic exotoxin Z (Tortora *et al*. 2013).

SAg8 Superoxide SAg8 binds to major histocompatibility complex II (MHCII) molecules on host cells to present antigens and T cell receptors, thereby releasing numerous cytokines. These cytokines have been demonstrated to play crucial roles in organ failure brought on by infection, shock, tissue destruction, fever, and hypotension (Lappin and Ferguson 2009). SpeA binds to the alpha subunit of MHCII; other superantigens bind to surrounding MHCII subunits, while SpeC contains binding sites for both the alpha and beta subunits. Many manifestations of scarlet fever and toxic rosacea syndrome appear to be caused by coccidioides exotoxin or erythrotoxin (Tortora *et al.* 2013).

Erythropoietic exotoxins Spes A, B, and C, known as streptococcal pyrogenic exotoxins, are known to disrupt the plasma membrane in subcutaneous capillaries and cause rashes from a single source (Tortora *et al.* 2013). In the etiology of necrotizing soft tissue infection and toxic shock syndrome, SpeA is a powerful antigen and cytotoxin caused by *Streptococcus pyogenes* bacteria. *Streptococcus* appears to produce pyrogenic exotoxins,

or "red toxins," which are the red toxins that cause many of the manifestations of scarlet fever and *Streptococcus* syndrome. There are different types of erythrocyte toxins; depending on the S. pyogenes strain, some strains may not produce venom (Proft *et al.* 2003). Phage encodes the SpeA, SpeC, and SpeH-M genes (Boyd *et al.* 2012), SpeB and SpeI genes are chromosomal, although (Llewelyn and Cohen 2002). Conversely, the SpeG gene attributable to nuclear chromosomes and lysogenic phages is a streptococcal toxic shock syndrome (Beres *et al.* 2006, Brosnahan and Schlievert 2011).

SpeA is a virulent agent that can invade and cause soft-tissue infection or bacteraemia caused by *S. pyogenes*, and the role of this toxin can be different in hosts that have a superior response to superantigens (Llewelyn and Cohen 2002). The Sped gene was previously regenerated as a damage factor associated with a disease that invades the body and invades tissues, therefore the production of SpeA by *S. pyogenes* has been connected to streptococcal toxic shock syndrome (Beres *et al*. 2006).

SpeC is an antigen produced by several Strep strains. pyogenes, closely related to streptococcal diseases such as toxic shock syndrome STSS, encodes a mature protein of 208 amino acids with a calculated molecular weight of 24,354. The mature amino acid sequence of SpeC is homologous to this type of amino acid sequence. An exotoxin. He then described five allelic variants, SpeCI-SpeC5, of the most common SpeC, SpeCI, and SpeC2 alleles in Streptococcus strains. pyogenic bacteria (Spaulding *et al.* 2013).

SpeA and SpeC induce activation of antigen-nonspecific T lymphocytes, inhibit antibody synthesis, induce fever, enhance the release of precursor cytokines, and possibly lead to multiorgan failure (Cunningham 2008). In streptococci, SpeA and SpeC are more prevalent. However, SpeA, which has four alleles, is a crucial virulence component in streptococcal infection (SpeAl-SpeA4), with the SpeA2 and SpeA3 alleles being the most common alleles in infection (Alba *et al.* 2006). SpeC streptococcal protein or cysteine B protease is one of the most important virulence factors in streptococci. The pyogenic cysteine protease is secreted as a 4 kDa zymogen that is cleaved to a 28 kDa mRNA under reducing conditions (Chuan and Jiunn 2008).

SpeC is homologous to the amino acid sequences of exotoxins of type A (Spaulding *et al*. 2013). Protective immunity against Streptococcus. Septic infection is achieved by antibodies against this protein (Walker *et al.* 2014.). M proteins released by bacteria into the circulation promote systemic activation of the coagulation cascade during infection, and soluble platelets can also be activated to form complexes with neutrophils and monocytes, leading to activation of these cells and induction of additional Inflammation again (Hraoui *et al*. 2010).

2.5.6 M protein

The cell wall of *S. pyogenes* contains an important and distinct antigen fragment called M-protein, which was replenished by Rebecca Lancefield about 90 years ago (1928). According to Walker *et al.* (2014), it is one and the most important among many cell wall virulence factors. The M protein is the most abundant protein and is encoded by the *emm* gene, and studies have shown that not all members of the genus *Pyogenes* contain it (Le Breton *et al*. 2013).

It is a highly polymorphic protein with a size of 41–80 kDa, and the structure of the M protein is similar to that of the *Staphylococcal* protein A. Despite extensive antigenic variability, all M proteins have an alpha-helical coiled configuration, which forms a fibrous structure about 50–60 nm long, extruding from the bacterial cell wall (Walker *et al.* 2005). Several studies have shown that M protein molecules, arranged in a hair-like structure under electron microscopy, are important virulence factors for *S. pyogenes*, discovered by Rebecca Lansfield more than 80 years ago (Metzgar and Zanpolli 2011).

M proteins released by bacteria into the circulation promote systemic activation of the coagulation cascade during infection, and soluble platelets can also be activated to form complexes with neutrophils and monocytes, leading to activation of these cells and induction of additional inflammation (Le Rhun *et al*. 2019). It has a hydrophobic region called the C-terminal that acts as a nodal anchor, and it binds to the bacterial cell wall through this region, which is rich in amino acids (proline and glycine) (Oehmcke and Herwald 2010). The M protein also consists of a highly variable region called N-Terminal

that confers antibody protection in this region, which explains the pathogenesis (Le Rhun *et al*. 2019).

The protein structure was studied, and it showed that the A repeat consisted of 14 amino acids, while the B repeat consisted of 25 amino acids, the C repeat consisted of 42 amino acids, and the D repeats, made up of a small number of amino acids, displayed some symmetry with each other (Metzgar and Zambolli 2011). The combination of these M-protein repeats facilitated its ability to be mutagenic and for bacteria to evade immune recognition (Oehmcke and Herwald 2010).

The N-terminal (amino-terminal) region is highly variable and responsible for serotype specificity; the M protein is involved in a different stage of the pathogenesis of *S. pyogenes* from adhesion, immune evasion, tissue colonization, resistance to phagocytosis, and the killing of polymorphous leukocytes by its binding With fibrinogen and complement regulatory proteins, it acts on the complementary shadowing of host cells (Le Breton *et al*. 2013).

M protein also confers phagocytosis resistance through non-immune binding of the FC region of IgG antibodies; in addition, M protein plays an important role in tissue colonization as it has been shown to inhibit gas attachment to keratinocytes in the skin mediated by Attached to the cell membrane are CD46 cofactor keratinocytes (Figure 2.1) (Metzgar and Zambolli 2011)

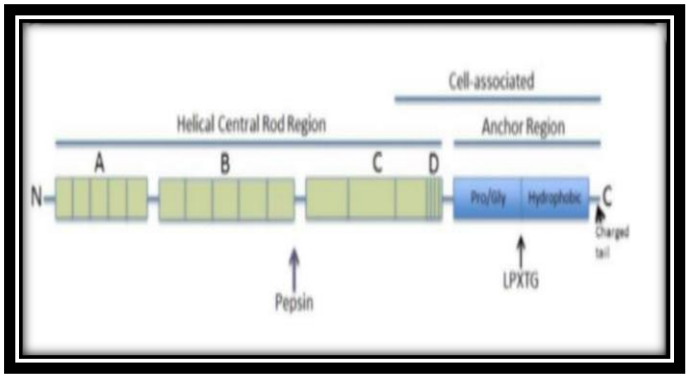

Figure 2.1 Shows the 6M protein sequence showing the four repeat blocks of the N-terminal region (Metzgar and Zampolli 2011)

2.5.7 C5a peptidase *scpA*

Bacterial cell membrane-associated surface protein, an important virulence factor affecting host cells, protects Streptococcus is derived from the phagocytosis of pyogenes and has been suggested to subvert host defenses, it is a major factor in the accumulation of neutrophils at sites of infection, delaying phagocytic infiltration and thus bacterial clearance from the mucosa and subcutaneously (Stevens and Kaplan 2000). A serine-like subtilisin, C5a-induced cleavage of C5a peptidase in the human terminal COOH region, significantly reduces the ability of anaphylatoxin to bind to polymorphonuclear neutrophil (PMNL) surface receptors, thus abolishing their chemical activity (Buckley *et al.* 2018). The C5a peptidase is thought to be crucial for bacterial colonization of the host by inhibiting the influx of PMNL and affecting the primary clearance of Strep. The pyogenic C5a peptidase is encoded in *Streptococcus*. *Streptococcus pyogenes* is caused by the chromosomal gene scpA, and the active site of the C5a peptidase is within the NH2-terminal half of the NH2-terminal O-chain polypeptide. However, how does it recognize and bind Strep it is unknown whether C54 is suppurative (Shumba *et al.* 2019). A C5a-binding protein was detected in Strep's 8-M urea extract. By ligand blot and biotin-treated C5 purulent known as C5a, as *Streptococcus* surface dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase GAPDH, a search of genomic databases revealed that the C5a-binding protein is also identical to the lymph node plasmin receptor (Buckley *et al*. 2018).

