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Molecular Detection of Transposable Elements Conferring to Antibiotic Resistance in Clinical Isolates of *Streptococcus pneumoniae*

A Thesis

Submitted to the Council of the College of Biotechnology, University of Al-Nahrain as a Partial Fulfillment of the Requirements for the Degree of Master in Biotechnology

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Dedication

For the person whom I miss in my life, the person who taught me success and patience my dearest father…

For my mother, that there are no words to describe her…

To my brothers and sisters whose love flows in my veins…

To my friends who taught me the soul of competition...

To my instructors who help me to be the person who I am today…

DUAA

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Praising to **Allah** who gave me health, strength and facilitated the ways for me to accomplish this work.

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Summary

Clinical samples were collected from patients suffering of respiratory tract infection attended to different hospitals in Baghdad, Iraq. Swab samples were obtained from pharyngitis and tonsillitis. In addition, sputum and cerebrospinal fluid . From these samples, total of 100 bacterial isolates were obtained after culturing on blood agar and chocolate agar. Among the total isolates, 51 isolates were primarily identified as *Streptococcus spp,* only 15 identified as *streptococcus pneumoniae* due to their ability to cause blood hemolysis type α-hemolysis and sensitivity to optochin. These isolates were identified according to their morphological, cultural and biochemical characteristucs. Results showed that these 15 isolates are Gram positive, negative for oxidase and catalase test, able to ferment inulin, lactose and trehalose but not for manitol and sorbitol. Furthermore, identification of these isolates was confirmed by using vitek-2 identification system. Susceptibility of the identified *S.pneumoniae* isolates to different antibiotics was examined. Results showed that these isolates were variable in the susceptibility pattern, where most of them 93% resistant to penicillin, then to each streptomycin and trimethoprim (87%), clindamycin (73%), kanamycin (67%), erythromycin, tetracycline and azithromycin (60%), ciprofloxcain and levofloxacin (53%). Genomic DNA was extracted from *S.pneumoniae* isolates by using extraction kit supplied by Promega\USA for the next step to detect transposons conferring antibiotic resistance in these isolates by using specific primers to amplify antibiotic resistance gene carried by these transposable elements. Results of amplification showed that 7 of these isolates harboring Tn*1545* conferring erythromycin resistance, 4 isolates harboring Tn*917* conferring erythromycin resistance, 5 isolates harboring

Tn*5397* conferring tetracycline resistance, 7 isolates harboring Tn*3872* conferring erythromycin resistance, 8 isolates harboring Tn*6002* conferring erythromycin resistance, and 4 isolates harboring Tn*916* conferring tetracycline resistance. Nucleotide sequence for each antibiotic resistance gene carried by these transposable elements was determined and compared by alignment with the symmetrical genes located on the same transposable elements in the standard strains of *S.pneumoniae* recorded in NCBI data base. Results of the alignment showed that 100% identity were recorded between sequences of antibiotic resistance genes located on genomic DNA of these isolates of *S.pneumoniae* and the nucleotide sequence of the same genes carried by standard strains of *S.pneumoniae*.

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List of Abbreviations

Abbreviation	Meaning
Cbps	Choline binding proteins
CLSI	Clinical laboratory standards institute
dNTP	Deoxynucleotide triphosphate
IS	Insertion sequence
MDR-SP	Multidrug resistant *S.pneumoniae*
MEF	Middle ear fluid
MICs	Minimum inhibitory concentrations
MRSA	Methicillin resistant *Staphylococus aureus*
Nan	Neuraminidase
OS	Oligosaccharides
PCR	Polymerase chain reaction
PG	Peptidoglycan
NCBI	National Center for Biotechnology Information
STM	Signature tagged mutagenesis
MLSB	Macrolide –Lincosamide- Streptogramin B
TAs	Teichoic acids
TEs	Transposable elements
TMP-SMX	Trimethoprim sulfamethozazole
Tn	Transposon
VRE	Vancomycin –resistant enterococci
WHO	World Health Organization
WTA	Wall teichoic acid

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1. Introduction and literature review

1.1. Introduction

Streptococcus pneumoniae is a medically important pathogen that causes a number of community acquired infections, including chronic bronchitis, otitis media, acute bacterial sinusitis and pneumonia. Pneumococcal pneumonia is a major cause of morbidity and mortality in developing countries (Falade and Ayede, 2011; Feldman *et al*., 2013) Host genetic factors that alter the immune response and/or environmental factors such as concurrent viral infection might increase susceptibility to disease (Von Mollendorf *et al.*, 2015).

