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The Genetic Diversity of Group A *Streptococcus* Isolated from Patients with Pharyngitis

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَأَنْزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُنْ

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صدق الله العلي العظيم

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Supervision Certification

We certify that this thesis, entitled " **The Genetic Diversity of Group A Streptococcus Isolated from Patients with Pharyngitis** " has been prepared under our supervision by " **Zainab Tariq Ali Najy** " at the Department of biology / College of Science for Women/ University of Babylon, as a partial requirement for the degree of **Master** in **Biology**.

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Dedication

To my family, *The first to teach me the alphabet, the first to hold my hand and accompany my steps, those who spent their lives for the love of me, who gave everything for me until I reached my wish.*

To my dear mother, *Professor Dr. Orouba Al-Birmani, who has always embraced me with the love of her great heart and her great kindness, who has always embraced me with the tenderness of her kind soul.*

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Summary

Streptococcus pyogenes (group A β-hemolytic Streptococcus (GAS)) is an important Gram-positive bacteria causes a wide spectrum of clinical diseases ranging from mild pharyngitis to life-threatening invasive infections.

It is the most common bacterial etiology for acute pharyngitis and accounts for 5 to 15% of all adult cases and 20 to 30% of all pediatric case.

A total of 125 clinical specimens , were collected from patients suffering from acute pharyngitis infection. who admitted to two main hospitals of Babylon Governorate : Al-Hilla General Teaching Hospital and Al-imam Al-Sadq General Teaching Hospital during the period extending from October, 2021 to January, 2022. The specimens were cultured in blood agar media, Columbia blood agar and Brain Heart Infusion agar media for isolation of S.*pyogenes.* Out of 125 pharyngeal swabs , only 40 isolates of S.*pyogenes* were detected by culture , biochemical test and specific gene (*spy1258*).

The diagnosis was confirmed for 40 bacterial isolates from *S.pyogenes* by means of the Vitek2 compact technique (Identification ID). Polymerase chain reaction (PCR) was performed using specific primer on genomic DNA from S.*pyogenes* . PCR amplification of *spy1258* gene indicated that 31/40 (77.5%) of S.*pyogenes* isolates prossess this gene. Streptococcal mitogenic exotoxin Z (*SMEZ*) gene is also studies and it was observed in 14/40 (35%) isolates of S.*pyogenes* .

Random amplified polymorphic DNA (RAPD) fingerprinting is a PCR based technique which has been successfully used to appear genetic variation between closely related strains within the same species, it is more reliable technique for the typing of GAS isolates in epidemiological investigation. the current study aims to investigation genetic diversity of S. *pyogenes* isolates from acute pharyngitis patients by RAPD-PCR fingerprinting method.

The current study implicated for investigation the genetic diversity using the RAPD-PCR fingerprinting method which achieved by arbitrary primer OPA13. Amplification of genomic DNAs from the GAS isolates with OPA13 primer resulted polymorphic DNA segment, phylogenetic analysis showed a high degree of genetic diversity, classified GAS isolates into 4 main clusters. The DNA fingerprinting by using RAP-PCR analysis is an effective method for evaluation the genetic diversity of GAS isolates.

The S. *pyogenes* isolates showed positive results for *spy1258*, *smeZ* genes and RAPD-PCR . Then all isolates were subjected to the antibiotic sensitivity test (AST). The reading of the device showed the following ratios: ampicillin (85%), Levofloxacin (100%), moxifloxacin (100%), erythromycin (0%), clindamycin (70%), linezolid (100%), vancomycin (100%), tetracycline (25%), Tigecycline (100%), chloramphenicol (85%), Trimethoprim / sulfamethoxazole (90%), respectively.

By studying all the apparent clusters of GAS, this study concluded a joint sensitivity to the antibiotics (levofloxacin, linezolid and moxifloxacin).

In addition, this study found genomic diversity of GAS isolates suggested that the isolates are multicolnal in origin dependent on the differences in a susceptibility toward antibiotic in clusters. Although the clusters shared with antibiotic resistance for (Erythromycin, Clindamycin , Tetracycline).

The present study showed that group A streptococci (GAS) are genetically diverse and possess *smeZ* genes regardless of their invasiveness. Majority of the GAS exhibited no restricted pattern of virulotypes. Therefore, it can be suggested that virulotyping is partially useful for characterizing a heterogeneous population of GAS and the greater occurrence of this gene in cluster No.1 is probably due to that these isolates were collected over long period of the time and had been represented by a comparative large number of isolates.

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List of Abbreviations

Abbreviation	Meaning
AM	Ampicillin
ARF	Acute Rheumatic Fever
BENPEN	Benzyl penicillin
bp	base pair
C	Chloramphenicol
C°	Degree Celsius
CD	Clindamycin
CTX	Cefotaxime
CTX	Ceftriaxone
D.W	Distilled water
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
DNA	Deoxyribonucliec acid
dNTP	Deoxyribonucleotide Triphosphate.
dTTP	Deoxythymidine Triphosphate
E	Erythromycin
EDTA	Ethylenediaminetetraacetic Acid
GAS	Group A Streptococcus
GEN	Gentamicin
gm	Gram
HGT	Horizontal gene transfer

LE	Levofloxacin
LZ	Linezolid
mg	Milligram
$MgCl_2$	Magnesium Chloride
ml	Milliliter
µl	Microliter
MLST	Multi- Locus Sequence Typing
MLST	multilocus sequence typing
MO	Moxifloxacin
NF	necrotizing fasciitis
No.	Number
NS	Non-Significant
P	Probability
PCR	Polymerase chain reaction (PCR)
PFGE	plus field gel electrophoresis
pg	Picogram
Pmol	Picomole
PTA	peritonsillar abscess
RA	Rifampicin
RAPD	Random amplified polymorphic DNA
RHD	rheumatic heart disease
S	Significant
S. *pyogenes*	*Streptococcus pyogenes*
SAgs	superantigens

SLO	streptolysin O
SLS	Streptolysin S
SMEZ	Streptococcal mitogenic exotoxin Z
SMEZn	streptococcal mitogenic exotoxin Zn
SPE	Streptococcal pyrogenic Exotoxins
SPEs	streptococcal pyrogenic exotoxins
SSA	streptococcal superantigen
STSS	streptococcal toxic shock syndrome
Taq polymerase	Thermus aquaticus polymerase
TBE	Tris-Borate-EDTA
TE	Tetracycline
TE	Tris-EDTA buffer
TEI	Teicoplanin
TGC	Tigecycline
TR	Trimethoprim / Sulfamethoxazole
Tris-HCl	Trisaminomethane Hydrochloride
UV	Ultra Violet
VA	Vancomycin

Chapter One

Introduction

1. Introduction:

Streptococcus pyogenes or group A *Streptococcus* (GAS) are β-haemolytic and are one of the most virulent Streptococcus species, causing skin infections, pharyngitis, impetigo and other invasive diseases (Luis *et al*., 2020) .

Streptococcus pyogenes is a human-restricted pathogen with a profound and persistent global burden of disease. It is a ubiquitous human pathogen and is responsible for more than 500,000 deaths each year. Acute pharyngitis is the most common of all clinical syndromes due to S. *pyogenes*. The diverse clinical spectrum of S. *pyogenes* also includes severe diseases including scarlet fever, cellulitis, necrotising fasciitis, toxic shock syndrome and post-infectious glomerulonephritis, acute rheumatic fever, and its sequelae rheumatic heart disease (Anderson *et al*., 2022).

Streptococcus pyogenes or Group A *Streptococcus* (GAS) was the most predominant bacteria found in the case of bacterial pharyngitis. GAS accounted for between 15 and 30 percentages of sore throat particularly in children under 15 years old . The incidence decreased with increasing age and less than 10% of acute pharyngitis in adults were bacterial causes. Globally, over 600 million cases of GAS pharyngitis are estimated each year (Osowicki *et al*., 2021) .

Antimicrobial agents are used in virtually all infections due to beta-hemolytic streptococci once diagnosed, regardless of severity, since it reduces symptoms and prevents immunological complications associated with S. *pyogenes* and streptococcal pharyngitis. Although uniformly susceptible to penicillin for years, beta-hemolytic streptococci have recently developed resistance to such a drug (Barros, 2021).

Several antimicrobials are used for the treatment of S. *pyogenes* infections such as cephalosporins, Lincosamides (clindamycin), and macrolides (erythromycin, clarithromycin and azithromycin) are usually used as first line drugs against GAS infection among patients with beta-lactam allergies. In addition, fluoroquinolones (Ciprofloxacin, levofloxacin etc.), tetracyclines, linezolid and vancomycin are all used as alternative antimicrobials. Antibiotic resistance in *Streptococcus pyogenes* has been changing and it is mainly due to inappropriate usage of broad-spectrum antibiotics (Berwal *et al*., 2019).

Resistance to the antimicrobial classes macrolides, lincosamides (such as clindamycin) and streptogramin B in beta –hemolytic streptococci is primarily linked to the acquisition of target modification enzymes encoded by *erm* genes, mediating resistance to all three classes, or *mef* genes encoding efflux pumps targeting only macrolides. In GAS all these resistance genes are associated with mobile genetic elements, and dissemination of these elements occur by either clonal expansion or horizontal genetic transfer (Oppegaard *et al*., 2020).

Genotyping is an effective method for monitoring bacterial strains in microbiology research and sequence analysis of the emm gene is currently used for GAS genotyping (Gherardi *et al*., 2018).

In recent years, emm cluster analysis has also been widely used in GAS molecular epidemiology analysis. The type-specific M protein, encoded by the *emm* gene, and superantigens (SAgs), encoded by the sAg genes, are important virulence factors in S. *pyogenes* (Golińska *et al*., 2016).

Random amplified polymorphic DNA(RAPD) fingerprinting is a technique is used in molecular typing of *streptococcus spyogenes* and to estimate genetic diversity among them which has also been called arbitrarily primed PCR (Moghaddam *et al*., 2019).

In RAPD analysis, the genomic DNA is amplified with a single primer with an arbitrarily selected nucleotide sequence. Binding of the primer to the template DNA is favored by a low annealing temperature. The multiple PCR products are separated according to size by agarose gel electrophoresis. The resulting RAPD patterns of different isolates can then be compared (Nusifera and Alia, 2019).

Transcriptional regulators are specialized DNA binding proteins which play a crucial role in directing gene expression within bacteria for their adaptation and survival in different environmental conditions. . *Spy1258* is a putative transcriptional regulator gene (TetR/AcrR family) which is specific for S. *pyogenes* and can be used as a marker for its detection (Abraham and sistla, 2020) .

The strains of S. *pyogenes* are often tested for the expression of super antigens (speA) These are capable of binding to the major histocompatibility complex II molecules on antigen presenting cells and T cell receptors, leading to the release of large amounts of cytokines. These cytokines have been shown to play major roles in fever, tissue destruction, shock, hypotension, and organ failure associated with invasive Streptococcal infections , such as smez, Streptococcal mitogenic exotoxin (smeZ) are extracellular toxin and exhibit antigenic properties (Altun and Yapıcı, 2020).

All of the streptococcal superantigens are encoded by genes located within bacteriophages, excluding Streptococcal pyrogenic exotoxin G (SPE G) and SMEZ, which are encoded on the core chromosome (Pandey *et al*, 2019).

Aim the Study:

The present study aims to investigate the genetic diversity of the local isolates of *Streptococcus pyogenes* by using RAPD fingerprinting technique and its association with antibiotic susceptibility profile, this aim was achieved by the following objectives:

1- Isolation and identification of *Streptococcus pyogenes* from pharyngitis patients by cultures and biochemical test.
2- Evaluation of antibiotic resistance in S. *pyogenes* by using AST Vitek 2 compact system.
3- Extraction of bacterial genomic DNA by (Promega/ USA) kit.
4- Molecular characterization of *Streptococcus pyogenes* by amplification of *spy1258* gene using conventional PCR technique.
5- Genetic detection of Streptococcal mitogenic exotoxin Z (smeZ) gene.
6- The DNA genotyping of isolates was achieved by RAPD- PCR analysis.

Chapter Two

Literature Review

2. Literatures Review:

2.1 General Characteristics of *Streptococcus:*

Streptococcus species are Gram-positive, cocci, facultative anaerobic bacteria. Species of Streptococci can be further defined using the Lancefield grouping scheme, a serotyping classification. In the United States, group A *Streptococcus* (GAS) includes *Streptococcus pyogenes*, and GAS is estimated to cause 240,000 foodborne illnesses annually. Other *Streptococcus* groups have also been observed to occasionally result in foodborne illnesses (Tan *et al*., 2014)

When alpha hemolysis (α-hemolysis) is present, the agar under the colony is dark and greenish. *Streptococcus pneumoniae* and a group of oral streptococci (*Streptococcus viridans* or *viridans streptococci*) display alpha hemolysis. This is sometimes called green hemolysis because of the color change in the agar. Other synonymous terms are incomplete hemolysis and partial hemolysis. Alpha hemolysis is caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobin to green methemoglobin (Sohail, 2021).

Beta hemolysis (β-hemolysis), GAS produce sometimes called complete hemolysis, is a complete lysis of red cells in the media around and under the colonies: the area appears lightened (yellow) and transparent. Streptolysin, an exotoxin, is the enzyme produced by the bacteria which causes the complete lysis of red blood cells. There are two types of streptolysin. Streptolysin O (SLO) and Streptolysin S (SLS). Streptolysin O is an oxygen-sensitive cytotoxin, secreted by most Group A streptococcus (GAS), and interacts with cholesterol in the membrane of eukaryotic cells (mainly red and white blood cells, macrophages, and platelets), and usually results in β-hemolysis under the surface of blood agar. Streptolysin S is an oxygen-stable cytotoxin also produced by most GAS strains which results in clearing on the surface of blood agar. SLS affects immune cells, including polymorphonuclear leukocytes and lymphocytes, and is thought to prevent the host immune system from clearing infection. Streptococcus pyogenes, or Group A beta-hemolytic *Streptococcus pyogenes* (GAS), displays beta hemolysis (Shaikh *et al*., 2010).

2.2 General Characteristics of *Streptococcus pyogenes:*

Streptococcus pyogenes is a species of Gram-positive, aerotolerant bacteria in the genus *Streptococcus*. These bacteria are extracellular, and made up of non-motile and non-sporing cocci (round cells) that tend to link in chains. They are clinically important for humans, as they are an infrequent, but usually pathogenic, part of the skin microbiota that can cause Group A streptococcal infection. S. *pyogenes* is the predominant species harboring the Lancefield group A antigen, and is often called group A *Streptococcus* (GAS). However, both *Streptococcus dysgalactiae* and the *Streptococcus anginosus* group can possess group A antigen as well. Group A streptococci, when grown on blood agar, typically produce small (2–3 mm) zones of beta-hemolysis, a complete destruction of red blood cells. The name group A (beta-hemolytic) *Streptococcus* (GABHS) is thus also used (Bessen, 2009).

A variety of virulence factors are associated with the severity of GAS infection including streptolysin O and S (hemolysin), streptokinase, streptodornase, M protein and its related protein, hyaluronic acid capsule, hyaluronidase, the cysteine protease SpeB, superantigen proteins (SAgs), and several phage-encoded exotoxins. M-like protein is a term applied to the surface protein that resembles the M protein in its structure . Virulence factors are equally distributed within S. *pyogenes*; some are encoded by chromosomes, while others depend on the presence of mobile genetic elements. . M protein is the most analyzed virulence factor which can be used in the serotype classification of S. *pyogenes* (Helal *et al*., 2020).

Moreover, invasive S. *pyogenes* strains, which cause deep soft-tissue infections and fasciitis as well as streptococcal toxic shock syndrome (STSS) and sepsis, also produce highly specific toxins with special pro-inflammatory properties. Streptococcal pyrogenic Exotoxins (SPE) possess superantigen properties and bridge antigen-presenting cells with immune system effector cells, leading to their polyclonal activation. This activation leads to accelerated T lymphocyte proliferation and liberation of significant quantities of proinflammatory cytokines, leading to toxic shock (Golińska *et al*., 2016).

2.3 Pharyngitis:

Pharyngitis, or commonly known as sore throat or strep throat, is the most common manifestation of infection with *Streptococcus pyogenes* (GAS). Infection with this bacterium is diagnosed in 20 to 40% of pharyngitis cases in children and in 5 to 15% in adults (Shaikh *et al*., 2010).