2.5.8 Biofilm formation of *S. pyogenes*

The ability to create biofilms is an essential component of bacterial pathogenicity, and *Streptococcus pyogenes* infection pathogenesis makes use of this innate characteristic, it is an intricate assemblage of microbes that develop on solid surfaces. Biofilms on surfaces are characterized by the unique structure of tiny colonies surrounded by membranous tissue and the hygroscopic nature of microbial proteins, nucleic acids, and polysaccharides (Stoodley *et al*. 2002). The presence of biofilms is a basis for the physiology of chronic tonsillitis. The biofilm of a polysaccharide of bacteria was discovered within the folds of the infected tonsils in the majority of patients with

persistent upper respiratory infection, and a small group of bacteria occurred on the removed tonsils whose antibiotics failed to kill the bacteria and could be *S. pyogenes* that cannot be eliminated with antibiotics is due to the formation of biofilms (Baldassarri *et al*. 2006).

Five distinct stages have been identified in the biofilm development of *S. pyogenes*. The first stage consists of planktonic cells that transiently adhere to the surface. In this stage, only small amounts of extracellular components bind to the attached cells, and many cells are still capable of independent movement. The third and fourth stages involve the development and maturation of the biofilm structure, microcolonies are three-dimensional structures made of clusters of cells and water channels, and the cells within these small colonies begin to change organ functions, eventually leading to the formation of a biofilm. The fifth step entails separating individual cells or cell bundles from the fold and structure of biofilms. These free cells have a role in spreading, recolonizing, and replicating the development of biofilms. In *S. pyogenes*, mature biofilms are known to consist of proteins, DNA, and a polysaccharide-containing substance known as a glycocalyx (Doern *et al*. 2009).

Bacteria that live in biofilms, including streptococci. Pyogenic bacteria can persist for long periods of time on biotic and abiotic surfaces, including soft toys, books, cribs, and other hard surfaces, and contact with surfaces that have become a source of transmission increases the likelihood of exposure (Marks *et al.* 2014). Formation of *Streptococcus Pyogenic* biofilms are important for maintaining asymptomatic carrier status in the tonsils (Roberts *et al.* 2012) and *S. Purulent* biofilms have been observed in infections of the root canal, throat, necrotizing fasciitis, and skin (Teepica *et al.* 2015). However, the development of biofilms increases host defenses and antimicrobial therapy resistance, allowing microbes to flourish in unfavorable conditions (Stoodley *et al.* 2002), and the structure of biofilms can provide bacteria with mechanical stability and Provide a place for biofilm exchange. Genetic elements (Diaz *et al*. 2011).

According to estimates, biofilms are linked to more than 65% of human bacterial illnesses, and bacteria found in biofilms are 1,000 times more resistant to antibiotics than

bacteria found outside of biofilms (Fux *et al.* 2005). Biofilm-forming bacteria can be difficult to eradicate, leading to chronic infections and long-lasting alterations in the tonsil's lymphoid tissue (Brook *et al*. 2014). The biofilm is too large to be taken up by macrophages as a structure, so its presence in the tonsils can interfere with the normal function of the lymphoid tissue of the tonsils, eventually leading to chronic or recurrent infections (Ogra and Welliver 2008). Biofilms have been shown to provide a protective niche for immunological reactions and antibiotic therapy, frequently linked to recurring or persistent bacteria (Roberts *et al*. 2012).

2.6 Pathogenesis of *S. pyogenes*

These bacteria can be transmitted in a variety of ways, including directly through the respiratory system and droplets from an infected person's sneezing and skin contact with healthy people, as well as by touching surfaces and objects, dust particles, or food containing colonies, although its entry into the body is unlikely. With food, it is less common (Kemble *et al*. 2013). The first stage of infection begins with the adhesion of the cell wall molecules of *S. pyogenes* such as fimbrial 12, which are similar to the compact and matrix protein structures of the human extracellular matrix, including laminin and collagen fibronectin (Terao 2012).

The adhesion proteins Fn are among the invasion factors used by bacteria and include SOF, PFBP, FbaA, and B, FI, SfbI, F2, SII protein (Cue *et al*. 2000). *S. pyogenes* has the ability to infect large areas of body tissues, which do not cause the great damage they cause in the body, and as previously stated, the typical and optimal path for infection is the respiratory openings represented by the mouth and nose because they can be more harmful and dangerous when entered through wounds and bruises on the body and injury in deep tissues, and systemic diseases can occur through the bloodstream and lymphatic system (Carapetis *et al*. 2005), it is one of the most common pathogens in humans, and annually, more than 700 million people can develop skin ulcers and pharyngeal infections, and there are more than 650,000 cases of rosacea and toxic shock syndrome (Nizet and Arnold 2012). About 30 million cases of tonsillitis infection occur each year in the world, most of them in the United States of America, *S. pyogenes* cause about 18

million cases of very severe diseases that kill half a million people worldwide annually (Carapetis *et al*. 2005). Epidemiological data suggest that the pharynx, particularly the gooseberries, is the main and main reservoir of *S. pyogenes* bacteria, and that systemic infection may occur from the pharynx and into the bloodstream, possibly resulting in rheumatic fever RF and rheumatic heart disease RHD as a complication of untreated tonsillitis (Watkins *et al*. 2017).

The rate of bacterial tonsil infection, especially group A beta hemolytic streptococci (GABHS), is between 5–15% of adults and 15–30% of children aged 5–15 years, and this can change the percentage according to the aforementioned conditions, and the rate of injuries in developing countries is 5–10 times higher than in developed countries (Todar 2002). Infections of the throat and tonsils are often observed widely and frequently in winter and early spring, especially in temperate climates, but this does not negate the occurrence of infections in other seasons of the year because there are cases recorded at times other than these (Lee and Wessels 2006).

2.6.1 Tonsillitis and pharyngitis

Acute pharyngeal and tonsil infections are the most common in upper respiratory tract infections, which cause high and increasing rates of morbidity and mortality. Most likely, the cause is viral, and the other cause is bacterial, which includes the beta-hemolytic *Streptococcus* group, which is common in children under 16 years of age by 15–30% (Ozkaya *et al*. 2013). The disease causes sores in the throat, secretions in tonsillar exudates, and the tonsils enlarge (enlarged hyperemic tonsils), as well as some cases of enlarged lymph nodes and a severe rise in temperature that reaches 38.5m (Tzelnick *et al*. 2020). Usually the symptoms appear after 2–5 days, and infection of the pharynx and tonsils takes two different forms, namely the acute form, which occurs in the case of the initial infection with bacteria, and the chronic form, which occurs in the event of treatment failure and may recur several times during the year; it can reach 5–6 times, and in the event that recovery and improvement are not obtained despite the extensive use of antibiotics, doctors remove them (Cetin and Düzenli 2020).

2.6.2 Scarlet fever

S. pyogenes is a disease that primarily affects children aged 4 to 8. The clinical signs are similar to tonsillitis and include sore throats, fever, and skin redness that appears 18-48 hours after the illness and is usually small red spots in the neck and chest area that quickly spread to the abdomen, arm, and leg. Most of the clinical signs of disease are due to erythrogenic toxins secreted by *S. pyogenes*, which can cause death, as well as complications such as acute glomerulonephritis and endocarditis, which cause heart valves to malfunction and can lead to death (Bisnob 2010).

2.6.3 Skin infections

Cutaneous infections caused by *S. pyogenes* in common with *Staph.aureus* bacteria cause many infections, as different serotypes of beta-hemolytic streptococci attack the skin of the host causing impetigo and cutaneous infections. Among the skin infections that affect adults, especially those with low immunity, is Erysipelas, which is in the form of redness and infection of the cutaneous superficial tissue of the skin, then it spreads to the extremities and the face quickly and most likely with fever and weakness (Bisnob 2010).

2.7 Antibiotics

They are organic substances produced by microorganisms that have the ability to inhibit the growth of another organism or destroy and kill it; they are often isolated from fungi or bacteria (Patini *et al*. 2020). The most important mechanisms of action of antibiotics include inhibition and remodeling of the cell wall, interference with protein synthesis, interference with DNA synthesis and inhibition of metabolism (Katzung 2018). Since the discovery of antibiotics naturally produced from microbial sources, resistance has emerged rapidly, often shortly after their introduction into clinical use. The activity of antibiotics and resistance are influenced by some differences in the structure of gram-positive or gram-negative bacteria (Haque *et al*. 2018).