Increasing trend of antimicrobial resistance in bacteria that cause infectious diseases is a global problem, although resistance significantly varies between geographical regions. Today, common bacterial pathogens can be resistant to all known antimicrobial agents (Mediavilla *et al*., 2016; Skov and Monnet, 2016).

Infections caused by resistant *S. pneumoniae* can be difficult to treat, resulting in a greater risk of death. Pneumococci resistant to more than three separate classes of antibiotics are considered to be multidrug resistant (Appelbaum., 2002). The emergence of drug-resistant *S. pneumoniae* has occurred around the world (Naba *et al.*, 2010).

To date, multidrug-resistant *S. pneumoniae* (MDR-SP) have been isolated from both adults and children around the world (Appelbaum., 2002),They are resistant to penicillin, clindamycin, and erythromycin (Yatim *et al*., 2013), *S. pneumoniae* colonization rates are high in children aging less than 5 years (Yatim *et al*., 2013).

The carrier state is asymptomatic, and transmission of pneumococci in children can occur from any individual colonized with the microorganisms. The incidence of antimicrobial-resistant *S. pneumoniae* in the nasopharynx of children increases the risk of resistant strains that cause *S. pneumoniae* infection (Yatim *et al*., 2013). The evolution of antibiotic-resistant strains is attributed to antimicrobial acquisition or inappropriate use of

antibiotics in the community (Arason *et al*., 2010). In addition, *S. pneumoniae* is a bacterium that possesses a horizontal gene transfer. This mechanism allows the acquisition of antibiotic-resistant genes which increases the resistance to a variety of an antibiotic.

The emergence of antibiotic resistance among bacterial pathogens is a major problem in the treatment of infectious disease in both the community and in healthcare settings throughout the world. In industrialized nations, there has been a steady rise in the incidence of high-profile healthcare associated infections that have become resistant to one or more antibacterial agents making treatment increasingly difficult (Rossolini *et al*., 2010).

These include, but are not limited to, methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and multidrug-resistant *Streptococcus pneumonia* (Rossolini *et al*., 2010). In addition, there are pathogens, such as *Clostridium difficile*, which have risen to global prominence over the last few years and have the ability to acquire mobile genetic elements from enterococci, indicating the potential to acquire resistance to the last line of defense antibiotics, for example vancomycin and other glycopeptides (Cartman *et al*., 2010; Jasni *et al*., 2010). These resistances are commonly acquired on mobile genetic elements such as conjugative plasmids and conjugative transposons, which are capable of a broad host range transfer between pathogens (Weigel *et al.,* 2003; Jasni *et al*., 2010) and between commensal and pathogenic bacteria. For example, methicillin resistance, which is mediated by the product of the mecA gene and present in MRSA strains, most likely originates from *Staphylococcus fleurettii*, an animal commensal (Tsubakishita *et al*., 2010).

Tn*916*/Tn*1545* family is responsible for a large proportion of the antibiotic resistance in these different pathogens. These conjugative elements are responsible for the dissemination of many antimicrobial resistance genes (usually resistance to tetracyclines, but also macrolides, lincosamides and

streptogramins, kanamycin and mercury) to some of the most important Gram-positive pathogens.

According to that mentioned above, this study was aimed to investigate the spreading of antibiotic resistance carried by transposon in clinical isolates of *S. pneumonia*. This was achieved according to the following steps:

1. Collection of specimens from respiratory tract infections.
2. Identification of *S.peumonia* according to their morphological characteristic and biochemical tests.
3. Investigating the antibiotic resistance of bacterial isolates against several antibiotics tetracycline, kanamycine, erthromycine,clindamycin, penicillin, Azithromycin, streptomycin, Ciproflaxin, levofloxacin, Trimethoprim.
4. Deterring the percentage of bacterial resistance to antibiotics.
5. Molecular detection of transposable elements Tn*916*, Tn*6002*, Tn*917*, Tn*1545*, Tn3872 and Tn*5397* by amplification those elements using special primers.
6. Sequencing of amplified fragment to characterize the transposable elements.