The peak incidence of GAS pharyngitis occurs in children aged 5 to 15 years old. Pharyngitis is more common to occur during winter and spring and can be transmitted by direct or indirect contact with an infected person via large respiratory secretions droplets, or through contaminated objects and food Non-infectious auto-inflammatory complications of pharyngitis can include acute rheumatic fever and post-streptococcal glomerulonephritis, both of which are thought to result from immune responses to streptococcal infection (Wessels, 2016).

Clinical features of GAS pharyngitis vary according to the age of the patient. Predicting GAS pharyngitis from clinical findings is more difficult in younger than in older children especially because younger children often do not complain of sore throat (Karacan *et al*., 2007).

The number of pharyngitis cases is higher in children than in adults due to exposures in schools, nurseries, and as a consequence of lower host immunity. Cases of Streptococcal pharyngitis occur more frequently in later winter to early spring in seasonal countries due to many people rebreathing the same indoor air. Disease cases are the lowest during autumn (Efstratiou and Lamagni, 2016) .

Clinical manifestations frequently include fever, tonsillar exudates, painful cervical adenopathy, pharyngeal erythema, and ear pain. Uncomplicated infectious pharyngitis, both viral and bacterial, typically is self-limited to 5 to 7 days, is not progressive, is bilateral, does not have trismus, and does not have evidence of airway obstruction (stridor) (Topal *et al.,* 2020) .

In addition to throat pain, symptoms may include fever, chills, malaise, headache, and particularly in younger children abdominal pain, nausea, and vomiting , Pain with swallowing and the presence of swollen, tender anterior cervical lymph nodes are typical features. Abdominal pain and vomiting is common, especially in younger children. Cough, rhinorrhea, hoarseness, conjunctival irritation, and diarrhea are notably absent in

Streptococcal pharyngitis, and the presence of these symptoms should suggest a non-Streptococcal (usually viral) etiology (Igarashi *et al*., 2017).

Scarlet fever is caused by one or more of the pyrogenic exotoxins produced by pharyngeal strains of GAS. Signs and symptoms most indicative of GAS pharyngitis are tonsillar or pharyngeal exudates, tender anterior cervical nodes, fever or history of fever, and absence of cough (Ebell *et al*,. 2000).

One of the first basic stages of S. *pyogenes* etiology is epithelial adhesion and colonization (Iuchi *et al.*, 2020).

Although GAS pharyngitis is self-limited, treatment is indicated within 9 days of symptom onset to prevent ARF and suppurative complications (Kimberlin, 2018).

Antibiotic stewardship can be achieved by providing delayed antibiotic prescriptions (i.e., to be filled only for children with positive cultures). The optimal treatment for GAS pharyngitis continues to be penicillin or amoxicillin for 10 days, because all Group A streptococci are susceptible to penicillin . Amoxicillin can be dosed once daily and a suspension is available (unlike penicillin V) (Le Saux *et al.*, 2014).

For children with non-anaphylactic hypersensitivity reactions to penicillins, an oral amoxicillin challenge or cephalexin is recommended (Wong *et al.*, 2020) .

2.4 Epidemiology of Group A Streptococci:

Group A streptococci (GAS) are an exclusively human pathogen. causes significant disease worldwide and adds a large burden to national health care systems . GAS are one of the most common bacteria encountered daily, and they result in acute infections with a wide array of manifestations in both adults and children and is responsible for an estimated 9,000-12,000 deaths per year in the United States (Liang *et al*., 2012) .

In developed countries, around 15% of school aged children develop a case of pharyngitis (generally called strep throat) each year whereas in developing countries the number is five to ten times more (Ali, 2016).

For superficial diseases such as pharyngitis and tonsillitis, it is estimated that over 600 million cases of symptomatic GAS pharyngitis occur each year among people aged over 4 and around 550 million of these cases occur in developing countries . In Europe, they account for around 800 consultations per 10,000 patients annually with all the economic impact of days missed from work or school (Gerber *et al*., 2009)**.**

Studies examining carriage rates in healthy adults suggest low levels of carriage . Estimates of pharyngeal *S. pyogenes* carriage in healthy children vary considerably from 2% up to17% (Marshall *et al*., 2015).

In the United States, 11 million people get infected with pharyngitis caused by GAS each year. 15-30% of the pharyngitis cases in children are caused by GAS while many cases are also caused by viral infections Scarlet fever incidence has fallen considerably over the last century, but recent studies show increased incidence of outbreaks in some developed countries despite the fact it is no longer a life-threating disease (Efstratiou and Lamagni, 2017).

Group A Streptococcus (GAS) is responsible for causing around 700 million cases of pharyngitis annually worldwide. Increasing incidence of mild symptoms such as strep throat may lead to an invasive prevalence of conditions such as necrotizing fasciitis (NF; also called flesh-eating disease), streptococcal toxic shock syndrome (STSS) and other post-infectious immune-related diseases at the population level (Castro and Dorfmueller, 2021).

Acute rheumatic fever (ARF) is the result of an autoimmune response to pharyngitis caused by infection with the sole member of the group A Streptococcus (GAS), *Streptococcus pyogenes*. ARF leads to an illness that is characterized by various combinations of joint pain and swelling, cardiac valvular regurgitation with the potential for secondary heart failure, chorea, skin and subcutaneous manifestations and fever (Carapetis *et al*., 2016) .

2.5 Pathogenesis of *Streptococcus pyogenes:*

Streptococcus pyogenes typically colonizes the throat, genital mucosa, rectum, and skin. Of healthy individuals, 1% to 5% have throat, vaginal, or rectal carriage. In healthy children, such carriage rate varies from 2 to 17%. There are four methods for the transmission of this bacterium: inhalation of respiratory droplets, skin contact, contact with objects, surface, or dust that is contaminated with bacteria or, less commonly, transmission through food. Such bacteria can cause a variety of diseases such as streptococcal pharyngitis, rheumatic fever, rheumatic heart disease, and scarlet fever (Efstratiou and Lamagni, 2016) .

Entry ports for GAS after person to person transmission are oral cavity, skin and wounds. In particular, mucosal membranes of the oropharynx and non-intact skin are preferred colonization sites (Cunningham, 2000; Tan *et al.*, 2014).

In otherwise healthy individuals, GAS usually causes mild and self-healing purulent infections of mucosal membranes and skin, such as pharyngitis, impetigo and pyoderma. In patients with predispositions such as immune-suppression, diabetes and related diseases, or specific HLA-DR (MHC class II cell surface receptor) subtypes, occasionally severe and invasive life-threatening diseases occur. Necrotizing fasciitis and streptococcal toxic shock syndrome belong to these disease manifestations with high morbidity and mortality rates. Antibiotic therapy is mandatory, even for uncomplicated primary infections, to prevent secondary autoimmune sequelae like rheumatic heart disease or glomerulonephritis (Cunningham, 2000).

The GAS cell surface displays a variety of proteins and other macromolecules that facilitates the colonization of host tissues (Nobbs *et al*., 2009).

The initial attachment process has long been hypothesized to be a two-step process, with weak and/or long-range interactions followed by more specific, high-affinity binding. Weak, hydrophobic interactions mediated by lipoteichoic acids may contribute to initial adherence to host surfaces .This weak interaction, in turn, may permit longer distance first attachment events mediated through extending surface appendages such as pili, followed by multiple, higher affinity binding events such as protein–protein or lectin–carbohydrate interactions. The GAS cell surface incorporates numerous protein adhesins that allow GAS to colonize distinct tissue sites. Many of these adhesive proteins are covalently attached to the cell wall peptidoglycan by sortase enzymes (Brouwer *et al.*, 2016).

Group A Streptococcus (GAS) is well adapted to its human host, since it is equipped with a large set of virulence factors of all classes. The bacteria express surface proteins and secreted factors leading to (i) immunoglobulin and complement factor degradation (EndoS, Mac, C5a peptidase) and (ii) general complement inhibition (achieved by M protein, capsule expression and Sic), (iii) extracellular matrix and serum protein binding via multiple MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) (M protein, Cpa, Eno, Epf, up to five different firbronectin-binding MSCRAMMS), (iv) dysregulation of coagulation (plasminogen/plasmin binding, streptokinase Ska activity), and (v) cytotoxic and cytolytic activity toward various host cell types (Nga, SLS, SLO). Depending on the presence of phage-related chromosomal islands as variable parts of the accessory genome in the different GAS serotypes, a variable number of superantigens (SpeA-J, SmeZ) is expressed and secreted (Banks *et al*., 2002; Spaulding *et al*., 2013).

The presence of individual genes encoding virulence factors is GAS serotype-specific and expression depends on environmental conditions. Transcriptional changes during GAS cultivation and pathogenesis were reviewed (Fiedler *et al.*, 2010).

As successful pathogen, GAS tightly controls virulence factor gene expression to keep the number of exposed proteins for immune recognition to a minimum. Regulation occurs on multiple levels including the activity of stand-alone transcription regulators and two component signal transduction systems, catabolite control, control of mRNA decay, cis- or trans-acting regulation of small non-coding RNAs (Patenge *et al.*, 2013), and quorum sensing (Jimenez and Federle, 2014).

Apart from the well-studied GAS virulence traits and pathogenesis mechanisms, like host cell adherence/internalization, phagocytosis resistance, escape from phagocytic killing, host cell apoptosis induction and autophagy escape (Walker *et al.,* 2014).

Group A streptococci also impairs phagocytic mechanisms with the help of the factors, Mac (1 and 2) and SIC. Cytolysins which promote phagocytic lysis and apoptosis are also used by GAS. GAS resist effectors of phagocytic killing by D- anylation of (LTA) lipoteichoic acid, SpeB, SIC, M protein and GpoA (glutathione peroxidase) (Kwinn and Nizet, 2007).

The production of reactive oxygen species by pathogen-infected cells is often associated with high levels of cell death, and S. *pyogenes* induce apoptosis in infected human epithelial cells (Regnier *et al*., 2016).

NADase and streptolysin O (SLO) are two potent cytotoxins secreted by S. *pyogenes,* and multiple roles in virulence have been attributed to each protein. SPN is actively translocated into the host cells, resulting in cell injury and death via depletion of host cell NAD+. SLO is a cholesterol-dependent cytolysin that forms pores in host cell membranes. SPN and SLO inhibit phagocytosis, inhibit maturation of autophagosomes, impair neutrophil oxidative burst, and prevent the killing of S. *pyogenes* by a variety of host immune cells (Zhu *et al*., 2017) .

Moreover, S. *pyogenes* induce the transcription of the interleukin (IL)-1a, IL-1b, IL-6, and IL-8 genes and the release of prostaglandin E2 .The surface of S. *pyogenes* cells incorporates a number of proteins adhesins (e.g., pili, Sfb1 / PrtF1, and M protein) that allow S. *pyogenes* to colonize distinct tissue sites (Brouwer *et al*., 2016).

2.6 Antibiotic Susceptibility Profile of *Streptococcus pyogenes:*

Group A Beta Hemolytic Streptococcus (S. *pyogenes*) (GAS) is a main bacterial infectious agent associated to pharyngitis. Optimal therapeutic approach in these patients has been a matter of debate to avoid the complications of infection. There are some complications of these infections including acute rheumatic fever (ARF), peritonsillar abscess (PTA), and rheumatic heart disease (RHD) . Some therapeutic approaches have been defined to this disease management. Treating the patients with pharyngitis without testing ensures some advantage, but this approach could have some complication such as risk of anaphylaxis, and use of irregular antibiotic has been associated to antibiotic resistance Observing the patients without providing testing or treatments are avoided the risks associated with use of antibiotic, but early treatment with antibiotics may prevent other rare complications of untreated GAS pharyngitis, including PTA, ARF, and RHD (Doğan *et al.,* 2014) **.**

Most S. *pyogenes* infections were treated with penicillin and still being effectively used for emprical treatment . However, those patients with allergic to penicillin have been treated with erythromycin, amoxicillin, cotrimoxazole, chloramphenicol, tetracycline, azithromycin and clindamycin. Hence, current treatment guidelines discourage the empirical use of antibiotics due to unnecessary antibiotic exposure and drug resistance. Additionally, due to lack of β-lactamase enzyme production by S. *pyogenes*, it was considered universally susceptible to penicillin group and later generation of β-lactam antibiotics (kebed *et al*., 2021).

In an Iraqi study on the antibiotic resistance of S.*pyogenes,* it was found that the isolates were sensitive to both Vancomycin and Ceftrtiaxon. While, completely resistance to Ampicillin and Amikacin (Dakhil and Hamim, 2016).

Erythromycin and other macrolides are considered as alternative 8 treatment options. However, because of the widespread use of these antibiotics, there has been a global rise in the emergence of macrolide resistance among strains of S. *pyogenes,* though the rate varies from one 9-11 geographical region to another (Tsai *et al*., 2021).

Streptococcus pyogenes resists the activity of macrolides via two predominant mechanisms. Some resistant strains utilize an efflux mechanism, where the bacterium utilizes a membrane associated protein that pumps the macrolide out of the cell, severely reducing the antimicrobial effect. This membrane protein is specific for macrolides. When the efflux mechanism is present in a macrolide resistant strain it is referred to as the M phenotype. S.*pyogenes* strains expressing the efflux pump are resistant to erythromycin, but they are susceptible to clindamycin (a drug from the lincosamide family) and streptogrammin B. Macrolides, lincosamides, and streptogrammin B are destructive to bacteria by binding to ribosomes and interfering with protein translation. The efflux pump (M phenotype) is associated with the presence of the *mefA* gene.The second mechanism of resistance is the modification of the bacterial ribosomes. Bacteria with this mechanism produce an enzyme that adds a methyl (-CH3) group on the ribosome. This methylation slightly changes the shape of the ribosome and reduces the affinity of the drug for the ribosome. The characteristics displayed by this mechanism are referred to by the MLSB phenotype (Rowe *et al.,* 2009).

2.7 Molecular Typing Methods of *Streptococcus pyogenes:*

Epidemiological investigations of GAS infections and outbreaks usually include classification into one of ~150 serotypes (emm typing) and Multi-Locus Sequence Typing (MLST). Both methods rely on sequencing of one (emm typing) or seven (MLST) typing genes. Both methods are relatively fast and uncomplicated, but require specialized equipment and can be too expensive for routine use. In addition, they both have too low resolution to distinguish between closely related strains. Pulsed field gel electrophoresis (PFGE) has been for many years the method of choice used to investigate differences between strains at the genome level. But PFGE analysis requires specialized equipment, skilled personnel, and is difficult to analyze and compare (Karaky, 2012).

Borek and coauthors 2012, conducted other method for S.*pyogenes* typing named phage profiling (PP) is based on a simple assumption that a regular PCR reaction with Taq polymerase and relatively short elongation time is not able to yield long DNA fragments, such as ~40–50 due to the presence of integrated phage. Only fragments without any integrated DNA, or short fragments inserted between integration sites can be efficiently amplified. Twenty one prophage and ICE (integrative conjugative element) integration sites (named from A to U) are well defined within GAS core chromosome (Borek *et al.,* 2012).

2.7.1 Emm-Typing of *Streptococcus pyogenes:*

The M protein, the most well-characterised GAS virulence determinant, is a surface expressed coiled coil protein that extends up to 60 nm from the cell wall and interacts with numerous proteins in the host extracellular matrix and serum (Frost *et al.,* 2018).

The contribution to virulence of the M protein is predominantly attributed to immune modulatory effects, mediated by the binding of host proteins such as immunoglobulins (Ig) and fibrinogen (Smeesters *et al*., 2010).

The M protein has been shown to be immunogenic; however, vaccine development targeting this protein has been hindered by the large diversity in the most immunogenic region of the protein at the N-terminal. Despite this, one leading GAS vaccine candidate is based on multiple N-terminal M protein antigens (Dale *et al*., 2013; Steer *et al*., 2016).

The *emm* gene encodes for the M protein and the variability within the N-terminal sequence of the *emm* gene accounts for the different emm types among the GAS strains, and studies shows that there are more than 234 emm types exist (Ferretti *et al.,* 2016).

The variable sequence of the *emm* gene that encodes for the M sera-specificity lies adjacent to one of the amplifying sequences of the primers, hence, allowing for direct and sequencing (Gillespie and Hawkey, 2006).