2.8 Antibiotic Resistance

Some bacterial species have become important and frequent opportunistic pathogens in hospitals, characterized by resistance to multiple antibiotics that results in intractable disease associated with high morbidity and mortality (Obritsch *et al.* 2004). Infections caused by these community-acquired infections represent a major therapeutic challenge to treatment with antibiotics, so choosing the appropriate dose of antibiotics to start treatment is necessary to avoid complications (Lister *et al*. 2009). These bacterial genera have different patterns of resistance to different antibiotics. Moreover, under specific selective pressure, these bacteria may easily develop strong resistance either through horizontal transfer of resistance genes or through mutation in chromosomally encoded genes (Ekizoglu *et al.* 2016). The resistance of bacteria to multiple antibiotics depends on several different factors, the most important of which is the low permeability of the external surfaces of bacteria. Genetically, it shows a wide range of ethylants for antibiotic resistance through conjugation, transformation, and sprouting, as well as the ability of bacteria to acquire additional resistance genes from other bacteria through the transfer of genetic elements (plasmids, progeny, and winning genes) (Tam *et al.* 2010).

Many infections caused by resistant strains are usually found as secondary infections in patients with cancer, AIDS, COPD, cystic fibrosis, and even among diabetics (Hogardt and Heesemann 2011). The causes of antibiotic resistance are complex, involve different levels of human behavior and society, and affect everyone in the world (Levy and Marshall 2004). Numerous attempts have been made to explain antimicrobial resistance and the interventions needed to address this challenge. The increased resistance of bacteria and fungi to classical antibiotics has led to huge clinical problems, especially in immunodeficiency (HIV) and cancer patients. Furthermore, identifying and treating antibiotic-resistant microorganisms is difficult and expensive (Li *et al.* 2005). In addition, complications associated with antibiotic-resistant bacterial infections lead to high morbidity and mortality (Sefton 2002).

3. MATERIALS AND METHODS

100 samples were collected from tonsillitis patients aged 1 to 40 years of both sexes who applied to Al-harith National Hospital in Salah al-Din Governorate, Iraq during the period from 9/10/2021 to 30/01/2022, transport media swabs to ensure the vitality of isolation and to keep it as long as possible.

3.1 Methods

3.1.1 Antibiotic disks

Table 3.1 Antibiotics disk used in the study (CLSI 2020)

Antibiotics	Content (µg)	Abbreviation	Inhibition diameters		
			S	I	R
Amoxicillin	25	AMX	≥17	15-16	≤14
Ceftazidime	30	CAZ	≥25	19-24	≤18
Amoxicillin-Clavulanicac	30	AMC	≥18	14-17	≤13
Levofloxacin	5	LEV	≥22	15-21	≤14
Cefotaxime	5	CAX	≥19	15-17	≤14
Ampicillin	10	AMP	≥19	16-18	≤15
Meropenem	10	MEM	≥20	16-19	≤15
Imipenem	10	IMP	≥22	19-21	≤18
Gentamycin	10	CN	≥15	13-14	≤12
Chloramphenicol	30	C	≥17	15-16	≤14

3.1.2 Sterilization

3.1.2.1 Heat

All prepared media, as well as synthetic media, as well as most of the solutions that were used in the study, for which high temperature does not affect them or their properties, were sterilized by wet heat sterilization. As for the carrier for bacterial culture, the loop

was sterilized by flame, while the glassware that was used was sterilized by dry heat sterilization and using an electric oven at 165 ^{O}C for two hours (MacFaddin 2000).

3.1.2.2 Filtration

Most of the solutions affected by high temperatures are sterilized by micro-membrane filters, which have holes with a diameter of 0.22 microns (Benson 2002).

3.1.3 Preparation of stains solutions and reagents

3.1.3.1 Gram stain

Gram stain was prepared to detect the shapes of bacteria under the microscope and to show the positivity of these bacteria towards the dye (Forbes *et al*. 2007).

3.1.3.2 Capsular dye solution

It is made by dissolving 10 g of nigrosin in 100 mL of distilled water, boiling it for 20 min, filtering it twice, adding 0.5 mL of formaldehyde, and keeping it at 4°C until use (MacFaddin 2000).

3.1.3.3 Normal saline solution

Prepare by dissolving 0.85 g of Add 90 mL of NaCl in distilled water, then increase the volume to 100 mL and sterilize using an autoclave at a temperature of 121 °C and a pressure of 15 lb/in2 for the purpose of maintaining the cells for 15 min and pressing.

3.1.3.4 Mcfarland tube standard

It was prepared according to (CLSI 2020) and as follows:

I. Solution **A**: It Dissolve 1.75 g BaCl2 in 90 mL distilled water, then increase the volume to 100 mL to obtain the concentration of 0.048 mL/L.

II. Solution B: Add 1 mL of concentrated sulfuric acid H2SO4 to 90 mL of sterile distilled water and make up to 100 mL. When using MacFarland's solution, mix 0.5 mL of solution A with 99.5 mL of solution B for a bacterial cell count of approximately 1.5 x 108 cells/mL.

3.1.3.5 The solutions used to investigate the production of beta-lactamase enzymes by the standard iodine method

Penicillin G solution: It was prepared by dissolving 0.6 g of the antibiotic powder in 50 mL of bi-phosphate, and the volume was completed to 100 mL with distilled water. The solution was then sterilized by filtration, pH adjusted, and maintained at 7.2 at a temperature of 20 °C. Starch solution: add 1 g Place 100 mL of distilled water and 100 mL of starch in a water bath at 100 °C for 10 min, placing it in an opaque bottle and storing it at 4 °C. Iodine solution: Dissolve 2.03 g of iodine and 5.32 g of potassium iodide in 90 mL of distilled water, then make up to 100 mL, then place in an opaque bottle and store at 4 °C (MacFaddin 2000).

3.1.3.6 Catalase reagent

3 g of H2O2 was dissolved in 100 mL of sterile distilled water and stored in an opaque flask with 3% hydrogen peroxide solution for use in studies of the production capacity of catalase bacteria (Brown and Smith 2017).

3.1.3.7 Oxidase reagent

Prepare the reagent by dissolving 0.1 g of tetraethyl-p-phenylenediamine dihydrochloride in 10 mL of distilled water and placing it in a dark vial. This reagent is used to study the ability of bacteria to produce oxidase enzymee (Forbes *et al*. 2007).

3.1.3.8 Kovae's reagent

The reagent was prepared by dissolving the carrier for p-dimethylaminobenzaldehyde in 75 mL of isoamyl alcohol and 25 mL of concentrated HCl, and the detector was stored in an opaque vial until use, which was used in an indole production assay. used in refrigerator (Brown and Smith 2017).

3.1.3.9 Voges-proskauer reagent

The reagent consists of two solutions, according to what was mentioned by Brown and Smith (2017), which are: A- Solution α-naphthol: The reagent was prepared by dissolving 5 g of α-naphthol in 100 mL of 95% ethanol. B- Prepare a 40% KOH solution by dissolving 40 g of potassium hydroxide KOH in 100 mL of distilled water.

3.1.3.10 Methyl red reagent

The reagent was made by dissolving 0.1 g of methyl red in 300 ml of 950 ethanol and then adding distilled water to make 500 mL (Brown and Smith 2017).

3.1.3.11 DNase reagent

Then the preparation by adding 8.4 milliliters of HCl acid to 80 milliliters of distilled water and slowly and then completing the volume to 100 milliliters of distilled water in order to obtain 1 molar of hydrochloric acid, and it was used to investigate the ability of bacteria to produce the enzyme that decomposes for DNA (Brown and Smith 2017).

3.1.4 Preparation of culture media

Some general culture media was prepared according to the company's manufacturing instructions: pH adjusted with sodium hydroxide NaOH 0.1N or 0.1N hydrochloric acid

HCL, and then autoclaved at 121° C /1 bar for 15 min, it is then cooled and distributed into sterile tubes or Petri dishes (MacFaddin 2000).

3.1.4.1 Blood agar medium

Blood Akar medium was prepared by dissolving 40 g Akar in 1000 mL distilled water, then autoclaving at 121 °C and 1 bar for 15 min, then cooling to 50° C, blood 5% added, this medium was used for bacterial growth and for determination of blood cell lysis capacity (McFadden 2000).

3.1.4.2 Azide blood agar medium

Azide medium was used for the initial isolation, because it allows the growth of streptococci as well as staphylococci and prevents the growth of other species, and it can also be distinguished between the dissolution of Alpha streptococcus from the decomposition of the type Beta (Starr *et al*. 2013).

3.1.4.3 Tryptic soy agar medium

To encourage the growth of bacterial isolates, 40 g of trace soybeans were dissolved in 1000 mL of distilled water, 15 min later, and cooled to 50 °C. This medium was then prepared in accordance with the manufacturer's instructions (McFadden 2000).

3.1.4.4 Brain heart infusion broth liquid medium

It is prepared according to the manufacturer's instructions and autoclaved at 121°C and 15 lb/in2 for 15 min, this medium is used to activate bacteria (MacFaddin 2000).

3.1.4.5 Broth brain heart infusion solid medium

Prepared by adding 5% of human blood to the medium of sterilized heart and brain stems, sterilized with oxidizers, and cooled to a temperature of 50° C, and this medium was used to purify cyanobacteria (Brown and Smith 2017).

3.1.4.6 Muller-hinton agar

Mueller Hinton Agar prepared according to manufacturer's instructions 38 mg/L, and was used in the antibiotic susceptibility test of bacteria (Procop *et al*. 2016).

3.1.4.7 Sugar fermentation medium

Greens amidst Phenol Red Broth and sterilized by autoclave under normal conditions and at pH 7.2, then add to it one milliliter of sugar solution 1% for each of the different types of sugars, sterilized by filtering with micro-filters with holes 0.22 μm, as glucose sugars were used, Lactose, mannitol (Procop *et al*. 2016).

3.1.4.8 Urea agar

Prepare according to the manufacturer's instructions, by adding 5 mL of 20% urea solution, after cooling the medium in a German bath to 50° C, then distribute the medium on tubes that were placed diagonally to harden deep slop, and this medium was used to investigate the ability of bacteria to Production of urease enzyme (Procop *et al*. 2016).

3.1.4.9 luria-broth medium

Then the medium was prepared by dissolving its components in 950 mL of distilled water, which are 10 g of trypton, 5 g of yeast extract and 10 g of sodium chloride, and adjusting the pH to 7, then completing the volume to a liter of water, distilled, and sterilized by autoclaving in biofilm screening experiments (McFadden 2000).

3.1.4.10 Salt tolerance agar medium

This medium is considered semi-selective for cocci, as it was prepared by adding 6.5% sodium chloride to the heart and brain broth broth medium and sterilized by autoclave, Using this medium, bacteria's tolerance to salinity is tested (MacFaddin 2000).

3.1.4.11 Motility medium

This medium is prepared by adding 4 g of Acar to 100 mL of nutrient broth supplemented with 1000 mL of distilled water, then sterilized in an incubator at 121 °C and 1 bar for 15 min, dispensed into tubes, which are Method for detecting bacterial movement (McFadden 2000).

3.1.4.12 Maintenance media

Preservation and perpetuation medium for isolates was prepared by dissolving 4 g of heart-brain blot medium in 50 mL of distilled water, then the volume was completed to 100 mL, then 15% glycerol was added, then placed in an autoclave for the purpose of sterilization, Poured with labeled test tubes, preserved at 34 degrees until use (MacFaddin 2000).

3.1.5 Culture of samples

The collected samples were planted on a medium of liquid soy trypone with azide and violet, and a blood akar plate by passing swabs, swabs and rotating them on a quarter of the plate, and the rest of the plate is planned with a sterile conveyor (Vandepitteb *et al*. 2003). The plates were cultivated aerobically, and by saving 5-10% of carbon dioxide CO_2 gas, then it was placed in the incubator for 18-24 hours at a temperature of 37° C in the anaerobic growth cylinder Candle jar (MacFaddin 2000), and after making sure of the presence of growth on the dishes, one colony was removed, and mapped on the middle of

the blood traces, traces of blood azide to purify it, and then transferred to a glass slide, and the sample was stained with gram formula and examined under a light microscope.

3.1.6 Laboratory diagnosis

According to the references (McFaddin 2000, Forbes *et al*. 2007), the isolation and diagnosis of *S. pyogenes* was carried out as follows:

3.1.6.1 Microscopic examination and colonial morphology

The microscopic characteristics of the cells were studied by taking a single colony from each of the primary positive media and repeating the growth to acquire pure samples and then they were determined based on their morphological characteristics in the medium hemolysis, lactose fermentation, mannitol fermentation, colony shape, shape and arrangement of cells, size, color, borders and texture, and then inspected under an oil microscope after streaking out pure colonies on clean slides and gram-staining and observing how bacteria interact with the dye.

3.1.6.2 Biochemical test

The following biochemical selections were made to identify *S. pyogenes* from other isolates.

3.1.6.2.1 Catalase test

The enzyme catalase is responsible for releasing oxygen from hydrogen peroxide. Using sterile wooden sticks and H2O2, a portion of the newly formed bacterial colony was transferred for one day at 37° C, and placed on a clean slide with 3% H_2O_2 added to it, the immediate release of oxygen bubbles gives a positive result (MacFaddin 2000).

3.1.6.2.2 Oxidase test

We moisten the filter paper with drops of the prepared oxide immediately, then transfer part of the colonies with wooden sticks and place them on the filter paper, after the appearance of a purple color within 10-30 seconds, a positive result for the enzyme (Forbes *et al*. 2007).

3.1.6.2.3 Bacitracin sensitivity test

This selection is used to differentiate between the females and in order to distinguish the type A from the other groups, 1-5 bacterial colonies were taken and plotted on a blood agar plate and then incubated for 18-24 hours and then, a pure colony was taken and cultured on the Mueller-Hinton plate that was added it was 5% of fresh human being and then put the bastracin chances centrally in the plate and after incubating for 18-24 hours, at a temperature of 37° C, then read the results and the result depends on the area of inhibition around the disc (Sayyahfar *et al.* 2015). If the area of inhibition is less than 12 mm, the result is considered negative, so the isolates are from another group, and if the area of inhibition is more than 12 mm, the result is considered positive, so isolates from the Christian group A .

3.1.6.3 Screening for some virulence factors in *S.pyogenes*

3.1.6.3.1 Capsule test

The investigation of the capsule was carried out according to what was mentioned in the sources (MacFaddin 2000). The carrier is taken from a growing bacterial colony of 18 hours of age, from the center of the heart and brain sputum with solid blood, and then put it on the glass slide and mixed with nicrocin dye, covered with the lid and examined under the microscope, with the appearance of an area clearly a halo that is transparent and non-pigmented throughout the cell and this halo represents the capsular indicative of a positive test.

3.1.6.3.2 Hemolysin production

The blood agar medium was inoculated with the bacterial isolates that are under the current study and incubated at 37° C for 24-48 hours, forming *S. pyogenes* is an area that can be clearly seen around the colonies, which indicates complete hemolysis (B-hemolysis) (MacFaddin 2000),

3.1.6.3.3 Sugar fermentation test

Inoculation of the medium of conserving sugars with newly grown bacteria at the age of 24 hours, and then incubated at a temperature of 37° C for a period of five days, and the change in the color of the medium from red to small indicates the occurrence of the fermentation process (Forbes *et al.* 2007).

3.1.6.3.4 Urease test

Urea medium Inoculate with the prick method, then incubate the tube at 37°C for 24 hours, the medium color changes from yellow to pink to indicate the ability bacteria to transform urea by producing urease (Forbes *et al.* 2007).

3.1.6.3.5 β-lactamase production test

The standard rapid iodine method as reported in MacFaddin (2000) was used to detect the production of beta-lactamase enzyme, the working method included the following steps: Vege Tables of 24-hour-old bacterial cultures grow on nutrient agar media. Then a number of colonies were transferred by the loop to a hole of the Microtiter plat containing 100 mL of penicillin and mixed well with the solution. Incubated for 30 min at 37° C. Add 50 microliters of starch solution, and then mix well to prevent the contents of the pit. Adding 20 microliters of iodine solution results in a dark blue color resulting from the reaction of iodine with starch.

After a short time, less than a minute, after adding the reagents, the Sony changed rapidly from dark blue to white, so the result was calculated as positive, the test was repeated when a late positive result appeared after more than 5 min. The negative control experiment was conducted by following the previous steps, but without the use of bacterial colonies.

3.1.7 Antimicrobial susceptibility test

Sensitivity testing was done for isolates of *S. pyogenes* on MHA agar was carried out by using the Kirby-Bauer Disk Diffusion test method according to what was mentioned in the sources (CLSI 2020), then the bacterial suspension of *S. pyogenes* grown on a nutrient agar plate was prepared, then a sterile test tube was placed in each sterile test tube containing 5 milliliters of physiological saline, one isolate, then the suspension was mixed and the turbidity was compared with the turbidity of a standard McFarland tube. MHA and in different directions, in order to ensure the homogeneity of spreading throughout the dish, then we leave the dish for a quarter of an hour to absorb the moisture, then, it distributes the antibiotic Tablets on the plate using sterile forceps, then the dishes are incubated at a temperature of 37 °C for 24 hours, and then results are recorded by measuring the inhibitory areas that appeared around the Tablets and they were compared with the proven standard diameters that were mentioned in the sources (CLSI 2020). Bacteria are resistant, sensitive or moderately resistant to antibiotics.

3.1.8 Vitek 2 system

The Vitek system was used to make sure that the isolates that are under study are *S. pyogenes*, after doing the biochemical tests and then using the Vitek 2 system to confirm the result of the manual biochemistry test, this system is used to identify the microorganisms and was provided with the identification database required for all the tests which enables him to identify microorganisms that can provide improved efficiency and high accuracy in microbial diagnosis which saves time and the need for any additional tests, which will be safer for the user of the system. This system was performed according to the manufacturer's instructions (Biomerieux-France) (Fritsche *et al*. 2011).

3.1.9 Presrvation of bacteria isolates

The tubes containing Nutrient agar slant were inoculated with bacteria and then incubated at 37° C for 24 hours and then kept at 4° C until use, the isolates were also preserved for long periods using the culture medium of infusion of the heart and brain liquid, after the mechanism of cholesterol was added by 20%, then the tubes containing the medium were inoculated with bacteria and they were preserved at a temperature of 20° C below zero (Vandepitteb *et al.* 2003).