Large epidemiological studies of pharyngitis and invasive disease have been done using emm sequence typing, particularly in the USA, Canada, and Europe . The emm typing has added a huge change as a reliable epidemiological tool for GAS epidemiology and subdivision; it also has the potential to classify isolates that was difficult to type by serological methods. It is also able to determine the non-typeable and weakly antigenic isolates (Bessen *et al.*, 2015).

All *emm* types fall into three main groups with distinct molecular structures that correspond to the previously described emm pattern-typing (patterns AC, D, and E), based on the presence and arrangement of *emm* and *emm*-like genes within the S. *pyogenes* genome (McMillan *et al.*, 2013).

The emm pattern of GAS strains strongly correlates with the preferred epithelial site of infection i.e. pharynx versus skin Thus, emm pattern AC strains are considered throat specialists, whereas pattern D are skin specialists, and pattern E are generalists (Bessen, 2016).

The M protein is a fibrillar coiled-coil dimer that extends from the bacterial cell wall, and is considered an archetypal Gram-positive surface protein (Marraffini *et al.*, 2006).

M protein inhibits phagocytosis of GAS in the absence of opsonising antibodies, promotes adherence to human epithelial cells and helps the bacterium overcome innate immunity .The multifunctional nature of this protein is also evident from its interactions with numerous host proteins occurring along the entire length of the surface-exposed portion of M protein (Smeesters *et al.*, 2010).

Most of the M protein sequence consists of heptad repeat motifs in which the first and fourth amino acids are typically hydrophobic, and are core stabilizing residues within the coiled coil (Lupas *et al.*, 2017).

Heterogeneity in the amino acid sequence of the N-terminal part of M protein, resulting in antigenic diversity, forms the basis of GAS serotyping, which was used for many decades (McMillan *et al.,* 2013).

Accurate emm-typing may not be achieved because of overlap in sequence between the *emm* genes and closely related *emm*-like genes called mrp enn, and sph . The 3′ sequences of *emm*-like genes are sufficiently similar to *emm* genes such that the emm typing by using CDC1 & CDC 2 primers . The CDC 2 is able to anneal and facilitate amplification from these genes as well as *emm*. The non-specificity of primer CDC2 leads to observed double bands in gel electrophoresis and can result in poor-quality sequencing or non-typeable strains. A recent large global WGS study enabled better definition of emm and emm-like genes . Using this global database, we now propose an updated emm-typing protocol, using a new and more specific primer to replace the 3′-situated CDC2 for more effective and accurate typing of S. pyogenes strains globally (frost *et al.,* 2020).

2.7.2 Typing by RAPD –PCR:

RAPD-PCR is a simple, rapid, easy, and inexpensive method that can be performed in a moderate laboratory (Rai *et al.,* 2009) .

In this method, a single short primer (8-12 nucleotides) is used in each reaction which its melting temperature (Tm) is low . Primers can attach randomly to several DNA sequences in the genome (Nanvazadeh *et al*., 2013).

The number and the positions of binding primer sites are unique for each bacterial strain .Amplified segments of DNA in RAPD PCR technique are random. Differences between the generated RAPD patterns from the different DNAs indicate polymorphism between strains. Prior knowledge of the genome under research is not necessary . Therefore, this technique is suitable for molecular typing of unknown strains. The main disadvantage of RAPD-PCR method is low reproducibility of the results (Nandani and Thakur, 2014).

Low intra-laboratory reproducibility is because of very low annealing temperature in RAPD-PCR reaction and low interlaboratory reproducibility is as a result of the sensitivity of RAPD-PCR reaction to very little differences in reagents, protocols, and equipment . Most of the variation that is sometimes observed in RAPD-PCR pattern will be eliminated by the optimization of the RAPD reaction and PCR protocol (Moghaddam *et al.,* 2019).

2.8 Super Antigens of *Streptococcus pyogenes:*

Superantigens are an extraordinary family of nonglycosylated low-molecular-weight exoproteins. They are secreted by all human-pathogenic S. *aureus* and group A streptococci that we have tested (>8,000), with secretion dependent on a cleavable signal peptide . Superantigens have molecular sizes ranging from 19,000 to 30,000 Da (Mc Cormick *et al.,* 2001).

The proteins are unusually resistant to heat (for example, most remain biologically active despite boiling for 1 h), they are generally resistant to proteolysis (for example, by trypsin and pepsin) and acids (such as stomach acid), and they are highly resistant to desiccation (toxic shock syndrome toxin 1 [TSST-1] remains biologically active after being dried on petri dishes for more than one year) .Their biological toxicity and environmental stability have resulted in some superantigens being categorized as select agents of bioterrorism. group A streptococci also may produce large numbers of superantigens. These were originally known as scarlet fever toxins or erythrogenic toxins due to their abilities to cause the scarlet fever rash but have more been referred to as streptococcal pyrogenic exotoxins (SPEs) (Spaulding *et al.*, 2013) .

Group A streptococci can produce up to 11 serologically distinct superantigens .The streptococcal superantigens include SPE (serotypes A, C, and G to M), streptococcal superantigen (SSA), and streptococcal mitogenic exotoxin Zn (SMEZn) . All of the streptococcal superantigens are encoded by genes located within bacteriophages, excluding SPE G and SMEZ, which are encoded on the core chromosome (Pandey *et al.*, 2019).

Streptococcal mitogenic exotoxin (smeZ) are extracellular toxin and exhibit antigenic properties (Altun and Yapıcı, 2020).

These SAgs are capable of binding to the major histocompatibility complex II molecules on antigen presenting cells and T cell receptors, leading to the release of large amounts of cytokines. These cytokines have been shown to play major roles in fever, tissue destruction, shock, hypotension, and organ failure associated with invasive streptococcal infections (Igwe *et al.*, 2003).

The type-specific M protein, encoded by the *emm* gene, and superantigens (SAgs), encoded by the *sAg* genes, are important virulence factors in S. *pyogenes* . Currently, stains of S. *pyogenes* are often tested for the expression of *speA*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *smez*, and the enzyme-encoding *speB* and *speF* genes as genes encoding SAgs, even though speB and speF have been confirmed to encode cysteine protease and streptococcal DNase proteins (Strus *et al.*, 2017; Yu *et al.*, 2021).

SAgs contribute to GAS pathogenicity based on their immune stimulatory activity. SAgs gene distribution has been used as a method for the detection of genomic heterogeneity, the correlation between gene contents, and the determination of clinical manifestation (Helal *et al.*, 2020) .

Bacterial "stand-alone" response regulators are pivotal to the control of gene transcription in response to changing cytosolic and extracellular microenvironments during infection. The genome of group A Streptococcus (GAS) encodes more than 30 stand-alone RRs that orchestrate the expression of virulence factors involved in infecting multiple tissues, so causing an array of potentially lethal human diseases (Buckley *et al.*, 2020).

Transcriptional regulators are specialized DNA binding proteins which play a crucial role in directing gene expression within bacteria for their adaptation and survival in different environmental conditions. . *Spy1258* is a putative transcriptional regulator gene (TetR/AcrR family) which is specific for S. *pyogenes* and can be used as a marker for its detection (Khalaf *et al.*, 2020; Abraham and sistla, 2016).

Liu and other authors described *spy1258* gene that is exceptionally lay out in isolates of S. *pyogenes*. The using of PCR technique of *spy1258* gene assisted an amplification of DNA that extracted from GAS only, but never from the other Streptococcus species (Liu *et al.*, 2005).

Chapter Three

Materials and Methods

3. Materials and Methods:

3.1 Materials:

3.1.1 Equipments and Instrument:

The equipments and Instrument that used in this study are demonstrated in table (3-1).

Table (3-1) : Equipments used in this research

Equipments	Company\country
Aerobic incubator	Memmert\ Germany
Autoclave	Hirayama\ Japan
Benson burner	Membrane\ Germany
Centrifuge	GFL\ Germany
Conical flask (different size of flask)	Himedia \ India
Digital camera	Sony\ Japan
Disposable (Pteri Dish ,Syringe, Plane) and glassware	Citro\ China
DNA tubes100µl	Eppendorf \ Oxford
Eppendrof tube	Eppendrof \ Germany
filter paper	Citro\ China
Freezer	Kelon\ China
Gel electrophoresis system	Cleaver Scientific\ U.K
Graduated Glass Cylinder	Citro \ China
Hood	Labogene\ Denmark
Light microscope	Olympus\ Japan
Micropipettes(different size of Micropipettes)	Eppendrof\ Germany
NanoDrop	Implen \ Germany
PCR thermal cycler	Cleaver\ England

PCR tubes 50µl	Eppendorf \ Oxford
Plastic test tubes	Afco\ Jordan
Platinum wire loop	Himedia \ India
Refrigerator	Arcelik\ turkey
Sensitive balance	American science and surplus \ USA
Slides	Himedia \ India
Sterile freezing vial (1.5 ml)	Biofiedl \ Australia
Sterile swab	Afco\ Jordan
Test tube rack	Himedia \ India
Tips (Different sizes)	Jippo \ Japan
Ultra violet light transilluminaton	surplus \ USA
Vitek 2 compact systm	Biomerieux\France
Volumetric flask 100, 1000ml	Jippo \ Japan
Vortex	Gemmy\Twain
Water bath	GFL\ Germany

3.1.2 Chemical and Biological Materials:

The chemical and biological materials of present study are listed in table (3-2).

Table (3-2): Chemicals and biological materials with sources

Chemical Materials	**Company\ country**
Catalase reagent	Prondisa \ spain
Crystal violates ,Iodine ,Safranine	Sigma\ Germany
Distilled water	Oxoid \ England
Ethanol 70%	BDH\ England
Ethidium bromide, Loading dye (bromophenol blue), Agarose, Master mix	Promega \ USA

Glycerol	Fluka\England
Nuclease free water	Promega\USA
Oxidase reagent	Prondisa \spain
Tris-Borate-EDTA (TBE) buffer,TrisEDTA (TE)	Bioneer\Korea

3.1.3 Culture Media:

The biological materials of current study are found in table (3-3).

Table (3-3): Biological material and sources

Culture Media	Purpose of use	Company\country
Blood agar base	Detection of hemolysin production	Himedia\India.
Brain heart infusion agar	Better bacterial growth, purer colonies, and more numbers	
Brain heart infusion broth	It was used in preservation of bacteria	
Columbia agar base	A general medium for the growth of fastidious bacteria	
Muller-Hinton Agar	Antibiotic susceptibility test	

3.1.4 Antibiotics Cassette:

The antibiotics cassette of this study listed in table (3 - 4).

Table (3- 4): Antibiotics cassette

Antibiotic Cassette	**Symbol**	**Disc potenc**	**Company\country**
Ampicillin	AM	10 mcg	Biomerieux\France
Benzyl penicillin	BENPEN	10 mcg	
Cefotaxime	CTX	30 mcg	
Ceftriaxone	CTX	30 mcg	
Chloramphenicol	C	30 mcg	
Clindamycin	CD	2 mcg	
Erythromycin	E	15 mcg	
Gentamicin	GEN	10 mcg	
Levofloxacin	LE	5 mcg	
Linezolid	LZ	30 mcg	
Moxifloxacin	MO	5 mcg	
Rifampicin	RA	5 mcg	
Teicoplanin	TEI	30 mcg	
Tetracycline	TE	30 mcg	
Tigecycline	TGC	15 mcg	
Trimethoprim / Sulfamethoxazole	TR	5 mcg	
Vancomycin	VA	30 mcg	

3.1.5 Diagnostic Disk:

Diagnostic disk of this study is found in table (3 - 5).

Table (3-5): Main diagnostic Disk

Diagnostic disk	Potency	Company\country
Bacitracin	10µg	Himedia\India

3.1.6 Primers Used in PCR:

Primers of present study are found in table (3 – 6).

Table (3 - 6): Primers used in this Study

Genes Name	Primer sequence (5´- 3´)		Size bp	Company \country	Reference
Spy1258	F	AAAGACCGCCTTAACCACCT	407	Bioneer/ Korea	(Moghaddam *et al*., 2019)
	R	TGGCAAGGTAAACTTCTAAAGCA			
SmeZ	F	TTTCTCGTCCTGTGTTTGGA	246		
	R	TTCCAATCAAATGGGACGGAGAACA			
OPA13	CAGCACCCAC				

3.1.7 Commercial Kits:

The commercial kits of this study are listed in table (3 -7).

Table (3-7): The commercial kits used in the present study

Type of kits	Company/country
Gram stain kit	Sigma/ Germany
Vitek 2 System Kit	Biomerieux /France
DNA ladder 100bp - 2000bp	Promega/ USA
Green master mix 2X Kit	Promega/ USA
DNA extraction kit	Geneaid/UK

3.1.7.1 DNA Extraction Kit:

The genomic DNA extraction kit are found in table (3 -8).

Table (3-8): Genomic DNA extraction kit

Materials	Company/country
1- Cell lysis: -GB Buffer - GT Buffer	Geneaid/UK
2- DNA binding alcohols:- - Absolute ethanol alcohol	
3- Wash solution: - W1Buffer - Wash Buffer	
4- DNA Elution: - Preheated Elution Buffer	

3.1.7.2 DNA Ladder Marker Used in PCR (Promega/ USA):

DNA ladder marker of this study is found in table (3-9).

Table (3-9): DNA ladder marker used in PCR reaction

Materials
1-A ladder consists of 13 double –stranded DNA with size 100 bp -2500 bp.
2-Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orangeG, 10 mM Tris-HCl (pH 8) and 50 mM EDTA).

3.1.7.3 Master Mix Used in PCR (Promega / USA):

Master mix of this study is found in table (3 - 10).

Table (3-10): Master mix used in PCR reaction

Master Mix 2x
1_ DNA polymerase enzyme (Taq). 2_ dNTP 250 µm (dATP, dGTP, dCTP, TTP). 3_ $MgCl_2$ (1.5mM). 4_ Reaction buffer (pH 8). 5_ tracking dye.

3.2 Methods:

3.2.1 Subjects and Specimen Collection:

In this study, 125 throat swabs were collected from patients with pharyngitis of both sexes, ages range from 1to 60 years, who attended in to the ENT major hospitals in Babylon city (Al-Hilla General Teaching Hospital and Al-imam Al-Sadq General Teaching Hospital) during the period from October 2021 to January 2022.

These specimens were collected from patients who admitted Consultation Unit and were suffering from specific symptoms such as headache, cough, fever and runny nose, especially in children and under 15 years of age, pain with swallowing, swelling and vomiting in some cases, according to the doctor's diagnosis and by means of a clean and sterile swab for one use .

The samples were collected with all precautions taken to avoid any possible contamination. The throat swab is used principally to collect material from the faucial area in patients suffering from acute pharyngitis or tonsillitis, and in the detection of contact, convalescent, or chronic carriers of haemolytic streptococci.

The operator must have a clear view of the whole fauces in a good light and see the swab touching the affected surfaces.The patient should be sitting up or standing with the head tilted back; with children or nervous patients an assistant should support the back of the head with one hand, to prevent sudden or unexpected movement, and hold the patient's hands with the other.

3.2.2 Sample Handling Protocol:

The swabs were carefully taken with transported media to preserve bacteria, and then transferred to the laboratory of the College of Science for women . Samples were cultured on blood agar media, MacConkey agar, Columbia blood agar and Brain Heart Infusion agar and incubated at 37°C for 24 hours. After incubation, Colonies of different morphology were isolated and bacteria were stained with gram stain and examined under the light microscope. If we suspected of these Bacteria *Streptococcus pyogenes* are we confirm by Vitek 2 Compact system.