3.2 Genetic Materials and Methods

3.2.1 DNA extraction kit

The kit used for DNA extraction, shown in the Table 3.2.

Table 3.2 The kit used to extract DNA and the components of each kit and the company

Kit Name	**Contain**
Presto™ Mini Gdna Bacteria Kit	GT Buffer
	Collection Tube
	Proteinase K
	GB Buffer
	W1 Buffer
	Wash Buffer
	Elution Buffer
	GD Colum
	Top DNA polymerase 1U
AccuPower® PCR PreMix	dNTP (dATP, dCTP, dGTP, dTTP) each: 250µM
	Tris-HCl (pH 9.0) 10mM
	KCl 30mM
	MgCl2 1.5mM
	Stabilizer and tracking dye

3.2.2 DNA primers

The primers were used to perform the PCR assay, and these primers were used to investigate some types of virulence genes specific to pyogenes bacteria. They are *scpA* , *speA* - *emm* and *gyrB* (Imöhl *et al*. 2017). The primers were designed for this study in Canada by IDT, shown in the Table 3.3.

Table 3.3 DNA Primers

Primer		Amplicon Primer sequence (5′-3′)	Amplicon
gyrB	F	GAA GTC ATC ATG ACCGTT CTG CAY GSN GGN GGN AAR TTY GG-3’	900 bp
	R	TGT AAA ACG ACG GCC AGT TCN GCN NAR YTT NCC NGG	
emm	F	TATTCGCTTAGAAAATTAA	914 bp
	R	GCAAGTTCTTCAGCTTGTTT	
speA	F	AGGTAGACTTCAATTTGGCTTGT	576 bp
	R	GGTGACCCTGTTACTCACG	
scpA	F	GCTCGGTTACCTCACTTGTCC	622 bp
	R	CAATAGCAGCAAACAAGTCACC	

3.2.3 Polymerase chain reaction

The assay was carried out in order to establish a confirmatory diagnosis of *S. pyogenes* isolates, as well as to investigate some types of virulence genes, namely *scpA emm* and *gyrB* enzyme gene, according to the method of Nagano *et al*. (2003) as follows:

3.2.3.1 Bacterial DNA extraction

The DNA extraction was carried out for *S. pyogenes* isolates, using the The genomic DNA extraction kit provided by Geneaid company in Korea, extracted as follows according to the company's instructions:

1. Transferring 1 mL of suspension to each yarn of the *S.pyogenes*. The cultures were grown on broth for heart and brain infusion, then placed in sterile 1.5 mL Eppendorf tubes and then transferred to a centrifuge at a speed of 15000 r/min in

order to collect the bacterial cells, and dispose of the mixed liquid with a mixture vortex for 5 seconds.

2. 200 mL of lysozyme buffer 20mg/mL were added and the mixture was mixed with Vortex for 5 seconds.
3. Incubate the mixture at room temperature for 10 min. During incubation, stir the tubes to ensure complete analysis of the cells in the mixture.
4. Add 200 µL of the kit-prepared GB Buffer solution and 20 µL of ProteinaseK to the lysed cell mixture and mix well with the mixture (vortex for 5 seconds).
5. The mixture was then incubated at 360°C for 10 min using a water bath.
6. Add a volume of 200 microliters of absolute ethanol to the dissolved mixture and mix the mixture thoroughly with a vortex mixer for 10 seconds.
7. Transfer the mixture from the Alpendorf tube to a 2 mL collection tube containing the column of the filter used to purify the DNA, the GD filter column included with the kit.
8. Place the collection tube and column containing the mixture in a centrifuge and spin at 1500 rpm to remove cell-degrading products.
9. Then, discard the lysed cell precipitation solution and transfer the GD column with DNA to a new dedicated collection tube.
10. Then add 400 µL of Buffer W1 prepared in the kit to the column containing the DNA wash. Place the tube in a centrifuge and spin at 15,000 rpm for 30 seconds.
11. Then the precipitate was disposed of, and after that, an amount of 600 microliters of the working solution containing absolute ethyl alcohol Wash buffer prepared with the kit was added to the column containing the DNA in order to get rid of the fat inside the column, the tubes were placed in the centrifuge and rotated At 15,000 revolutions per minute for 30 seconds.
12. Then get rid of the precipitate, and then return the tubes to the centrifuge again to dry the columns, and rotated at 15,000 rpm for 3 min.
13. Then transfer the columns containing the DNA to sterile Alpendorf tubes and add 50 µL of the solution Elution Buffer equipped with the kit to the center of the column, and leave for 5 min, after which the tubes were placed in the centrifuge, and it rotated at a speed of 15,000 cycles for 30 min A second time to dissolve the

DNA, and then keep it at -20° C until it is used in Polymerase Chain Reaction PCR assay.

3.2.3.2 DNA examination

Then detecting the extracted DNA using the Nanodrop spectrophotometer THERMO, USA, which is related to detection and measuring the concentration of nucleic acids, as this was done by detecting the nucleic acid by determining the concentration of the nucleic acid ng\ul DNA, and then measuring the purity of the DNA, during the reading of the absorbance, at a wavelength ranging from 280/260 mm. we use the device as follows:

Turn on the Nanodrop device, and then choose the DNA measurement program, the type of DNAM, and zeroing the scale substrate twice, by placing 2 microliters of (Elution Buffer) and using a sterile micropapite on the surface of the scale substrate and doing the miniaturization, and then we clean the substrate using the blotting paper of the device. Put 1 microliter of each extracted DNA sample on the substrate, and then press the Ok button to perform the DNA concentration measurement process. The purity of the extracted DNA samples was determined by reading the absorbance at 260/280 nm wavelengths using a nanodroplet spectrophotometer, as DNA was considered pure when the absorbance ratio was 1.8.

3.2.3.3 PCR master mix

The mixture was prepared for the polymerase chain reaction using the AccuPowers PCR PreMix kit, which was prepared from before the Canadian company ABM, and according to company instructions as follows:

1. The mixture was prepared for the polymerase chain reaction by the method of Diplex PCR in the PCR tubes that were equipped with the kit and containing the components for the polymerase chain reaction, and the rest of the other

components were added to the mixture according to the instructions of the company (Table 3.4).

Table 3.4 Components and volumes of the Monoplex PCR master mix

Mixture Contents	Volume (µl)
Master Mix	25
Forward Primer	3
Reverse Primer	3
Template DNA	5
Nuclase -Free Water	14
Total	50

2. After completing the preparation of the polymerase chain reaction mixture, the tubes were closed and the mixing was carefully done by a vortex device for 10 seconds.
3. The tubes are weighted to the PCR Thermocycler to perform the thermal cycles PCR thermocycler conditions.

3.2.3.4 Pcr thermocycler dna

The test was carried out for the polymerase chain reaction using a PCR thermocycler, as shown in Table 3.5.

Table 3.5 Conditions Used in PCR Thermal Amplifier Machine

PCR Step	Repeat cycle	Temperature	Time
Initial denaturation	1	95° C	5 min.
Denaturation	30	95° C	30 sec.
Annealing		63° C	30 sec.
Extension		72° C	1 min.
Final extension	1	72° C	5 min.
Hold	-	4° C	Forever

3.2.3.5 Gel electrophoresis

Electrophoresis was carried out using agarose gel at a rate of 1.5%, by reading the reaction result of the PCR product as follows: Make 100 mL of TBE spin-buffer solution, and add 1.5 grams of agarose gel. Melt the gel in an electric oven for 15 min at a 1X concentration. The gel was cooled to 350 degrees, and then the dye that changes color when exposed to radiation was added. The dye is Ethidium bromide. The mixture was stirred well. Take the gel into a mold that contains tiny teeth. Leave the gel to solidify for 15 min at room temperature after pouring it into the mold. Then remove the comb, and place the solidified agarose gel into the heating amplifier tray with samples. PCR product was added to the samples using two loading buffers on paper wrapped around parafin. One drop was added for every four drops of PCR product. The mixture was then put into the wells of the gel pad. Measure the amount of DNA that was amplified in the PCR machine, and place that amount of DNA in the first well. After the gel is loaded, the circuit is filled with TBE Buffer at a concentration of 1 X. The gel is then immersed in the solution, and the relay cover closed. The relay is turned on for an hour at a voltage of 100 volts and a current of 80 amps. Use the UV light, after the migration process is done, to see if the output of the gel with the thermal amplifier in it is good.

4. RESULTS AND DISCUSSION

4.1 Isolation

From September 10, 2021 to January 30, 2022, we collected 100 samples from the Al-Harith National Hospital in Salah al-Din Governorate, Iraq, and outpatient clinics.The samples were from tonsils, and the swabs were planted directly on selective media in the middle of liquid soy trypton with azide and crystal violet to inhibit the growth of negative bacteria, and fungi and other germs that can be found in the mouth were incubated at a temperature of 37 °C in anaerobic conditions, and 80% gave positive growth.