3.2.3 Questionnaire:

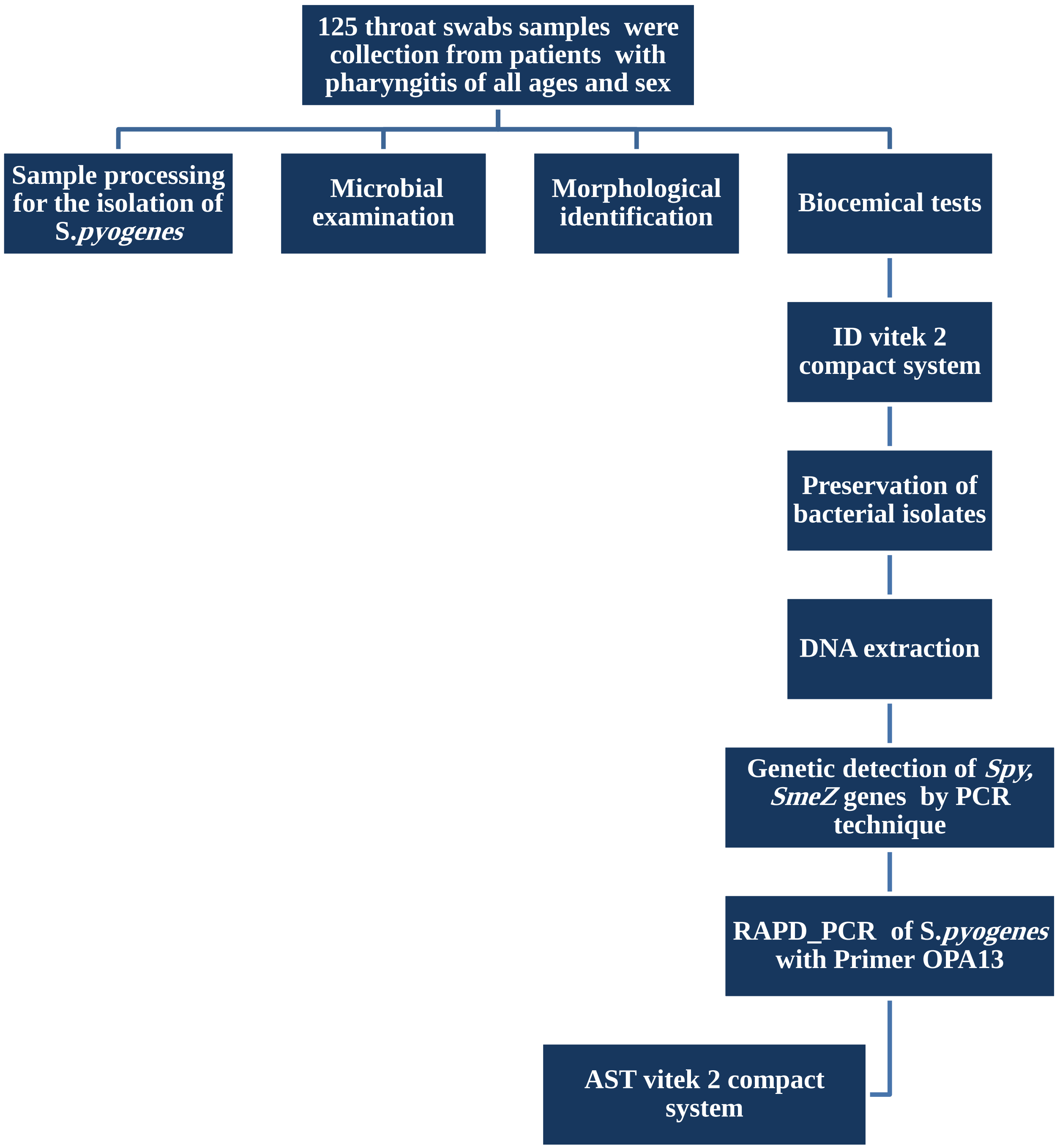
In this questionnaire, the name, age, sex, signature and whether the patient took antibiotics and the advisory status papers diagnosed by the doctor were taken. Pregnant women and people with chronic diseases were excluded in this study.

3.2.4 Ethical Approval:

The necessary ethical approval from ethical committee in Al-Hilla General Teaching Hospital and Al-Imam Al-Sadq General Teaching Hospital was obtained. Moreover, agreement from the family and patients for sampling and carrying out this work was obtained and this study was cross sectional study .

3.2.5 Study Design:

The current study is summarized according to the scheme (3-1) shown below:



Figure(3-1): Study design

3.2.6 Preparation of Solutions and Reagents:

3.2.6.1 Catalase Reagent:

This reagent is important for diagnosing S. *pyogen* bacteria. It is prepared by adding 3 ml of H_2O_2 to 100 ml of distilled water and then kept in a dark container where it detects the ability of the bacteria to produce the enzyme catalase (Forbes *et al.*, 2007).

3.2.6.2 Oxidase Reagent:

This reagent prepared immediately by dissolving 1gm of tetramethyl para phenyldiamine di-hydrochloride in 100ml distilled water in a dark bottle. Is used to detect the presence of oxidase enzymes produced by a variety of bacteria. The oxidase test is based on bacterial production of an intracellular oxidase enzyme and some organisms may produce more than one type of oxidase enzyme (Forbes *et al.*, 2007).

3.2.6.3 Gram Stain Solutions:

In this study, gram stain solutions from a company Sigma/ Germany were used, and the test was conducted according to the instructions mentioned by the company, and this included four solutions crystal violate, iodine, absolute alcohol, and safranine. It was used to diagnose colony characteristics by light microscopy and to distinguish between Gram-positive and Gram-negative bacteria (Forbes *et al.*, 2007).

3.2.7 Preparation of Culture Media:

All bacterial culture media that mentioned in table (3-3) were prepared according to the manufacturer's instructions and then sterilized by an autoclave at a temperature of 121 °C for 15-20 minutes.

3.2.7.1 Blood Agar Medium (pH: 7.1):

The blood agar medium was prepared according to the recommendations of the manufacturer, by dissolving 40 g of blood agar in 1 liter of distilled water, then sterilizing the medium with an autoclave. The purpose of using this medium is to provide a culture medium rich in the necessary requirements for the growth of fastidious bacteria such as S. *pyogenes* to determine their ability to hemolysis (Russell *et al.*, 2006) .

3.2.7.2 Muller - Hinton Agar Medium:

Muller_ Hinton agar medium was prepared according to the manufacturer's instructions by adding 42 g to 1000 ml of distilled water. And then put it in the autoclave and after the sterilization process, it is left to reach a temperature of 50 degrees Celsius and then fresh human blood is added to it for, the purpose of using it to test the sensitivity of the S. *pyogenes* bacteria to the anti-bacitracin (MacFaddin, 2000).

3.2.7.3 Brain Heart Infusion Agar Medium:

This medium was prepared by dissolving 40 gm of medium to 1000 ml distilled water. And then put it in the autoclave and after the sterilization process, it is left to reach a temperature of 50 degrees Celsius and then fresh human blood is added to it for (MacFaddin, 2000).

3.2.7.4 Brain Heart Infusion Broth With 5% Glycerol:

This medium was prepared by adding (15ml) of glycerol to (85ml) of BHI broth before autoclaving. It was used in preservation of bacteria for many months in deep freeze (MacFaddin, 2000).

3.2.7.5 Columbia Agar Base:

The Colombia agar medium is prepared according to the recommendations of the manufacturer by adding 30 grams of the medium to a liter of distilled water, and then placing it in the sterilization device, then leaving it to reach a temperature of 50 degrees Celsius, and then adding fresh human blood for the purpose of using it to growth fastidious bacteria.

3.2.8 Laboratory Diagnosis for Isolation and Identification of *Streptococcus pyogenes:*

3.2.8.1 Colonial Morphology and Microscopic Examination:

A single colony was taken from each primary positive culture and its identification depended on the morphological properties (colony size, shape, color and nature of pigments, translucency, edge, elevation and texture). Bacterial smear stained with Gram stain was used to check the cellular morphological properties of bacterial cells, including Gram reaction, shape and arrangement (Fang *et al*., 2018).

3.2.8.2 Biochemical Test:

This biochemical test use to help identification of S.*pyogenes* .

3.2.8.3 Catalase Test:

Brain heart infusion agar was marked with the selected bacterial colonies and incubated at 37º C for 24 hours, after that, the growth was transferred by using the wooden stick and was put on the surface of a clean slide, then a drop of (3% H2O2) was added. The positive result indicated if gas bubble formation (Gillespie, 2014).

3.2.8.4 Oxidase Test:

This test depends on the presence of certain bacterial oxidases enzyme that would catalyze the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-ρ-phenylene-diaminedihydrochloride), the dye was reduced to a deep purple color . A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10 seconds (Dinu and Apetrei, 2020) .

3.2.8.5 Hemolytic Reaction:

Blood agar medium was streaked with a pure culture of bacterial isolate to be tested and incubated at 37Cº for 24-48 hrs. The appearance of a clear zone surrounding the colony is an indicator of β- hemolysis while the greenish zone is an indicator of α- hemolysis (Mogrovejo *et al.,* 2020).

3.2.8.6 Bacitracin Sensitivity Test:

Bacitracin test is used to determine the effect of a small amount of bacitracin on an organism. To perform a bacitracin sensitivity test, a single pure colony blot is taken and then spread on a Muller- Hinton agar medium with 5% fresh human blood in all directions. Then one tablet of the antibiotic is taken with sterile forceps and placed on a plate and placed in an incubator at a temperature of 37 degrees Celsius for 24 hours. a zone of inhibition surrounding the disc indicates the susceptibility of the strain (Abraham and SiSTlA, 2016).

3.2.9 Vitek 2 Compact System:

The Vitek 2 System was used to confirm the result of the manual biochemical test, in recent times this system used to identify microorganisms (Kareem *et al.*, 2020).

It was supplied with the required identification data base for all routine identification tests that provide an improved efficiency in microbial diagnosis which reduce the time and the need to do any additional tests , that will be safe for the user of system . This system was performed according to the manufacturer's instructions (Biomerieux-France). This system consists of:

1-A personal computer.

2-Reader/incubator that consisting of multiple internal components including: card cassette, card filler mechanism, cassette loading processing mechanism, card sealer , bar code reader, cassette carousel and incubator.

3-The system also contains: transmittance optics , waste processing, instruments control electronics and firm ware .

This system was performed according to the manufacturer's instructions (Biomerieux-France):

1-Three ml of normal saline were placed in plane test tube and inoculated with a loopfull of single colony of overnight culture.

2-The test tube was inserted into a dens check machine for standardization of colony to McFarland's standard solution (1.5×10^{8} cell/ml).

3-The standardized inoculums were placed into the cassette.

4-Then a sample identification number was entered into the computer software via barcode . Thus the VITEK 2 card was connected to the sample ID number.

5-The cassette was placed in the filler module , when the cards were filled, transferred the cassette to the reader/incubator module.

3.2.10 Preservation and Subculture of Frozen Bacterial Isolates:

Fresh 24 hr. Nutrient broth culture of isolates was frozen in 15% glycerol brain–heart infusion broth and storage at -20ºC until required. Frozen stock cultures stored at -20°C were sub-cultured on fresh blood agar plates and then incubated in aerobic or micro aerobic condition at 37°C for 24hours (Oskouei *et al.,* 2010).

3.2.11 Preparation of Molecular Materials:

3.2.11.1 Preparation of 1X TBE Buffer:

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1: 9 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. Thus each 100ml of 10X TBE added to 900ml of sterile distal water to produce final concentration,1X TBE (Sanderson *et al.,* 2014).

3.2.11.2 Preparation of Agarose Gel:

It was prepared by dissolving agarose (1.5 gm) in 100 ml of TBE buffer (10x), after boiling, leave the solution to cool until 50º C, then adding 0.5 µl of ethidium bromide, mixed well and poured into a tray of gel electrophoresis (Xi and Mihajlovic, 2019)

3.2.11.3 Ethidium Bromide Solution:

It was prepared by dissolving 0.05gm of ethidium bromide in 10ml distilled water and stored in a dark reagent bottle (Sambrook and Rusell, 2001).

3.2.12 Genotyping Assays of *Streptococcus pyogenes:*

3.2.12.1 DNA Extraction:

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (UK).Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a Thermal Cycler according to the following steps:

1-Cultured bacterial cells were transferred to 1.5 ml microcentrifuge tube, centrifuged for 1minute at 14-16,000×g and the supernatant was discarded.

2-A volume of 200 µl of Gram Buffer was added to 1.5 ml microcentrifugetube then 200µl of lysozyme buffer was added to the Gram Buffer then vortex to completely dissolve the Lysozyme.

3-A volume of 200µl of Gram Buffer in the 1.5 ml microcentrifuge tube ,incubated at 37^{o}C for 30 minutes. During incubation the tube was inverted every 10 minutes.

4- A volume of 20 µl of proteinase K was added then mixed by vortex, incubated at 60^{o}C for at least 10 minutes. During incubation the tube was inverted every 3 minutes.

5-A volume of 200 µl of GB Buffer was added to the sample and mix by vortex for 10 minutes.

6-The sample lysate was incubated at 70ºC for at least 10 minutes. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 µl per sample) was pre-heated to70ºC (for step 5 DNA Elution).

7-Following 70ºC incubation, 5 µl of RNase A (10mg ml) was added to the clear lysate and mixed by shaking vigorously.

8-The lysate was incubated at room temperature for 5 minutes.

9-A volume of 200 µl of absolute ethanol was added to the clear lysate and immediately mixed by shaking vigorously, the precipitate was broken up by pipetting.

10-A GD Column was placed in a 2ml collection tube.

11-All of the mixture was transferred (including any precipitate) to the GD column, centrifuged at 14000-16000 xg for 2 minutes.

12-The 2 ml collection tube was discarded containing the flow-through and the GD column was placed in a new 2 ml collection tube.

13-A volume of 400 µl of W1 buffer was added to the GD Column, Centrifuged at 14000-16000 g for 30 seconds.

14-The flow-through was discarded and placed the GD column back in the 2ml collection tube.

15-A volume of 600 µl of wash buffer (ethanol added) was added to the GD column, centrifuged at 14000-16000 xg for 30 seconds.

16-The flow-through was discarded and placed the GD column back in the 2ml collection tube, Centrifuged again for 3 minutes at 14000-16000 xg to dry the column matrix.

17-The dried GD column was transferred to a clean 1.5 ml centrifuge tube.

18- Avolume of100 µl of preheated elution buffer or TE was added to the center of the matrix, centrifuged at 14000-16000 xg for 30 seconds to elute the purified DNA.

19-Then detection of DNA by horizonta gel electrophoresis,and concentration measured by Nanodroop DNA.

3.2.12.2 Detection of DNA Concentration or Purity by Nanodroop:

The extracted DNA was checked by using Nanodrop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260/280nm) as following steps:

1.After opening up Nanodrop software, chosen the appropriate application (Nucleic Acid, DNA).

2.A dry wipe was taken to clean instrument pedestals several times. Then carefully pipette 2 µl of ddH2O on to the surface of the lower measurement pedestals for blank system.

3.The sampling arm was lowered and clicked OK to initialized the Nanodrop, then cleaning off the pedestals and 1 µl of extracted DNA carefully pipette onto the surface of the lowered measurement pedestals, then concentration and purity of extracted DNA was checked (wilfinger *et al.*,1997).

3.2.13 Rehydration of Primers:

Lyophilized primer pairs were rehydrated by DNA rehydration solution 1X (pH 9) Tris- EDTA buffer (TE-buffer). Initially, primer storage-stock tube prepared and then the working solution would prepared from primer stock tube. According to the manufacturer's recommendations (Bioneer/Korea), TE buffer was added to produce 100 picomole/microliter concentration of primer stock solution. Molecular working solution was prepared from stock as 1:10 (v/v) by dilution with TE buffer to get 10 picomole/microliter.

3.2.14 The Mixture of Polymerase Chain Reaction (PCR):

PCR master mix preparation PCR master mix was prepared by using (Promega/ USA kit) and this master mix was done according to company instructions as the following table (3-11).

Table (3- 11): Contents of the reaction mixture of PCR

Contents of reaction mixture	Volume
Green master mix	12.5µl
Forward primer	2.5µl
Reveres primer	2.5µl
DNA template	5µl
Nuclease free water	2.5µl
Total volume	25 µl

After that , these PCR master mix component that mentioned in Table above placed in standard (PCR kit) that contains all other components which needed to PCR reaction such as (Taq DNA polymerase , dNTPs , Tris - HCl pH : 8 , KCl , $MgCl_2$, stabilizer , and loading dye) . Then , all the PCR tubes were transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes . Then placed in PCR Thermocycler (Cleaver/England).

3.2.14.1 PCR Thermocycler Conditions:

PCR thermocycler conditions were done for each gene independent as following table (3-12) depend on instructions.

Table (3-12): PCR condition for *spy* and *smeZ* genes

PCR steps	PCR condition	repeat	Reference
Initial denaturation	95°C for 5 min	1	(Degaim *et al*., 2019)
Denaturation	95°C for 30 sec	35 cycle	
Annealing	55°C* for 30 sec 56°C** for 30 sec		
Extension	72°C for 30 sec		
Final extension	72°C for 30 min	1	
Hold	4°C forever	—	

**Spy* gene, ***SmeZ* gene

3.2.15 RAPD- PCR Mix Protocol:

RAPD-PCR technique was performed for Amplification was carried out using three random primer for RAPD (OPA13). This method was carried out according to described by (Moghaddam *et al.,* 2019).