They were then cultivated and grown on the medium of solid heart and brain necrosis with blood, and in a manner of planning to obtain pure and typical colonies of the genus Cocci from 80 isolates, 10 (12.5%) isolates belonging to the genus *S. pyogenes* were identified. This was confirmed by biochemical tests and agricultural characteristics. It was 12.5%, and the result was close to Al-Ani (2016)'s isolation percentage of 12%, as well as similar to Mohammad *et al.* (2005)'s isolation percentage of 13%, and the study's results were not identical to Muthusamy *et al.* (2012), who obtained a rate of 5.1%.

The reason for the different percentages and differences in isolation between studies may be due to the age of the children involved in the study, as well as the different methods used to isolate the bacteria and the different goals of the study, all of which contributed to the differences in counts between groups. which the study relied, the use of antibiotics indiscriminately, and the wrong season for collecting samples also affect the percentage of isolated bacteria, as some seasons have a higher rate of infection than others due to climatic conditions, cases of overcrowding, and the economic and social status of patients. Results show the numbers and percentages of samples taken from tonsillitis infections and distributed by gender.

The results showed that the infection rate of males was higher than that of females, at a rate of 58 (58%) and 42(42%) respectively from tonsillitis infections. Many studies indicate that males are more susceptible to ear infections than females (Ughasoro *et*

al. 2021). Moreover, our results also disagree with the results obtained by Al-Tameemi *et al.* (2020), which show that females are more susceptible to infection than males.

As in Table 4.1, the results show the numbers and percentages of samples taken from tonsillitis infections and their distribution according to age groups. The patients' ages ranged from 1–10 years. The highest infection rate among the age groups was 76% for the age group 1–10, 20% for the age group 11–20, and 3% for the age group 21–30, while the lowest rate was 1% for the age group 31–40. The result agrees with Al-Tameemi *et al.* (2020), where they found during their study that the children most at risk of developing tonsillitis are between the ages of 1 and 9 years.

Children and teens between 5 and 15 years old have the highest incidence of pharyngotonsillitis caused by bacteria, according to Khadilkar and Ankle (2016). This infection occurs more often in children due to their increased contact with other kids and adults at home, day care, and school. The varying bacteria in the oral mucosa can increase the likelihood of pharyngotonsillitis occurring (Lamaro *et al.* 2009). According to some studies, recurring pharyngotonsillitis is important in deciding whether to perform a tonsillectomy. 75.5% of patients seen at the clinic reported having had pharyngotonsillitis multiple times, according to Stuck *et al.* 2008. Along with tonsillar hypertrophy, sleep apnea, nocturnal snoring, and breathing pauses can be indications for tonsillectomy (Table 4.1) .

Table 4.1 Distribution of sources of infection and percentage of isolated bacteria according to age groups

Source isolation / **Age groups (year)**	**Tonsillitis**	
	No	**%**
1-10	76	76%
11-20	20	20%
21-30	3	3%
31-40	1	1%
Total	100	100%

The results of the study showed a clear superiority in the incidence of tonsillitis in children, as most of the 80 (80%) isolates were isolated from the age group 1–10 years. The reason could be related to the increased activity of children at this age, which gives an opportunity for more exposure to infection than other ages. Besides this, children mix and communicate with each other in the classroom as well as play together, which leads to transmission and infection in this age period (Saleh 2009).

Tonsil infection often appears in the first 10 years of life, and antibiotic treatment is often inadequate or inappropriate, resulting in persistent, resistant, recurrent and chronic infections (Khadilkar and Ankle 2016). It was found that the frequency of tonsillectomy was more in people between the ages of 10 and 18 years (Abouzied and Massoud 2008).

They also found that in all age groups, chronic tonsil infection was an indication for surgery and noted that rates of tonsillectomy increased by 58% among children aged 4 to 15 years (Koshy *et al*. 2014). He explained that the rate of tonsillectomy was 91.3 per 10,000 children among children aged 7-15 years (Locker *et al*. 2010).

4.2 Diagnosis

4.2.1 Cultural characteristic

The colonies growing on the previously mentioned culture media appeared as spherical colonies, small in size, with complete convexity to the edges, and white color, surrounded by an area of 2-3 mm that completely dissolved red blood cells of the type beta, due to their ability to produce the hemolysin enzyme, and the growth conditions were not Honia by adding CO2 at a temperature of 37 ^{o}C for 24 hours.

4.2.2 Microscopic characteristic

The result of the microscopic examination using Gram stain to identify the cells, the result was the cells in the form of pool chains with a wet or smooth surface showing the colonies in white color Gram positive stained in purple (Cunningham 2008).

4.2.3 Biochemical test

4.2.3.1 Catalase test

This examination is very important in order to differentiate between the genus *Streptococci* that does not produce the enzyme catalase from the other genera that produce it and these genera such as *Micrococcus, Staphylococci* and *Neisseria* and the result was negative for all isolates for this examination, and the developing colonies did not form bubbles of O_2 gas on the culture medium after adding a drop of hydrogen peroxide at a concentration of 3%.

4.2.3.2 Oxidase test

The results of the detection of oxidase in all isolates showed a negative result when part of the colonies were transferred with wooden chopsticks and placed on a filter paper on which drops of the prepared reagent were placed simultaneously, and they were mixed well enough that the purple color did not form within half a minute, indicating their inability to produce the oxidase enzyme.

4.2.3.3 Indole test

All isolates that are under the current study showed that the indole ring was not formed, as the positive test result was the red ring as a result of the hydrolysis of the amino acid tryptophan and its conversion to indole.

4.2.3.4 Methyl red test

All the diagnosed bacterial isolates gave a positive test for the methyl red test, which indicates the ability of the bacteria to analyze glucose sugar.

4.2.3.5 Voges proskauer test

All isolates showed a negative assay for the selection of Voges Proskauer VP, which indicates the inability of bacteria to form the neutral compound Acetyl-methyl carbinol from partial glycolysis.

4.2.3.6 Bacitracin susceptibility test

This assay is important to differentiate between group A streptococci from other groups, beta-hemolytic, as 99% of group A isolates are sensitive to pasteracin, and all isolates showed clear sensitivity to this assay with an inhibition zone of 18 mm (Figure 4.1).

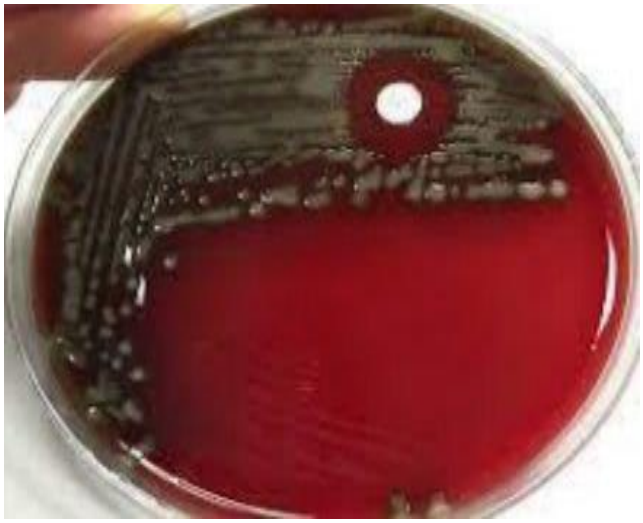

Figure 4.1 Shows bacitracin sensitivity test for isolates of *S. Pyogenes*

4.2.3.7 Motility test

After the tubes containing the movement medium were inoculated by stabbing, and then the tubes were incubated at a temperature of 37° C for one day, no growth outside the stabbing was recorded for all isolates, which indicates the inability of bacteria to move.

4.2.3.8 Sugar fermentation test

As for the fermentation test for sugars, the results of all isolates showed a positive result of the fermentation test for glucose-lactose, and the color of the medium turned yellow.

4.2.3.9 Urease test

All isolates showed a negative test for the urea enzyme test, and the color of the medium did not change from yellow to pink, indicating the inability of bacteria to decompose urea by producing urease enzyme. The results of these biochemical tests are in agreement with those of Hussein (2021) (Table 4.2).

Table 4.2 Biochemical tests and microscopic examination of *S. Pyogenes*

Type of test	Result
Cocci the shape of the cells	Cocci form of chains
Gram stain	+
Bacitracin sensitivity test	+
Coagulase	-
Catalase	-
Oxidase test	-
Indole test	-
Methyl red	-
Lactose fermentation	+
Glucose fermentation	+
Motility	-
Voges Proskauer test	-
hemolysis	Beta

4.3 Detection of Some Virulence Factors Associated with Pathogenicity of Bacteria

4.3.1 Capsule test

All yarns have been shown to contain the capsule through microscopy and by using a dye called Necrosin, as the cells were in the form of bacilli surrounded by a halo.

4.3.2 Production test enzyme DNAse

All isolates of *S. pyogenes* gave a positive result for the DNase test, as these isolates showed transparent halos around the bacterial colonies indicating the positive test.

4.3.3 Hemolysis enzyme production test

After inoculation of the blood agar medium with bacterial isolates of *S. pyogenes* and incubation at 37°C for 48–24 hours, a region of complete lysis of red blood cells was formed that can be clearly seen around the colonies, and the ten bacterial isolates of the genus *S. pyogenes* showed a positive result of 100%.(Figure 4.2).