Table (3-13): Protocols of PCR reaction mixture volumes

Contents of reaction mixture	Volume
Green master mix	12.5µl
Primers	2.5µl (10 pMol)

DNA template	5µl
Nuclease free water	5µl
Total volume	25 µl

3.2.15.1 PCR Thermocycler Conditions:

PCR thermocycler conditions were done for primer independent as following table depend on instructions .

Table (3-14): PCR condition for OPA13 primer

PCR steps	PCR condition	Repeat	Reference
Initial denaturation	94°C for 4 min	1	(Moghaddam *et al.*, 2019)
Denaturation	94°C for 1 min	1	
Annealing	40°C* for 30 sec	40 cycle	
Extension	72°C for 2 min	1	
Final extension	72°C for 7 min	1	

*OPA13 primer

3.2.15.2 Detection of Amplified Products By Agarose Gel Electrophoresis:

Successful PCR amplification was confirmed by agarose gel electrophoresis. Agarose gel was prepared by dissolving 1.5gm of agarose powder in 100ml of TBE buffer (pH:8) previously prepared (90 ml D.W.

were added to 10 ml TBE buffer) in boiling water bath , allowed to cool to 50º C and ethidium bromide at the concentration of 0.5 µg/ml was added (Sambrook and Russell, 2001).

The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray.

The tray was fixed in an electrophoresis chamber which was filled with TBE buffer that covered the surface of the gel 5µl of DNA sample was transferred into the assigned wells in agarose gel, and in one well the 5µl DNA was mixed with 1µl of loading buffer.

The electric current was allowed at 60 volts for 80 min. UV transilluminaterin 280 nm was used for the observation of DNA bands, and the gel was photographed by using a digital camera.

3.2.15.3 RAPD_PCR Gel Analysis:

Analysis of fingerprinting gel images was done by (**Paleontological Statistics version 4.0)** and to build phylogenetic tree using UPGMA (unweighted pair group method with arithmetic mean) method.

Chapter Four

Results and Discussion

4. Results and Discussion:

4.1 Isolation of *Streptococcus pyogenes:*

In this study, a total of 125 throat swab were collected from patients suffering from pharyngitis from both sex with age (1 - 60 years) . They were collected from the ENT unit Al-imam Al-Sadq General Teaching Hospital, peace be upon him, and Al-Hilla General Teaching Hospital, during the period from October 2021 to January 2022.

All the samples were subjected to microaerobic culturing on blood agar medium, brain heart infusion agar and Columbia agar , and it was found that out of the total of (125) samples, 116/125 (92.8%) samples showed positive bacterial culture. No growth was noted in 9/125 (7.2%) samples which may be return to the presence another microorganisms need specials condition for growth like viruses , fungi and other agents. . Among (116) bacterial growth, 40 (32%) of them were identified as *streptococcus pyogenes* .

Table (4-1): Prevalence of *Streptococcus pyogenes* isolates from patients with acute pharyngitis

Total NO. of specimens	**Positive bacterial culture**	**NO. of S.*pyogenes* isolates**	**No growth**
125	116 (92.8%)	40 (32%)	9 (7.2%)

This percentage of isolates obtained from pharyngitis swabs is almost similar to the study conducted by (Gottlieb *et al.*, 2018) who found that The most common bacterial infection associated with pharyngitis is Group A beta-hemolytic streptococci, which causes 5% to 36% of cases .

In a local studies (Khalaf *et al.*, 2020) concerned with isolating S.*pyogenes* from pharyngitis patients of the percentage of it were 30% and 52.2% respectively.

The reasons for the discrepancy in the rates of infection with GAS are not relatively understood and may be due to the different conditions of the study in terms of demographic of patients registered in each study.

The incidence and prevalence of both invasive and non-invasive GAS infections in developing countries are largely unknown. Systematically collected data are essential for a functioning disease-control program and, thus, the measurement of incidence and temporal trends are an essential first step toward reducing the burden of GAS disease in developing countries (Whitelaw *et al.*, 2018).

4.2 Distribution of Patients According to Age:

In this study, 125 throat swabs were collected for patients with pharyngitis, and the ages of the patients ranged from 1 to 60 years. The results revealed that the wide age of detection of GAS in age ranging from (1-10) years and low age of detection of GAS in age ranging from (31- 40) years .The ages of *Streptococcus pyogenes* were isolated as shown in the following table:

Table (4-2): Distribution of S. *pyogenes* isolates according to age groups

Age (years)	**S. *pyogenes***	**Percentage %**
1-10	11	27.5%

11-20	8	20%
21-30	5	12.5%
31-40	2	5%
41-50	5	12.5%
51-60	9	22.5%
Total	**40**	**100%**

The results showed that children aged 1 to 10 years were the most susceptible to infection with S. *pyogenes*, and that pharyngitis disease was dangerous and rapidly spreading among school-age children.

Streptococcus pyogenes group A (GAS) is spread from person to person via respiratory droplets with a short incubation period of 2∼5 days. GAS pharyngitis peaks in the late winter and early spring months when children are predominately indoors for school and sports. Colonization is also higher in winter months, and while up to 20% of school age children are colonized with GAS in their throat during this time, colonization has not been shown to contribute to the spread of disease. In low- and middle-income countries and other situations in which crowding is common (e.g., schools), outbreaks of pharyngitis are common. Group A *Streptococcus pyogenes* (GAS) pharyngitis can occur at all ages and it is most common in school-aged children with a peak at 7∼8 years of age (Norton and Myers, 2021) and for adults the rate was 2% across all seasons (Danchin *et al.*, 2007) .

Group A Streptococcus pyogenes (GAS) pharyngitis can occur at all ages and it is most common in school-aged children with a peak at 7-8 years of age. Pharyngitis caused by GAS is rare in children less 3 years and become much less common in late adolescence (Shulman et al., 2012; Chua et al., 2016).

In 2010, there were 1.814 million emergency department visits for pharyngitis, of which 692,000 were for patients under the age of 15. Most cases of pharyngitis occur in children under the age of 5. Adults can also develop the disorder but at a lower rate. Globally, pharyngitis rates are very high chiefly in countries where antibiotics are overprescribed (Faden *et al.*, 2017).

Pharyngitis due to S. *pyogenes* is very common in school-aged children. A meta-analysis based on a systematic review of 29 studies provides a prevalence of information about this condition. When studies of children of all ages with sore throat were analyzed, there was a pooled prevalence of 37% (95% confidence interval (CI) 32-43%) of children who were found to have a positive diagnostic test performed on a pharyngeal swab for S. *pyogenes*. This analysis also demonstrated that the prevalence of S. *pyogenes* carriage among well children with no signs or symptoms of pharyngitis was 12% (95% CI 9–14%) (Shaikh *et al.*, 2010).

About hundred millions people develop serious S. *pyogenes* infection every year. It cause about 660,000 invasive infections and 616 million cases of pharyngitis that result in 163,000 death from 2009 to 2014 (Imöhl *et al*., 2017) .

In African countries, S. *pyogenes* was isolated from children with acute pharyngitis and the its' prevalence was as high as 66.7, 28, 23, and 11.3% in Nigeria (Uzodimma *et al*., 2017), Egypt (Sultan and Seliem, 2018) , Kenya (Osowicki *et al*., 2019) and Ethiopia (Tesfaw *et al*., 2015) respectively.

4.3 The Diagnostic Characteristics of *Streptococcus pyogenes:*

Diagnosis of S.*pyogenes* depends on several phenotypic characteristics, including colonial morphology, beta-hemolytic activity, biochemical tests, bacitracin sensitivity test, and microscopic examination as shown in table (4-3).

Table (4-3): Diagnostic features of *Streptococcus pyogenes*

Test	Results
Growth on blood agar	Small, soft, pin-like colonies of gray color
Hemolysis	Beta –haemolytic
Morphology	Cocci (short chain)
Gram stain	Gram positive
Catalase	Negative
Oxidase	Negative
Bacitracin	Sensitivity

The samples was streaked on blood agar base , followed by microaerobic incubation at 37ºC for 24-48 hr. Laboratory diagnosis of *Streptococcus pyogenes* depends primarily on culturing the bacteria from clinical samples and their identification by phenotypic traits.

The Culturing of throat swabs remains the gold standard and reference method for the diagnoses of *Streptococcus pyogenes* that cause pharyngitis (Spellerberg and Brandt, 2016).

The group A streptococci are fastidious organisms that need complex nutritional requirements. It must contain essential nutrients to provide the optimal growth of bacterial cultures, especially in the blood agar. It gives a preliminary diagnosis of S. *pyogenes* through the clarity of the area of blood hemolysis. The shapes of Bacterial colonies on the blood agar are small, grey, pinhead-like, with a lysis area of 2-3 mm (ferretti *et al.*,2001). *Streptococcus pyogenes* are implicated in many diseases, including pharyngitis, which is more prevalent. The bacterial cells arrangement in the form of chains, double or single cocci (Yoshino *et al.*, 2010).

4.4 Rapid Identification of *Streptococcus pyogenes* by Vitek 2 System:

After the laboratory diagnosis of bacterial colonies developing in the biochemical test as summarized. Bacterial isolates were identified and confirmed using Vitek 2 compact auto analyzer system, and the results obtained from the device were analyzed using a program analyzed using compact software. This device depends on the standard biochemical identification for bacterial isolates.

The results of this system showed that the probability rate for S.*pyogenes* isolates more than 85% Appendix No. (1- 30).

Vitek 2 compact system provides a more accurate identification of microbes with fast results, high confidence, a good degree of characteristics, and little time and effort spent compared to techniques for manually identifying and diagnosing microbes (Kareem *et al*., 2020).

4.5 Confirm Diagnosis of *Streptococcus pyogenes* by *spy1258* Gene by PCR Technique:

To confirm the diagnosis of *Streptococcus pyogenes* DNA was extracted from all suspected isolates that previously identified S. *pyogenes* by brain heart infusion agar, Columbia agar, biochemical tests and Vitek 2 system (ID) , so the conventional PCR was carried out using these DNA samples for the amplification of specific (*spy1258*) primer . *Spy1258* gene is present in S. *pyogenes* and this gene is specific for *Streptococcus pyogenes* , So it can facilitated downstream analyses such as molecular detection .The results recorded that all isolates 31/40 (77.5%) were produced the specific 407 bp DNA fragment when compared with allelic ladder ,as shown in Figure (4-2).

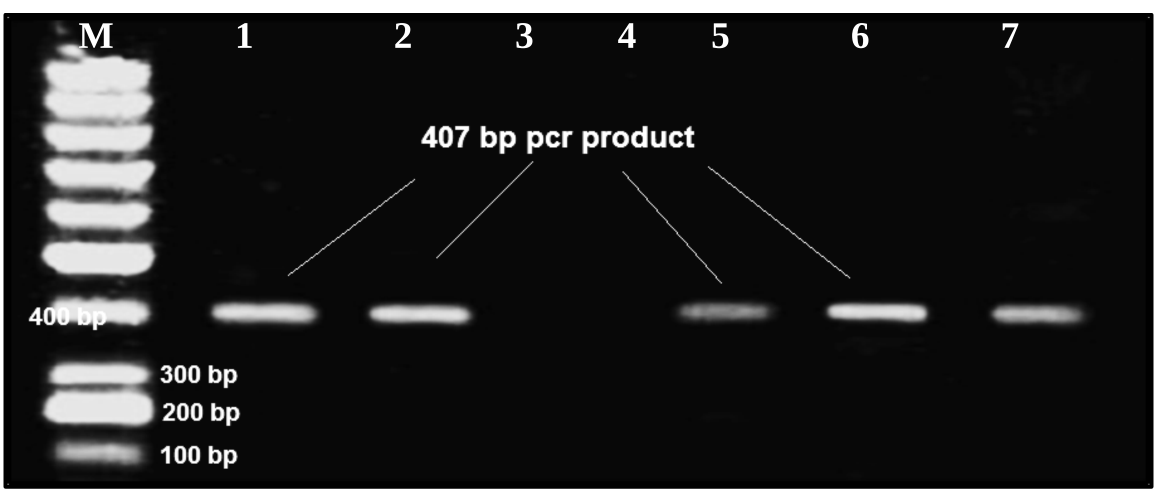


Figure (4-2): 1.5 Agarose gel electrophoresis image at 75V for 1 houre that showed PCR products analysis of *spy1258* gene in *Streptococcus pyogenes* isolated from clinical samples . Where M : is DNA marker (100-1000bp) and Lane (1-7) showed (1,2,5,6 ,7) at (407 bp), while lane 3 and 4 showed negative result

The polymerase chain reaction (PCR) technique directing to transcriptional regulator genes supplied the rapid and dependable manner for detection of a pathogenic microbes (liu *et al*., 2005).

A *spy1258* gene was one of reputed transcriptional regulator gene (TetR/AcrR family) that was precise for GAS and could be used as a marker for the detection of this bacteria (Brahmadathan and Gladstone 2006 ; Kumar *et al*., 2011).

A previous local study using PCR for amplification of *spy 1258* gene , Al-Saadi and other authors were observed that a specific DNA fragment of the expected size (450bp) was generated from all S .*pyogenes* except one isolate was documented as S. *pyogenes* (Al-Saadi *et al*., 2015).

Similar study recorded that 61% of Group A Streptococcal tonsillitis harbor *spy1258* (Degaim *et al*., 2019).

Although the spy 1258 is the most widely common primer used for molecular detection of S. *pyogenes* . Orsud *et al*., 2020 recorded that, out of more than 200 strains , 13% of them were positive by PCR using spy 1258 primer and many isolates which are typical for S. *pyogenes* by microscopic characteristics, colony features and biochemical tests, the authors Refer the reason of the low sensitivity of *Spy 1258* primer and the variability in S. *pyogenes* genome sequence to the necessitate developing new primers according to the environmental and geographical distribution Isolation, identification and biochemical profile of pathogenic and opportunistic bacteria from sore throat (Orsud *et al.*, 2020) .

The study validated by (Zhao *et al*., 2015) documented that the *spy1258* gene used for accurate and precise identification of GAS strains. It was noted that identical gene sequences of *spy1258* were completely absent in other bacterial genomes available at Gen Bank. Many studies have used this particular gene for the rapid detection of S. *pyogenes* from various clinical samples(Abraham and SiTlA, 2016).

The difference in the percentage of the presence of the *Spy1258* gene is due to the possibility of the presence of genetic variation resulting from mutations in GAS isolates .

4.6 Detection of *smeZ* Gene:

The smeZ virulence factor gene was investigated in S. *pyogenes* . It was found that *smeZ* gene was observed in 14/40 (35 %) isolates of S. *pyogenes* out of 40 isolates of this bacteria .The positive result were detected by 246 bp bands when compared with allelic ladder, detected by using PCR technique of gel electrophoreses process are shown in figure (4-3).

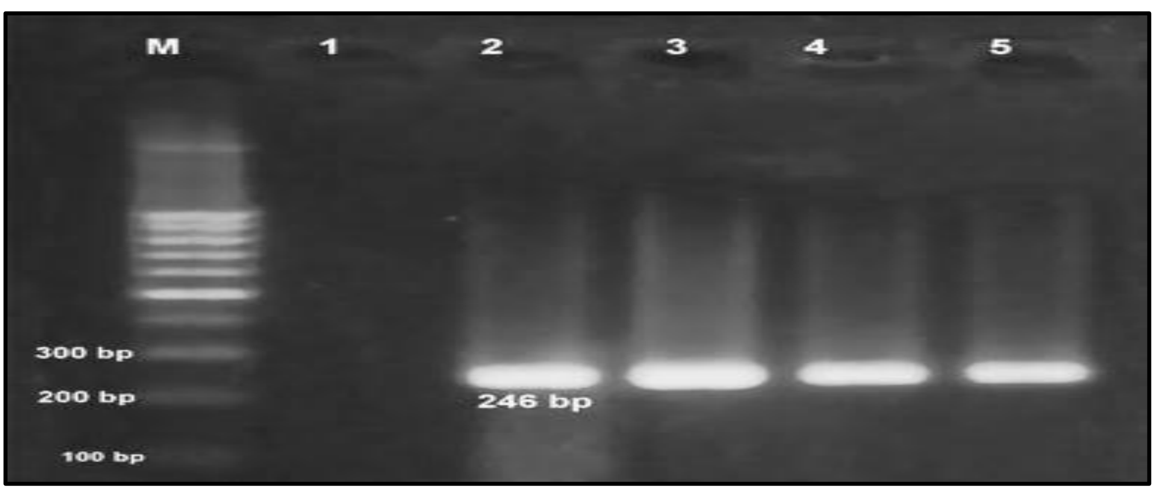


Figure (4-3): 1.5 Agarose gel electrophoresis image at 75V for 1 houre that showed PCR products analysis of *SmeZ* gene in *Streptococcus pyogenes* isolated from clinical samples . Where M : is DNA marker (100-1500bp) and Lane (1-5) showed (2,3,4,5) at (246 bp), while lane 1 showed negative result .