Figure 4.2 The assay shows the production of hemolycin in *S. Pyogenes*

4.3.4 Biofilm production test

The results of the current study showed that all isolates are capable of biofilm formation at 100%, and these results were identical to a number of studies, where Cross *et al*. (2020) indicated that all isolates of *S. pyogenes* obtained from pharyngeal infection samples have the ability to form biofilm, which led to an increase in the rates of antibiotic resistance and an increase in necrosis and tissue inflammation. As for Alamiri *et al.* (2020) and his group, they studied membrane formation on living surfaces and were able to measure the biomass formed from the biofilm formation of *S. pyogenes* on epithelial and keratinocyte cells and the extent of that biomass's resistance to antibiotics.

4.3.5 Beta-lactamase production test

The ability of bacteria to produce beta-lactamase enzymes for all isolates of *S. pyogenes* that are under the current study was investigated, and the standard iodine method was adopted, which gives a preliminary survey of all types of beta-lactamase enzymes produced by the isolates. It is also a quick way to get results. Penicillin G was used as a basis for the examination; the enzyme works to open the beta-lactam rings, leading to the formation of penicilloic acid, resulting in a change in the color of the index from red to yellow due to the change in the acidic function Ph. Three isolates gave a positive result at a 30% rate, and the rest of the isolates gave a negative result for the examination, indicating that they are not capable of producing -lactamase enzymes, making them sensitive to penicillin antibiotics, which were the first choice for treatment. Studies and research indicate that *S. pyogenes* bacteria are not able to resist B-lactam group antibiotics except in the case of genetic mutations as a result of repeated and indiscriminate use of antibiotics (Vannice *et al*. 2020).

4.4 Antimicrobial Susceptibility Test

The sensitivity of the bacterial isolates under the current study was tested according to CLSI (2020) using the method of diffusion of 10 antibiotics; these antibiotics were used

because of their widespread and frequent use among people in the treatment of many infections caused by infection with bacteria *S. pyogenes*. Table 4.3 shows the percentages of resistance and sensitivity for Streptococcus bacteria to the antibiotics used in the current study, as well as the differences between them.

All isolates showed complete resistance to each of Amoxicillin and Ampicillin, as these results were identical to the findings of Vannice *et al.* (2020). The emergence of antibiotic resistance is one of the problems facing the medical community and is usually the result of the widespread and indiscriminate use of antibiotics during recent decades, as many bacterial strains have acquired resistance to them. Resistance to antibiotics may arise as a result of genetic changes that bacteria adopt after infecting the host in sites where the best antibiotics are not working, and sometimes they surround themselves with abscess cavities or biofilms as defensive means. Resistance to antibiotics arises as a result of genetic changes that occur in the bacterial cell due to genetic mutations or because of resistance genes carried on plasmids and transposons (Alamiri *et al.* 2020).

Whereas the current study found that all of the spinners of *S. pyogenes* were sensitive to the antibiotic Amoxicillin clavulanic acid, the reason for the increase in the efficiency of these antibiotics could be attributed to the synergistic action after adding clavulanic acid, which led to an increase in the antibiotic's efficiency after mixing it with the antibiotic. These antibiotics are designed to treat gram-positive bacterial infections (Ubukata *et al*. 2020). With regard to cephalosporins, represented by cefotaxime and ceftazidime, the resistance rates were 40% and 20%, respectively. Vannice *et al.* (2020) observed isolates of *S. pyogenes* that have shown resistance towards the group of cephalosporins, and their ratios are different. As for the carnitines represented by Meropenem and Imipenem, all isolates showed complete sensitivity; the percentage reached 100% toward both antigens because of its high ability to inhibit most beta-lactamase enzymes (Mirsalehian *et al.* 2017), and the emergence of resistance to these antibiotics is a matter of great concern because it is the last resort.

As for Gentamicin, the isolates showed a resistance of 40%, and these results were close to what was reached by Khademi *et al.* (2021), if he obtained a resistance rate of 36.9%

to Gentamicin in his study on *S. pyogenes* isolated from infected children and adults. The results shown in Table 4.3 showed that all isolates showed a complete sensitivity of 100% towards the group of quinolones represented by the antagonist quinolones represented by Levofloxacin, and the results of the current study were identical to those of Deshwal *et al*. (2020).

In a study conducted by Berwal *et al*. (2019), isolates of *S. pyogenes* and those isolated from respiratory tract infections in intensive care units showed a resistance of 5.3% to Levofloxacin. Said *et al*. (2020) also managed to obtain isolates sensitive to levofloxin. During his study in the city of Dohuk, he studied patients with pelvic inflammatory disease, which explains the deadly action of this group of antibiotics and their high efficiency against Streptoccus bacteria, starting by inhibiting the DNAgyrase enzyme, causing the accumulation of pieces of double strands cut in the genome of the bacterial cell, which causes a disability in the basic movements of DNA and RNA polymerase along the DNA template (Khan *et al*. 2018).

With regard to chloramphenicol, the isolates under the current study were recorded the resistance rate is the highest, it reached 50%, and the results agreed with what Willms *et al*. (2021) reached if they obtained isolates resistant to Chloramphenicol (Table 4.3).

Table 4.3 Antibiotic susceptibility patterns of *S. Pyogenes*

Antibiotics	**Content (µg)**	**Abbreviation**	**Inhibition diameters**			***S.pyogenes***	
			S	**I**	**R**	**S**	**R**
Amoxicillin	25	AMX	≥17	15-16	≤14	0%	100%
Ceftazidime	30	CAZ	≥25	19-24	≤18	80%	20%
Amoxicillin-Clavulanicac	30	AMC	≥18	14-17	≤13	100%	0%
Levofloxacin	5	LEV	≥22	15-21	≤14	100%	0%
Cefotaxime	30	CTX	≥19	15-17	≤14	60%	40%
Ampicillin	10	AMP	≥19	16-18	≤15	0%	100%
Meropenem	10	MEM	≥20	16-19	≤15	100%	0%
Imipenem	10	IMP	≥22	19-21	≤18	100%	0%
Gentamycin	10	CN	≥15	13-14	≤12	60%	40%
Chloramphenicol	30	C	≥17	15-16	≤14	50%	50%

R=Resistant S= Sensitive

4.5 Molecular Detection of Virulence Factors

4.5.1 Genetic investigation of the virulence gene m protein *emm*

After removing the isolates from the thermal polymerase PCR device and placing them in the electrophoresis device, the results showed that all ten bacterial isolates were diagnosed as containing the virulence gene M Protein emm, with a percentage of 100% and a size of 914 bp. The results in this study were not in agreement with the study conducted by Khalaf *et al.* (2020), which obtained a percentage of 61.5%, and they were almost similarWith the findings of the researcher, Arêas *et al.* (2014), he obtained a percentage of 94.5%, and this percentage, which was reached in this study, is the highest among the previous percentages (Figure 4.3).

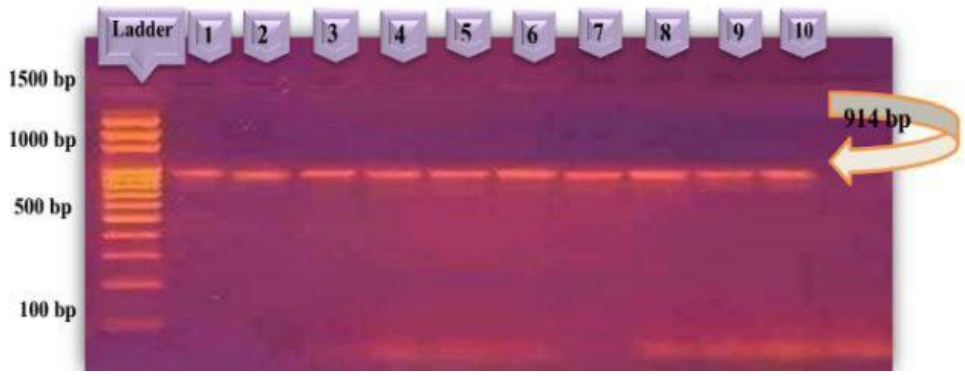

Figure 4.3 Agarose gel electrophoresis showing the results of polymerization of the virulence gene M Protein *emm* of *S.pyogenes* from 1-10 represent bacterial isolates

4.5.2 Genetic investigation of the virulence gene c5a surface peptidase *scpA*

The results of the current study, in which the virulence gene C5a surface peptidase scpA was investigated, showed that the bacterial hyphae of *S. pyogenes* possessed the gene scpA and the ratio was 100%, all isolates of 10 pyogenes contain this enzyme and the size is 622bp, the results of the current study were the highest percentage because it got a percentage of 100%, and this study was not identical to the study conducted by Hamim *et al.* (2017), in which he obtained a percentage of 59.9% (Figure 4.4).

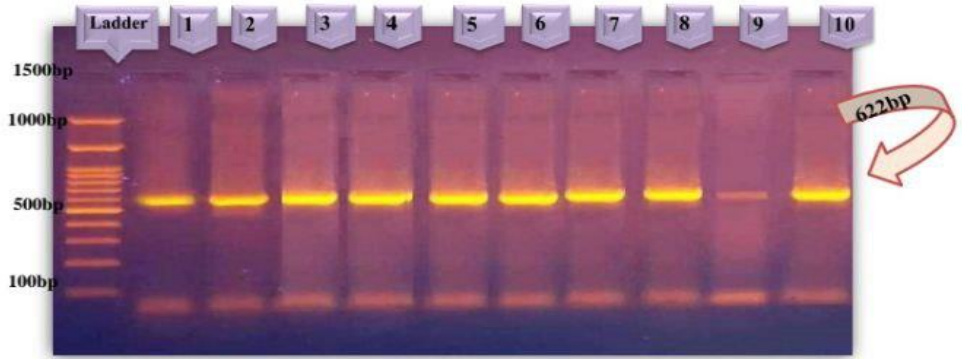

Figure 4.4 Agarose gel electrophoresis showing results of virulence gene C5a peptidase scpA polymerization of *S.pyogenes* from 1-10 represent bacterial isolates

4.5.3 Genetic investigation of the virulence gene streptococcal superantigens *speA*

The results in this study showed that bacterial isolates of gene *SpeA* were very high, and all isolates recorded that they contained 100%, *SpeA* and the size of pb576m, when compared with previous studies, they are inconsistent with Wang *et al*. (2012) who scored 12.7 %, and also inconsistent with Beres *et al*. (2006) and the percentage was 18.4%. and also inconsistent with the study conducted by Nabat *et al*. (2019) and got a percentage of 10.8, and it was similar to the results of Hussein (2021) and it got 100%. Previous studies showed that the presence of gene *speA* in bacterial strains that have a high pathogenic capacity to infect the skin and infection of the tonsils (Figure 4.5) (Wang *et al*. 2012).

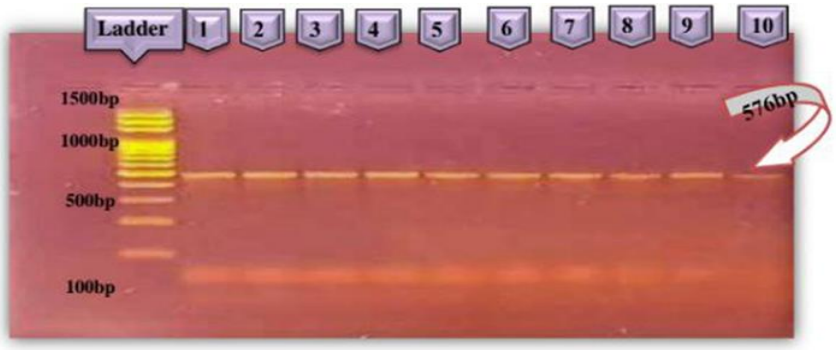

Figure 4.5 Agarose gel electrophoresis showing results of virulence gene gene *Streptococcal* Superantigens *speA* polymerization of *Strep.pyogenes* from 1-10 represent bacterial isolates

4.5.4 Molecular detection of gyrase enzymes gyrb

The reason for choosing the enzyme *gyrB* and not choosing the method adopted and used previously in the molecular analysis 16 S rRNA gene is because it was filled with many previous studies on the same topic and it is constantly repeated, and the *gyrB* genes are more differentiated and distinguished than that of S rRNA16, besides therefore, the high discrimination strength of DNA within *S. pyogenes* has been confirmed, and is suiTable for phylogenetic analysis of closely related *Streptococcus* strains (Itoh *et al*. 2006). Hence, we conclude that *gyrB* genes are effective alternative targets to classify the genus *Streptococcus*.

The results of the current study showed that they were positive for all ten isolates and recorded 100%, with a size of pb900. This study agrees with what was reached by Hussein (2021), whose results recorded 100%, of *gvrB* gene for *S.pyogenes* (Figure 4.6).

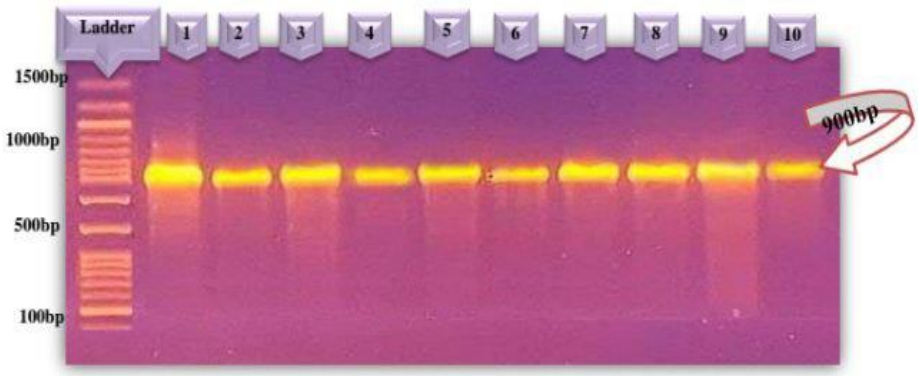

Figure 4.6 Agarose gel electrophoresis showing results of virulence gene Gyrase enzymes *gyrB* polymerization of *S.pyogenes* from 1-10 represent bacterial isolates

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

1. That male has a higher incidence of tonsillitis than female.
2. The highest percentage of infection detected at age group 1-10 years.
3. The highest isolation rate recorded for *S. pyogenes* among children age 1-10 years.
4. All the bacteria isolate were highly sensitive to Amoxicillin clavulanic, Imipenem, Meropenem, and Levofloxacin and highly resistance to Amoxicillin and Ampicillin.
5. All isolates possessed, in a percentage that are under study, the virulence genes (M protein *emm*, peptidase enzyme C5a *scpA* and exotoxin *SpeA*.

5.2 Recommendation

1. The need to alert patients to adhere to and take the full course of treatment even after feeling better so that bacteria do not appear or resist treatments.
2. The need to conduct a drug susceptibility test for bacterial isolates before starting treatment for the infected, so that they can be given the appropriate and effective antibacterial.

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APPENDICES

APPENDIX 1. Laboratory instrument and equipment

APPENDIX 2. Chemical and biological materials

APPENDIX 3. Culture media

APPENDIX 4. Ethics committee

APPENDIX 1. Laboratory instrument and equipment

The device name	The manufacture company
Autoclave	Labtech -Korea
Balance	Sartorious-U.K
Benson burner	Memmert-Germany
Candle jar	Gallen kan /England
Centrifuge	Olympus /Japan
Gel electrophoresis apparatus	Bio-rad /Italy
Hood	Olympus /Japan
Incubator	Gallenkam/ England
Refrigerator	Ishtar -Iraq
Light microscope	Olympus / Japan
Millipore filter	Sartorius membrane filter Gm bH, W. / USA
Nanodrop spectrophotometer	Thermo/ Taiwan
Oven	Memmert-Germany
pH-meter	Henna - China
Thermocyclerapparatus (PCR)	Fisher scientific /Germany
Vitek-2 system	bioMérieux /France
Vortex	Scientific Industries Inc / USA
Water bath	Thermo/Taiwan
Water distillatory	H-jurgens-CO./Germany

APPENDIX 2. Chemical and biological materials

Chemical and biological materials	The manufacture company
Absolute ethanol 96%	BDH -UK
Agar-agar	Siga -England
Agar-agar	Siga-England
Agarose	BDH -England
Agarose	BDH -England
Commercial Kovac's reagent	CDH -India
DNA ladder 100*bp*	Bioneer -Korea
Ethanol 70%	GCC (England)
Ethidium bromide	Bio basic INC/USA
Gram stain	Syrbio -S.A.R
HCL	BDH-England
Hydrogen peroxide	GCC -England
Lysozyme	Bio basic INC/USA
Methyl red	Siga -England
Peptone	Difco-U.S.A
Primers	IDT -Canada
Sodium chloride	BDH -England
Tetramethyl p-phenyl diamine-dihydrochloride	BDH - USA
α-Naphthol ($C_{10}H_8O$)	BDH - USA

APPENDIX 3. Culture media

The culture media	The purpose of its use	Origin
Azide blood agar medium	For primary isolation because it allows the growth of streptococcus as well as staphylococcus and prevents the growth of other species	Himedia -India
Blood agar base	Detection of hemolysis production	
Brain infusion brotheart	It was used to activate and preserve bacteria	
Luria-Bertani Broth	A growth and activator of bacteria in genetic experiments	
MacConkey agar	The medium was used to ferment lactose from non-lactose	
MR-VP broth medium	It is used to detect partial and complete hydrolysis of glucose	
Muller-Hinton agar	It is used to test the sensitivity of bacteria to antibiotics	
Nutrient agar	general soothing	
Nutrient broth	general soothing	
Peptone water medium	It is used to detect the indole ring	
Triple-sugar iron agar	It was used to distinguish bacteria on the basis of fermentation of sugars and production of hydrogen sulfide	
Urea agar	It is used to investigate the ability of bacteria to produce urease	

APPENDIX 4. Ethics committee