The superantigen genes are the main virulence factors and closely related to the pathogenicity of GAS. Yet, 14 superantigen genes, including *speA, speC, speG, speH, speI, speJ, speK, speL, speM, smeZ*, and *ssa*, were found to be distributed among various strain (Imöhl *et al*., 2017).

Group A Streptococcus superantigens, except *speG, speJ,* and *smeZ* encoded by chromosome, *speA, speC, speH, speI, speK, speL, speM,* and ssa are encoded by phage, which is the main driving force for pathogenic strains to obtain pathogenic factors through transfer. The transfer and mutation of genes can produce highly pathogenic GAS strains, which affect the epidemic situation of the GAS disease, resulting in different distributions of the S. *pyogenes* superantigen gene spectrum in different periods and geographical areas.

A study by (Li, *et al*., 2020) on pharyngitis patients found 96.97% of GAS isolates harbored superantigen *smeZ* genes.

Molecular epidemiology and antimicrobial resistance of group a Streptococcus recovered from patients in Beijing, China.

In the previous studies from Portugal showed that *smeZ* 96% was common in GAS (Friães *et al*., 2012).

A local study by Degaim *et al*., 2019 recorded that 50% of GAS isolates from tonsillitis patients harbor *smeZ* superantigen gene .

Molecular Study of *spy1258* and *smeZ* genes in Group A Streptococcal Tonsillitis . (Schmitz *et al*., 2003) showed the distribution rate of *smeZ, speA, speC, speH, speJ* and *ssa* genes were associated with movable elements, and the *smeZ* allele was found in 95.8% strains.

All the above studies showed that the distribution of superantigen gene profiles were region dependent of the study and prevalence of *smeZ* gene in GAS isolates may be associated with transferred this gene by different elements through chromosomal DNA.

4.7 The Genetic Diversity of *Streptococcs pyogenes* Isolated from Pharyngitis by RAPD-PCR Technique:

4.7.1 RAPD Fingerprinting Analysis:

Amplification of genomic DNAs from the GAS isolates with OPA13 primer, based on the number, intensity and size range of RAPD bands. In RAPD pattern consisting of (1 - 20) distinct DNA fragments with size ranging from (100 - 2000bp) among the isolates, it was found that one isolate formed no band as shown in figure (4 - 4) .

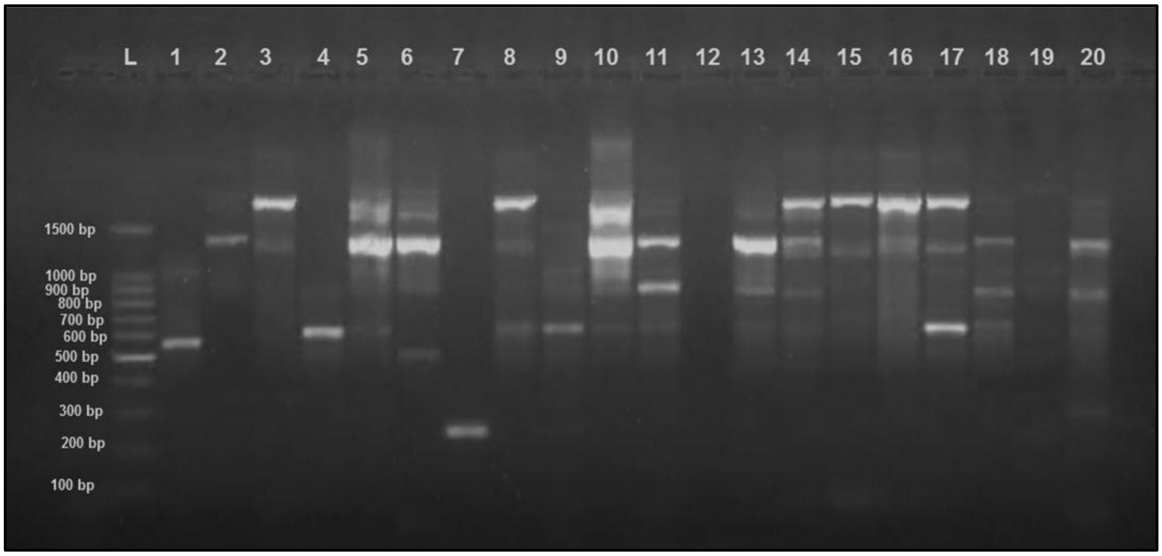


Figure (4-4): 1.5 % Gel electrophoresis of RAPD –PCR products using OPA13 primer. M: is DNA marker 100-2000bp, Lane 1-20: GAS samples resulted the DNA polymorphic segments at length size 250-above 2000bp visualized by Ethidium bromide under UV transelluminator

In phylogenetic analysis, this study showed a high degree of genetic diversity in GAS isolates they were classified into 4 main clusters.

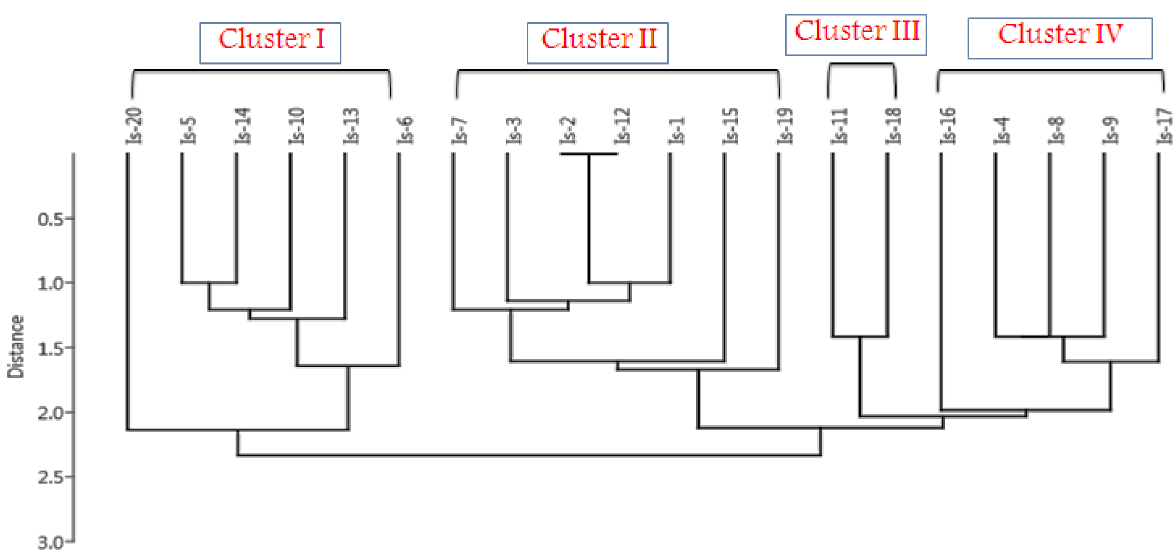


Figure (4-5) : RAPD-PCR dendrogram phylogenetic tree analysis of *Streptococcus pyogenes* isolates by using (Paleontological Statistics version 4.0). The Cluster analysis using (algorithm Ward's method) were showed 4 clusters polymorphic variants between 20 S. *pyogenes* isolates

Table (4-4): RAPD-PCR cluster analysis and genetic diversity for S. *pyogenes* isolates

No.	No. of Isolate
I	**Is-5, Is-6, Is-10, Is-13, Is-14, Is-20**
II	**Is-1, Is-2, Is-3, Is-7, Is-12, Is-15, Is-19**
III	**Is-11, Is-18**
IV	**Is-4, Is-8, Is-9, Is-16, Is-17**
Totel : 4	**20**

The most common bacterial cause of pharyngitis is infection by Group A *β*-hemolytic *streptococcus* (GABHS), commonly known as strep throat. 5 – 15% of adults and 15 – 35% of children in the United States with pharyngitis have a GABHS infection (Mustafa and Ghaffari, 2020).

In a local studies (Khalaf *et al*., 2020) concerned with isolating S.*pyogenes* from pharyngitis patients of the percentage of it were 30% and 52.2% respectively **.**

The reasons for the discrepancy in the rates of infection with GAS are not relatively understood and may be due to the different conditions of the study in terms of demographic of patients registered in each study.

The incidence and prevalence of both invasive and non-invasive GAS infections in developing countries are largely unknown. Systematically collected data are essential for a functioning disease-control program and, thus, the measurement of incidence and temporal trends are an essential first step toward reducing the burden of GAS disease in developing countries (Whitelaw *et al*., 2018).

The molecular typing of GAS isolates by using RAPD pattern, it classified the isolates into four main cluster which were determined by converting RAPD data into algorithm Ward's method and analyzed by Paleontological Statistics version 4.0 to produce a phylogenetic tree .

The pharyngeal GAS isolates in the present study revealed a high degree of genetic variations which can be generated by mutations. A previous study noted that the RAPD and PFGE techniques could be efficient tools in epidemiological studies of GAS (González *et al*., 2003; Pakbin *et al*., 2021).

Introduced that RAPD-PCR-High resolution melting curve (HRM) analysis as a potential alternative method to differentiate non-dysenteriae Shigella species from clinical samples. They found RAPD-PCR-HRM assay more sensitive and specific than ERIC-PCR-HRM as the potential of alternative method for differentiation of non-dysenteriae Shigella (Pakbin *et al*., 2021).

4.8 Antibiotics Profile of *Streptococcus pyogenes* by Vitek2 Compact Technique:

All isolates of streptococcus bacteria that were identified by biochemical tests and Vitek 2 compact system (ID) were subjected to antibiotic sensitivity testing using the Vitek 2 Antimicrobial Susceptibility Tests (AST). The results were obtained as shown in the table (4-5) and illustrated in appendix (1-30).

Table (4-5): Antibiotics susceptibility profile of bacterial isolates

Type of antibiotic	Symbol	Sensitive		Intermediate		Resistant	
		No	*%*	*No*	*%*	*No*	*%*
Ampicillin	AM	17	85	0	0	0	0
Levofloxacin	LE	20	100	0	0	0	0
Moxifloxacin	MO	20	100	0	0	0	0
Erythromycin	E	0	0	0	0	8	40
Clindamycin	CD	14	70	0	0	6	30
Linezolid	LZ	20	100	0	0	0	0
Vancomycin	VA	20	100	0	0	0	0
Tetracycline	TE	5	25	2	10	11	55
Tigecycline	TGC	20	100	0	0	0	0
Chloramphenicol	C	17	85	1	5	2	10
Trimethoprim / sulfamethoxazole	TR	18	90	1	5	1	5

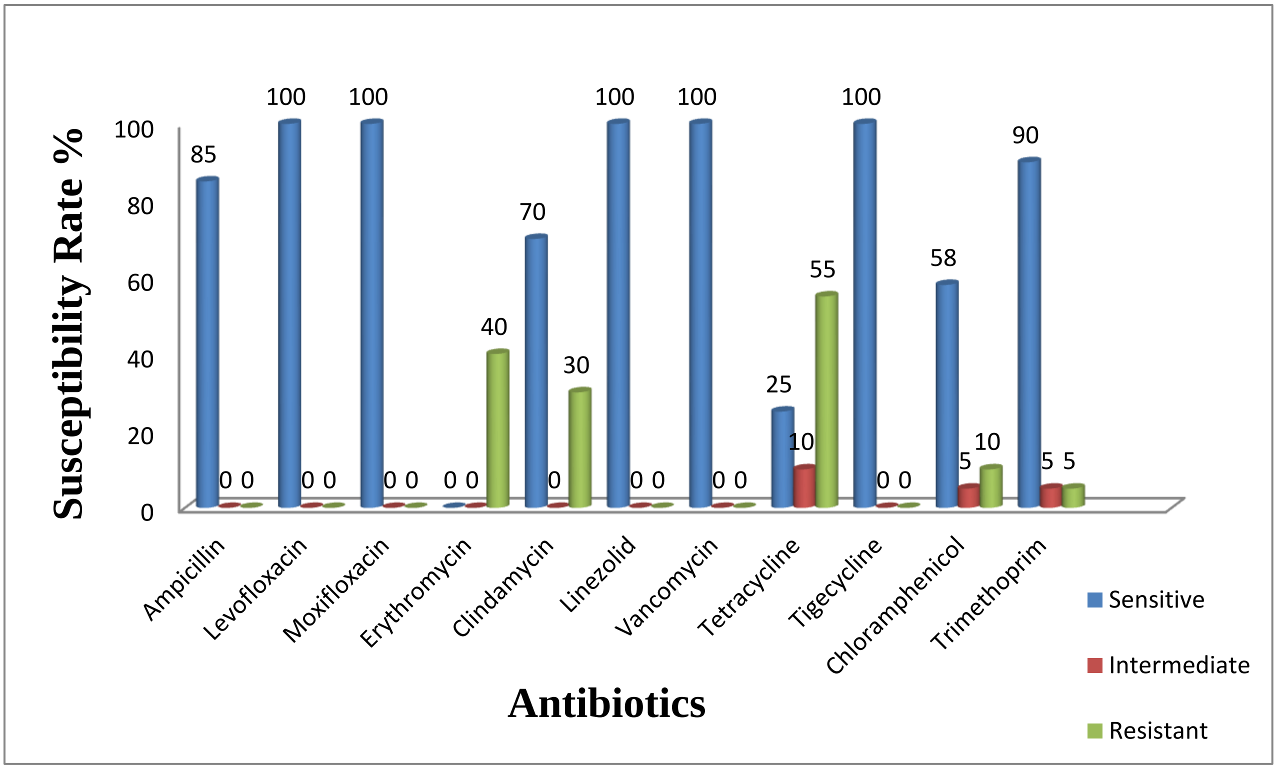


Figure (4-6): Antibiotic susceptibility profile of *Streptococcus pyogenes* isolates by Vitek2 compact technique

These study investigated the antimicrobial properties of 20 isolates of S.*pyogenes* using Vitek 2 compact technique.

The current study showed the sensitivity of GAS isolates toward ampicillin is 85% . It agrees with the Iraqi study conducted by (Mahdi *et al*., 2017) at a percentage (88.88%) , also, converged with the ratio obtained by Kebede *et al*., 2021 who found that all 282 GAS isolates are sensitive for ampicillin.

Another studies recorded a high sensitivity rates were in USA (Gutiérrez-Jiménez *et al*., 2018), Asia (Kumar 2017; KHANDEKAR 2019), Europe (Espadas-Maciá *et al*., 2018) , African countries including Egypt (Abd El-Ghany *et al*., 2015) , Kenya (Kunga 2018) , Ethiopia (Tesfaw *et al*., 2015).

Ampicillin is a penicillin beta-lactam antibiotic used in the treatment of bacterial infections caused by susceptible, usually gram-positive, organisms. the bactericidal activity of Ampicillin results from the inhibition of cell wall synthesis.

penicillin and ampenicillin remains the drug of choice for most GAS infections. This might be due to lack of β-lactamase production by S.*pyogenes.* (Fang *et al*., 2020).

No confirmed reports of GAS resistance to β-lactam antibiotics have been documented, although treatment failures have been reported Sela and Barzilai 1999 . Vannice *et al*., 2020 explained that reason for GAS resistance to penicillin the development pbp2x mutation confers reduced susceptibility to β-Lactam Antibiotics .

The current study showed that the sensitivity of GAS isolates to levofloxacin is 100% . This ratio is similar to the study that explained that more than 98.5% of the GAS strains were sensitive to levofloxacin (Liang *et al*,. 2012).

And with (Shen *et al*., 2018) conducted similar data with present study. This percentage is also consistent with a study conducted by (Kebede *et al*., 2021) , where the sensitivity of Levofloxacin was 97%.

Levofloxacin is a third-generation fluoroquinolone antibiotic with wide-spectrum and potent in vitro antimicrobial activity against aerobic gram-negative and -positive microorganisms . Levofloxacin is one of the major antimicrobial agents for the treatment of community-acquired lower respiratory tract infections (Grossman *et al*., 2005).

The moxifloxacin- sensitivity GAS isolates is relatively high in this study by 100%. This result is consistent with the study carried out by the (Tateda *et.al*., 2019), where he showed that S.*pyogenes* was highly susceptible to moxifloxacin.

The fluoroquinolones are a class of broad-spectrum antimicrobial agents, therefore, they are highly active against both aerobic Gram-positive by inhibiting the DNA gyrase and topoisomerase enzymes (Brar *et al*., 2020).

The result of the study showed erythromycin -resistance GAS at 40%, and this is related to the Italian study erythromycin-resistant S. *pyogenes* was found in 35% of the children admitted to hospital with throat infections. And a Turkish study showed, the rate of erythromycin resistance was determined as 74.5% out of 51 samples (BOZLAK *et al*,. 2021) .

Erythromycin is a bacteriostatic antibiotic, it prevents the further growth of bacteria rather than directly destroying it. This action occurs by inhibiting protein synthesis. Erythromycin binds to the 23S ribosomal RNA molecule in the 50S subunit of the bacterial ribosome (Farzam *et al*., 2021). Another study documented that , Out of (14) isolated samples of S. *pyogenes* , only (3) were resistant to the antibiotic erythromycin by (21.4%) (Helal *et al*,. 2020).

The sensitivity of GAS isolates toward Clindamycin in this study is 70%, it is similar to those conducted by Camara *et al*. (2013) which was 76.4%. The percentage of the current study is lower than data conducted by a local study, it was 96.3% (Mahdi *et al.*, 2017).

Clindamycin prevents peptide bond formation, thereby inhibiting protein synthesis by reversibly binding to 50S ribosomal subunits (Struzycka *et al.*, 2019).

Clindamycin -resistance pattern in this study was 30%, This percentage is close to the study conducted by Oppegaard *et al*. (2020) who found the resistant rate of GAS to clindamycin was 25% .

The resistance to clindamycin antibiotic occurs by two primary mechanisms: target site modification or efflux pumps. Inducible resistance can result in treatment failure, as inducible clindamycin resistance is undetectable unless macrolides are also present (Lewis *et al*., 2014).

Clindamycin resistance in the United States is on the rise, from an estimated 0.5% in 2003 (Richter *et al*., 2005) to currently as high as 15% in pediatric populations (DeMuri *et al*., 2017). Isolates from invasive infections are more commonly resistant, increasing from 2% to over 23% in this time (Fay *et al*., 2021). The resistance rates are geographically variable; in China, resistance may approach 95.5% (Stevens and Bryant, 2017), where over a similar period, northern Europe rates approximated 1% (Bruun *et al*., 2021).

Despite the rapid change in resistance trends and the emergence of potentially hypervirulent, resistant strains, the recommendation remains: continue the use of protein synthesis inhibitors such as clindamycin when necessary, but to be mindful and vigilant for resistant isolates (Stevens *et al*., 2014).

In this study, GAS isolates showed a high sensitivity to the antibiotic linezolid by 100%, , similar data conducted by Zurenko *et al*. (2014) recorded that S.*pyogenes* isolates were sensitive for tedizolid and linezolid at (>98%) .

Linezolid is the first available oxazolidinone to inhibit bacterial protein synthesis by interfering with translation. Linezolid binds to a site on the bacterial 23S ribosomal RNA of the 50S subunit, which prevents the formation of a functional 70S initiation complex. It is indicated for gram-positive infections (Azzouz and Preuss, 2022).

Inoculum effects were seen using linezolid on isolated S.*pyogenes* .The model has proven robust and largely in agreeance with published data . This is the result of the study carried out by Australian researchers (Marum *et al*., 2021).

The sensitive proportion of S.*pyogenes* toward Vancomycin is 100%, the current finding is comparable to results of other studies, the sensitivity percentages were 81.4% and 100% respectively (Fahad 2018; Agrawal *et al*., 2014). Vancomycin is a glycopeptide antibiotic that exerts its bactericidal effect by inhibiting the polymerization of peptidoglycans in the bacterial cell wall (Koyama *et al*., 2012).

The resistance of GAS pharngitis to the tetracycline in the current study is 55%, this is consistent with the Brazilian study which proved the resistance of S.*pyogenes* to tetracycline antibiotic by 61% (Barros, 2021).

Tetracyclines are an important class of broad-spectrum antibiotics that prevent bacterial growth by inhibiting protein biosynthesis. This large family includes compounds with bacteriostatic activity and a wide range of uses, from Gram-positive and Gram-negative (Rusu and Buta, 2021).

The result of the current study showed the sensitivity of GAS to the tigecycline is 100%. Betriu *et al.*, (2014) whose finding that at 0.06 µg/ml MIC , 90% of the tested isolates were inhibited.

Tigecycline is a broad-spectrum antibiotic derived from minocycline and was the first glycylcycline class antibiotic approved for clinical use . tigecycline has increased antibacterial potency due to its higher binding affinity with the 70S ribosomes. This effect inhibits the delivery of the thermo-unstable ternary complex elongation factor (EF-Tu) GTP·aminoacyl-tRNA to the ribosomal A (aminoacyl) site and eventually perturbs polypeptide translation (Jenner *et al*., 2013).

Streptococcus pyogenes isolates are sensitive to the chloramphenicol antibiotic by 85% in the current study, and this is consistent with the study conducted by Chinese study in (2021) which proved that more than 90% of the isolates are resistant to chloramphenicol (Liang *et al*., 2021).

Chloramphenicol is a ribosome-targeting antibiotic that binds to the peptidyl transferase center (PTC) of the bacterial ribosome and inhibits peptide bond formation (Chen *et al*., 2021). The proportion of bacteria resistance to antibiotics is relatively low in this study by 10% and this result is consistent with the study carried out by the researchers (Kebede *et al*., 2021), where he showed that the S. *pyogen* bacteria have the ability to resist antibiotics because they have resistance genes the proportions of antibiotics resistances to chloramphenicol (14.3%).

In this study, the sensitivity of GAS to the trimethoprim antibiotic is 90%, it is corresponds to the percentage in the Norwegian study , which showed a sensitivity rate at 94% and the sensitivity rate of GAS to trimethoprim was (81.48%) in the Iraqi study (Mahdi *et al.,* 2017).

Trimethoprim / Sulfamethoxazole is a sulfonamide (antimicrobial drug class) that works directly on the synthesis of folate inside microbial organisms, e.g., bacteria. Sulfamethoxazole achieves this directly as a competitor of p-aminobenzoic acid (PABA) during the synthesis of dihydrofolate via inhibition of the enzyme dihydropteroate synthase. Trimethoprim is a direct competitor of the enzyme dihydrofolate reductase, resulting in its inhibition, which halts the production of tetrahydrofolate to its active form of folate. The combination of these two agents is meant to create a synergistic anti-folate effect; tetrahydrofolate is a necessary component for synthesizing purines required for DNA and protein production (Kemnic and Coleman, 2021).

The rate of bacterial resistance to the trimethoprim in this study was 5%, and this percentage corresponds to the of Norwegian study, which recorded that one GAS isolate was resistant to trimethoprim-sulfamethoxazole (Oppegaard *et al*., 2020)

This discrepancy in the percentage of infection with bacteria is due to many reasons Some of the main contributors in the emergence and spread of highly resistant bacteria for health-care associated infections (HAIs) are the intensive and prolonged use of antibiotics in the hospital setting (Dixit *et al*., 2019).

4.9 RAPD-PCR Fingerprinting and Antibiotic Susceptibility Pattern:

After the RAPD_ PCR reaction was performed for 20 bacterial isolates of *Streptococcus pyogenes*, the clusters was cross-linked with antibiotic – susceptibility profile to reveal the genetic diversity of these isolates, as follows in Table (4-6).

Table(4-6): The antibiotic susceptibility profile of S.*pyogenes* in different clusters variants

No.of Cluster	Isolate No.	Anti. Sensivity	Antibiotics Resistance
I	Is-5, Is-6, Is-10, Is-13, Is-14, Is-20	LE,MO,LZ,C,TR	E,CD,TE
II	Is-1, Is-2, Is-3, Is-7, Is-12, Is-15, Is-19	AM,LE,MO,LZ,TR	
III	Is-11, Is-18	AM,LE,MO,LZ,C,TR	
IV	Is-4, Is-8, Is-9, Is-16, Is-17	AM,LE,MO,LZ,C	

By studying all the apparent clusters of GAS, this study concluded a joint sensitivity to the antibiotics (levofloxacin, linezolid and moxifloxacin). As a result. The finding of this study about resistance to macrolides and tetracycline in these isolates, which may be presence resistance genes possessed like ermB and tetM (Yu *et al*., 2021) .

By studding genomic diversity of GAS isolates suggested that the isolates are multicolnal in origin dependent on the differences in a susceptibility toward antibiotic in clusters. Although the clusters shared with antibiotic resistance for (E,CD,TE).

Other study by De Melo and coauthors (2003). Correlates between the genetic diversity which studied by PFEF with antibiotic susceptibility pattern .

From an epidemiological point of view, it was frequently demonstrated that the prevalence of macrolides-resistant S. *pyogenes* correlated well with the total consumption of antibiotics in particular geographical areas (Munita *et al*., 2016)

Besides antibiotic consumption, changes in the clonal composition of the S**.** *pyogenes* population may also be an important cause for fluctuations in macrolide resistance rates (Montes *et al.*, 2014).

Macrolide resistance among GAS isolates with two major resistance mechanisms. First by Target modification by ribosomal methylases encoded by *ermA* and *ermB* genes confers the macrolide resistance phenotype. The second by The expression of efflux pumps, encoded by the *mefA* gene, is related to the M phenotype and confers resistance only to macrolides. Macrolide resistance among beta-hemolytic streptococci has been reported in different geographical regions at varied rates (Barros, 2021).

This results may be due to Horizontal gene transfer (HGT) allows bacteria to exchange their genetic materials (including antibiotic resistance genes, ARGs) among diverse species (Sun *et al.,* 2019) .

Horizontal gene transfer (HGT) has caused antibiotic resistance to spread from commensal and environmental species to pathogenic ones, as has been shown for some clinically important ARGs (Von Wintersdorff *et al.*, 2016).

4.10 RAPD_PCR Pattern and *smeZ* Gene Distribution of *Streptococcus pyogenes* Isolates:

The *smeZ* gene was investigated in S.*pyogenes* . It was observed in 14 (35%) isolates out of 40 isolates of this bacteria. By distributing the prevalence of *SmeZ* gene on bacterial isolates, the current study found the highest percentage was 6 (42.9%) for the first cluster, 4 (28.6%) for the second cluster, 1(7.1%) for the third cluster, and 3(21.4%) for the fourth cluster.

Table (4-7): Showing RAPD_PCR pattern and *SmeZ* gene distribution of GAS isolates

No. of Cluster	Clusters	*SmeZ* gene
I	Is-5, Is-6, Is-10, Is-13, Is-14, Is-20	6 (42.9 %)
II	Is-1, Is-2, Is-3, Is-7, Is-12, Is-15, Is-19	4 (28.6 %)
III	Is-11, Is-18	1 (7.1 %)

IV	Is-4, Is-8, Is-9, Is-16, Is-17	3 (21.4 %)
Total		14 (100%)

Streptococcus pyogenes can express several different superantigens that can vary in their potency, thus differences in mitogenicity within and between emm-types can be influenced by the complement of superantigen genes, as well as differences in expression . SMEZ is the most potent streptococcal superantigen described, although produced in small amounts compared to other superantigens (Yang *et al*., 2005), and the *smeZ* gene is present in the majority of S. *pyogenes* strains, with over 40 different alleles (Maripuu *et al*., 2008).

The high virulence potential of smeZ in Streptococcal toxic-shock syndrome (STSS) has been demonstrated by both in vitro and in vivo studies. (Proft & Fraser 2003) showed that disruption of smeZ in a GAS isolate led to abrogated mitogenic responses in human cells and a complete inability to elicit cytokine production from human cells, despite the presence of other superantigen genes.

Two earlier studies have proposed linkage equilibrium between the emm-genotype and the smeZ allele of the GAS isolates (Proft *et al.,* 2000; Rivera *et al.,* 2006).

(Maripuu *et al.,* 2008) support the suggestion of a link between the emm-genotype and the smeZ allele. Despite the presence of diverse superantigen gene profiles within emm-genotypes, found only one exception to the absence of allelic variation in smeZ within the same emm-genotype. Two of the four emm isolates lacked the *smeZ* gene and the other two encoded different smeZ alleles. However, isolates of different emm-genotypes were found to encode the same smeZ allele. The high degree of polymorphism detected in the *emm* and *smeZ* genes among GAS isolates mirrors the high immunogenic pressure and the importance of these two genes for the virulence of GAS isolates.

In addition, described emm1/G16 pattern strains that differed for the presence of smeZ from emm1 strains isolated in Portugal (Friães *et al.*, 2012).

Maribuu (2011) had been studied Sag profile distribution within emm-genotyping , who was found that the primary differences in the *Sag* gene profile of isolates with the same emm – genotypes involved the addition or loss of one or two phage-encoded *Sag* genes.

The present study showed that group A streptococci (GAS) are genetically diverse and possess *smeZ* genes regardless of their invasiveness. Majority of the GAS exhibited no restricted pattern of virulotypes. Therefore, it can be suggested that virulotyping is partially useful for characterizing a heterogeneous population of GAS and the greater occurrence of this gene in cluster No.1 is probably due to that these isolates were collected over long period of the time and had been represented by a comparative large number of isolates.

Chapter Five

Conclusions and Recommendations

5. Conclusions and Recommendations:

The current study has come to the following conclusions:

5.1 Conclusions:

1- The confirmed identification of S.*pyogenes* by PCR detection of *spy 1258* gene is a gold standard method followed the culture.

2- *Streptococcus pyogenes* bacteria are 100% sensitive to the antibiotic levofloxacin, Moxifloxacin, Linezolid, Vancomycin and Tigecycline so these antibiotic are more reliable treatment for pharyngitis associated with GAS infection.

3- Genotyping by RAPD- fingerprinting technique is a useful method of the genetic diversity investigation of S.*pyogenes* in purpose to epidemiological studies and associated with antibiotics susceptibility profiles.

4- Genomic diversity of GAS isolates suggested that the isolates are multicolnal in origin dependent on the differences in a susceptibility toward antibiotic in clusters. Although the clusters shared with antibiotic resistance for (Erythromycin, Clindamycin , Tetracycline).

5.2 Recommendations:

The current study recommends, based on the finding the following :

1- Further studies to investigate the genetic relationship between GAS isolates from different sources of infection.

2- Appropriate and early diagnosis with optimal management for the pediatric patients under 10 years of age with acute pharyngitis to reduce the risk of conversion to recurrent or chronic disease.

3- Introduce the comparative study for the assessment more the efficiency methods in determination the genetic diversity of GAS pharyngitis like the emm-typing or multilocus sequence typing (MLST) and plus field gel electrophoresis (PFGE). Additionally to the RAPD_ Fingerprinting methods.

4- Introduce the comparative study for the diagnosis S.*pyogenes* by different methods like molecular detection by PCR amplification of 16S r RAN gene and sequencing additionally to the *spy1258* gene and by the culture – dependent methods.

5-Further and continuous molecular epidemiologic studies are needed to increase understanding of possible associations of virulence determinants and their variants that facilitate host-pathogen interactions. This understanding may help in guiding the design of vaccines against GAS infections.

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Appendix

Appendix (1)

Patient Name: Said, Anas20 | Patient ID: 60
Location: | Physician:
Lab ID: 60 | Isolate Number: 1

Organism Quantity:
Selected Organism : Non or low reactive biopattern

Source: Oral Swab | **Collected:**

Comments:	

Identification Information	**Analysis Time:** 7.80 hours	**Status:** Final
Selected Organism	-1% Probability **Non or low reactive biopattern** **Bionumber:** 000000000000030	
ID Analysis Messages		

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	-	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	-	29	TyrA	(-)	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	-	39	ILATk	-	42	LAC	-	44	NAG	-	45	dMAL	-	46	BACI	-
47	NOVO	-	50	NC6.5	-	52	dMAN	-	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	-															

Appendix (2)

Patient Name: Said, Anas5 | Patient ID: 61
Location: | Physician:
Lab ID: 61 | Isolate Number: 1

Organism Quantity:
Selected Organism : Unidentified Organism

Source: Oral Swab | **Collected:**

Comments:	

Identification Information	**Analysis Time:** 7.78 hours	**Status:** Final
Selected Organism	**Unidentified Organism** **Bionumber:** 001000200000031	
ID Analysis Messages		

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	-	9	BGAL	-	11	AGLU	-
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	-	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	-	39	ILATk	-	42	LAC	-	44	NAG	-	45	dMAL	-	46	BACI	-
47	NOVO	-	50	NC6.5	-	52	dMAN	-	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (3)

HAMMURABI LAB.
Microbiology Chart Report

bioMérieux Customer:

Printed January 24, 2022 6:52:47 PM AST

Patient Name: Said, Anas6
Location:
Lab ID: 62

Patient ID: 62
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Unidentified Organism

Source: Oral Swab

Collected:

Comments:	

Identification Information	**Analysis Time:**	7.78 hours	**Status:**	Final
Selected Organism	**Bionumber:**	**Unidentified Organism** 000000200000030		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	-	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	-	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	-	39	ILATk	-	42	LAC	-	44	NAG	-	45	dMAL	-	46	BACI	-
47	NOVO	-	50	NC6.5	-	52	dMAN	-	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	-															

Appendix (4)

Patient Name:
Location:
Lab ID: 26122121

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability **Bionumber:**	**Streptococcus pyogenes** 071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (5)

Patient Name: | Patient ID:
Location: | Physician:
Lab ID: 26122126 | Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source: | **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability	**Streptococcus pyogenes**		
	Bionumber:	071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (6)

Patient Name: | Patient ID:
Location: | Physician:
Lab ID: 26122119 | Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source: | **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability	**Streptococcus pyogenes**		
	Bionumber:	071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (7)

Patient Name: | Patient ID:
Location: | Physician:
Lab ID: 30122112 | Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source: | **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability	Streptococcus pyogenes		
	Bionumber:	071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (8)

Patient Name: | Patient ID:
Location: | Physician:
Lab ID: 26122122 | Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source: | **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability	Streptococcus pyogenes		
	Bionumber:	071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (9)

Patient Name: | Patient ID:
Location: | Physician:
Lab ID: 26122112 | Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source: | **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability	**Streptococcus pyogenes**		
	Bionumber:	071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (10)

Patient Name: | Patient ID:
Location: | Physician:
Lab ID: 30122113 | Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
BP Infection Site:

Source: | **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.80 hours	**Status:**	Final
Selected Organism	85% Probability	**Streptococcus pyogenes**		
	Bionumber:	071412367773631		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	(-)	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix (11)

Patient Name:
Location:
Lab ID: 26122118

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	8.73 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	4		Clindamycin	>= 1	R
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	4		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	>= 8	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (12)

Patient Name:
Location:
Lab ID: 26122119

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.90 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	0.5		Clindamycin	>= 1	R
Ampicillin	<= 0.25	S	Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	0.25	S
Gentamicin			Tetracycline	4	I
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	>= 8		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (13)

Patient Name:
Location:
Lab ID: 26122116

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	10.93 hours		Status:	Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	4		Clindamycin	<= 0.25	S
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	0.5	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	4	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (14)

Patient Name:
Location:
Lab ID: 26122117

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.92 hours		Status:	Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	>= 8		Clindamycin	<= 0.25	S
Ampicillin	8	S	Linezolid	<= 2	S
Cefotaxime	>= 8		Teicoplanin		
Ceftriaxone	4		Vancomycin	0.5	S
Gentamicin			Tetracycline	<= 0.25	S
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	4	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	160	R

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (15)

Patient Name:
Location:
Lab ID: 26122113

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.95 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	>= 8		Clindamycin	<= 0.25	S
Ampicillin	>= 16		Linezolid	<= 2	S
Cefotaxime	>= 8		Teicoplanin		
Ceftriaxone	>= 8		Vancomycin	0.5	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug   *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (16)

Patient Name:
Location:
Lab ID: 2612218

Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Collected:

Source:

Comments:	

Susceptibility Information	Analysis Time:	11.32 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	2		Clindamycin	<= 0.25	S
Ampicillin	4		Linezolid	<= 2	S
Cefotaxime	TRM		Teicoplanin		
Ceftriaxone	1		Vancomycin	0.5	S
Gentamicin			Tetracycline	0.5	S
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug   *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (17)

Patient Name:
Location:
Lab ID: 26122114

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	10.47 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	2		Clindamycin	<= 0.25	S
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	4		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	0.5	S
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	2	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (18)

Patient Name:
Location:
Lab ID: 2612215

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Collected:

Source:

Comments:	

Susceptibility Information	Analysis Time:	11.30 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	2		Clindamycin	>= 1	R
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	TRM		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	4	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	>= 8	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (19)

Patient Name:
Location:
Lab ID: 2612217

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time: 11.32 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	>= 8		Clindamycin	<= 0.25	S
Ampicillin	>= 16	S	Linezolid	<= 2	S
Cefotaxime	>= 8		Teicoplanin		
Ceftriaxone	>= 8		Vancomycin	0.5	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (20)

bioMérieux Customer:

Patient Name:
Location:
Lab ID: 2612219

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Collected:

Source:

Comments:	

Susceptibility Information	Analysis Time: 11.33 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	4		Clindamycin	<= 0.25	S
Ampicillin	8	S	Linezolid	<= 2	S
Cefotaxime	4		Teicoplanin		
Ceftriaxone	4		Vancomycin	0.5	S
Gentamicin			Tetracycline	4	I
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S.

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (21)

Patient Name:
Location:
Lab ID: 2612211

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.27 hours		Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	>= 8		Clindamycin	<= 0.25	S
Ampicillin	>= 16	S	Linezolid	<= 2	S
Cefotaxime	>= 8		Teicoplanin		
Ceftriaxone	>= 8		Vancomycin	1	S
Gentamicin			Tetracycline	TRM	
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	>= 16	R
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug   *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (22)

Patient Name:
Location:
Lab ID: 2612212

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.28 hours		Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	0.25		Clindamycin	<= 0.25	S
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	4		Teicoplanin		
Ceftriaxone	1		Vancomycin	0.5	S
Gentamicin			Tetracycline	0.5	S
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	8	I
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug   *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (23)

Patient Name:
Location:
Lab ID: 2612213

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time: 9.08 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	>= 8		Clindamycin	>= 1	R
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	0.5	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	>= 8	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (24)

Patient Name:
Location:
Lab ID: 2612214

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time: 11.28 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	1		Clindamycin	>= 1	R
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	2		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	40	I

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (25)

Patient Name:
Location:
Lab ID: 26122111

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.97 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	>= 8*		Clindamycin	<= 0.25	S
Ampicillin	TRM	S	Linezolid	<= 2	S
Cefotaxime	TRM		Teicoplanin		
Ceftriaxone	TRM		Vancomycin	0.5	S
Gentamicin			Tetracycline	0.5	S
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin	TRM	
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent with correction

Appendix (26)

Patient Name:
Location:
Lab ID: 26122112

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.22 hours		Status:	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	4		Clindamycin	<= 0.25	S
Ampicillin	4	S	Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	0.5	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	4	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (27)

Patient Name:
Location:
Lab ID: 26122115

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time:	11.93 hours		Status:	Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	>= 8		Clindamycin	<= 0.25	S
Ampicillin	>= 16	S	Linezolid	<= 2	S
Cefotaxime	>= 8		Teicoplanin		
Ceftriaxone	>= 8		Vancomycin	0.5	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	1	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	>= 16	R
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (28)

Patient Name:
Isolate: 26122120-1 (Qualified)

Patient ID:

Card Type: AST-ST03 Bar Code: 5421616503706339 Testing Instrument: 00000A726B5A (AL-NUKHBA LAB)
Setup Technologist: Laboratory Administrator(Labadmin)

Organism Quantity: **Selected Organism: Streptococcus pyogenes**

Comments:	

Identification Information			
Organism Origin	Technologist		
Selected Organism	Streptococcus pyogenes		
	Entered: Dec 26, 2021 19:02 CST	By:	Labadmin

Analysis Messages:

High level resistance to gentamicin (MIC of > 128 mg/L), is generally caused by the production of a bifunctional APH(2')-AAC(6) enzyme that determines loss of synergism of all aminoglycosides (except streptomycin and arbekacin) with b-lactams and glycopeptides irrespective of MIC values.

Susceptibility Information	Card: AST-ST03		Lot Number: 5421616503	Expires: Apr 30, 2022 13:00 CDT	
	Completed: Dec 26, 2021 22:14 CST		Status: Final	Analysis Time: 11.40 hours	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Benzylpenicillin	1		Clindamycin	<= 0.25	S
Ampicillin	2		Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.25	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	2	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings:	**Last Modified:** Jan 7, 2021 15:14 CST	**Parameter Set:** Global CLSI-based+Phenotypic 2019
Confidence Level:	Inconsistent	

Appendix (29)

Patient Name:
Location:
Lab ID: 2612216

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:

Susceptibility Information	Analysis Time:	11.30 hours		Status:	Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	2		Clindamycin	>= 1	R
Ampicillin	4		Linezolid	<= 2	S
Cefotaxime	1		Teicoplanin		
Ceftriaxone	1		Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.5	S	Chloramphenicol	4	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	>= 8	R	Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Inconsistent

Appendix (30)

Patient Name:
Location:
Lab ID: 26122110

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes

Source:

Collected:

Comments:

Susceptibility Information	Analysis Time:	11.33 hours		Status:	Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Benzylpenicillin	TRM		Clindamycin	<= 0.25	S
Ampicillin	TRM		Linezolid	<= 2	S
Cefotaxime	TRM		Teicoplanin		
Ceftriaxone	TRM		Vancomycin	1	S
Gentamicin			Tetracycline	TRM	
Levofloxacin	2	S	Tigecycline	<= 0.06	S
Moxifloxacin	0.12	S	Chloramphenicol	2	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin		
Erythromycin	TRM		Trimethoprim/Sulfamethoxazole	<= 10	S

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent

الخلاصة :

Streptococcus pyogenes البكتيريا العقدية المقيحة هي المجموعة أ (GAS) محللة للدم نوع بيتا β-hemolytic Streptococcus . بكتيريا موجبة الغرام تسبب مجموعة واسعة من الأمراض السريرية التي تتراوح من التهاب البلعوم الخفيف الى الالتهابات الغازية التي تهدد الحياة .

Streptococcus pyogenes مجموعة أ (GAS) هي من المسببات البكتيرية الاكثر شيوعاُ لالتهاب البلعوم الحاد وتمثل ٥ _ ١٥ % من جميع حالات البالغين و٢٠ _ ٣٠ % من جميع حالات الاطفال .

تم جمع ١٢٥ عينة سريرية من مرضى يعانون من التهاب البلعوم الحاد . تم ادخالهم الى مستشفيين رئيسيين في محافظة بابل : مستشفى الحلة التعليمي العام ومستشفى الامام الصادق التعليمي العام خلال الفترة الممتدة (٢١ تشرين الاول ٢٠٢١ الى ٢٥ كانون الثاني ٢٠٢٢) . ثم تم استنبات العينات في (وسط أكار الدم ، وسط اكار الدم كولومبيا و Brain Heart Infusion agar) ، وعزل S.*pyogenes* .

من بين ١٢٥ مسحة بلعومية ، تم الكشف عن ٤٠ عزلة فقط من S.*pyogenes* عن طريق الزراعة والاختبار البيوكيميائي والجين المتخصص (*spy1258*) .

تم إجراء تفاعل البلمرة المتسلسل (PCR) باستخدام باديء متخصص على الحمض النووي الجيني من S.*pyogenes*. أشار تضخيم PCR للجين (*spy1258*) إلى أن ٤٠/٣١ (٧٧.٥ ٪) من عزلات S.*pyogenes* تمتلك هذا الجين.

تم أيضًا إجراء دراسات على جين (*SmeZ*) ، وقد لوحظ في١٤ / ٤٠ (٣٥ ٪) عزلة من *Streptococcus pyogenes* .

بصمة الحمض النووي متعدد الاشكال العشوائي (RAPD) اعتمدت بنجاح لاظهار التباين الجيني بين السلالات وثيقة الصلة داخل نفس النوع . هي تقنية اكثر موثوقية لتنميط عزلات GAS في التحقيق الوبائي . تهدف الدراسة الحالية إلى التحقيق في التنوع الجيني لعزلات *Streptococcus pyogenes* من مرضى التهاب البلعوم الحاد بواسطة طريقة بصمة RAPD-PCR .

تضمنت الدراسة الحالية التحري في التنوع الجيني باستخدام طريقة بصمة RAPD-PCR . التي تم تحقيقها من باديء اعتباطي OPA13 .

أظهر تضخيم الحمض النووي الجيني من عزلات (GAS) مع OPA13 نتائج من قطع من الحمض النووي متعدد الاشكال ، واظهر تحليل النشوء والتطور درجة عالية من التنوع الجيني ، مصنفة عزلات (GAS) الى أربع مجموعات رئيسية . تعد بصمة الحمض النووي باستخدام تحليل RAP-PCR طريقة فعالة لتقييم التنوع الجيني لعزلات (GAS) .

أظهرت عزلات *S.pyogenes* نتائج إيجابية للجينات *spy1258* و *smeZ* و RAPD-PCR . ثم خضعت جميع العزلات لاختبار الحساسية للمضادات الحيوية (AST) . أظهرت قراءة الجهاز النسب التالية: أمبيسيلين (٨٥٪) ، ليفوفلوكساسين (١٠٠٪) ، موكسيفلوكساسين (١٠٠٪) ، إريثروميسين (٠٪) ، كليندامايسين (٧٠٪) ، لينزوليد (١٠٠٪) ، فانكومايسين (١٠٠٪).) ، تتراسيكلين (٢٥٪) ، تيجيسيكلين (١٠٠٪) ، كلورامفينيكول (٨٥٪) ، تريميثوبريم / سلفاميثوكسازول (٩٠٪) على التوالي.

من خلال دراسة كل المجاميع الظاهرة لعزلات (GAS) ، توصلت هذه الدراسة الى حساسية مشتركة للمضادات الحياتية (ليفوفلوكساسين ، لينزوليد وموكسيفلوكساسين). بالاضافة الى ذلك ، وجدت هذه الدراسة ان التنوع الجيني لعزلات (GAS) اقترحت أن العزلات متعددة النسيلة في الأصل وتعتمد على الاختلافات في القابلية للتأثر بالمضادات الحيوية في المجاميع ، على الرغم من أن المجاميع تشترك في مقاومة المضادات الحيوية لـ (الاريثروميسين، كليندامايسين، التتراسيكلين).

أظهرت الدراسة الحالية أن المكورات العقدية المقيحة من المجموعة أ (GAS) *Streptococcus pyogenes* متنوعة وراثيًا وتمتلك جينات *smeZ* بغض النظر عن غزوها. لم تظهر غالبية GAS أي نمط مقيد للأنماط الفيروسية. لذلك ، يمكن اقتراح أن التنميط الفيروسي مفيد جزئيًا لتوصيف مجموعة غير متجانسة من GAS ، وربما يرجع التواجد الأكبر لهذا الجين في المجموعة رقم ١ إلى أن هذه العزلات تم جمعها على مدى فترة طويلة من الوقت وتم تمثيلها بواسطة عدد كبير مقارن من العزلات.

وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم للبنات
قسم علوم الحياة

التنوع الجيني للمكورات العقدية من المجموعة أ المعزولة من مرضى إلتهاب البلعوم

رسالة

إلى مجلس كلية العلوم للبنات في جامعة بابل
كجزء من متطلبات نيل درجة الماجستير
علوم الحياة

من قبـــل

زينب طارق علي الجنابي
بكالوريوس علوم حياة
جامعة بابل - ٢٠١٩ م

بـإشـراف
أ.م.د. عروبه كطوف البيرماني

٢٠٢٢ م
١٤٤٤هـ