1.2. Literature review

1.2.1. Respiratory Tract Infections

The respiratory tract constitutes a wide and critical frontier at the interface between the body and the environment. This complex organ system is divided into the upper airways and lower airways. The upper airways or upper respiratory tract includes the nose and nasal passages, paranasal sinuses, the pharynx, and the portion of the larynx above the vocal folds (cords). The lower airways or lower respiratory tract includes the portion of the larynx below the vocal folds, trachea, bronchi, and bronchioles. The lungs can be included in the lower respiratory tract or as a separate entity and include the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli (Man *et al*., 2017). Upper respiratory tract infections are less severe whereas lower infections are often associated with high mortality rates (van den Bergh *et al.*, 2012). Acute lower respiratory tract infections constitute the third leading cause of human death worldwide with 3.2 million deaths in 2015 (Figure 1-1), and the first cause of mortality in children under five years, according to the World Health Organization (WHO) (WHO, 2017).

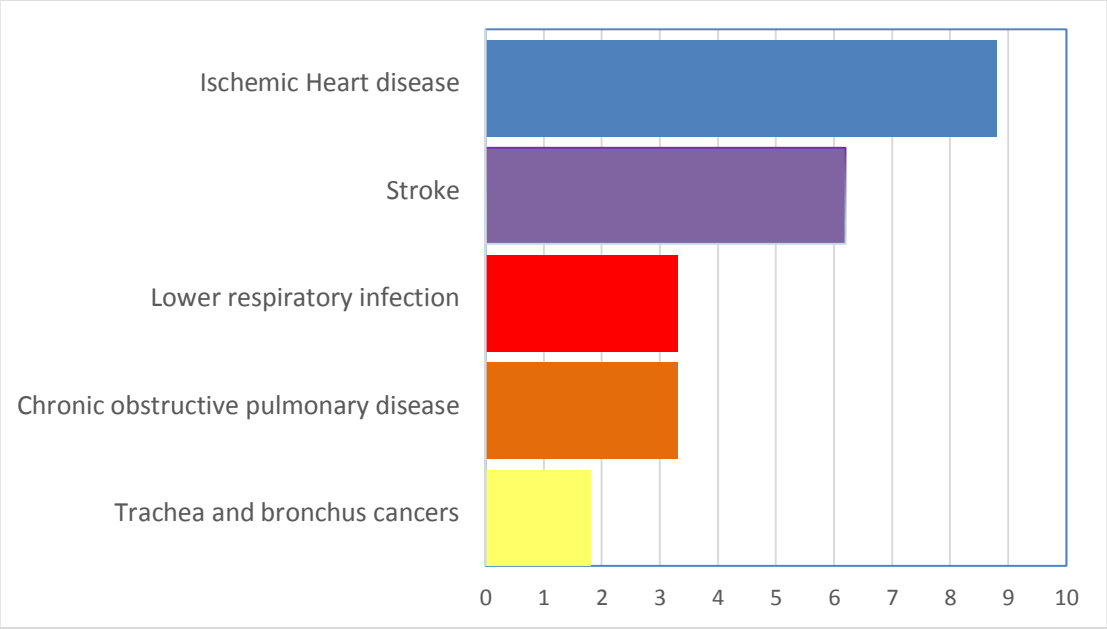


Figure (1-1): Causes of death worldwide. Lower respiratory infections constitute the third cause of death in the world population (red bar) being responsible for 3.2 million

of deaths in 2015. Heart diseases (Ischemic heart disease – blue bar - and strokes – pink bar) were the most cause of deaths. Among the 5 main causes of global death described, lower respiratory infections are the only transmissible infectious disease (WHO, 2017).

The diversity of pathological agents makes it difficult to prevent, diagnose and treat these diseases, contributing to high mortality rates (Shaughnessy *et al.*, 2016, Figueiredo-Mello *et al.*, 2017). These diseases can affect the general population, but severe cases and high mortality rates are found among children up to age 5, immunosuppressed adults and elderly (Nair *et al.*, 2010, Stupka *et al.*, 2009). Acute lower respiratory infections constitute a major global health burden due to the emergence of resistance to antimicrobial treatments, the presence of multiple pathogens and the recurrence of infections throughout life (Feldman and Anderson, 2014).

1.2.2. Respiratory bacterial infections

Etiological studies of acute lower respiratory tract infection identify a high prevalence of different types of bacteria, even more than viral detection. Among the most frequent bacterial causes of pneumonia are *Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus.* All these pathogens are asymptomatic bacteria; its carriage is well described in healthy individuals (Siegel and Weiser, 2015). Also, *Mycoplasma pneumoniae and Chlamydia pneumoniae* are opportunistic bacteria that are considered as important pathogens causing pneumonia and bronchiolitis (Esposito *et al.*, 2002, Arnold *et al.*, 2007).

1.2.3. Genus *Streptococcus*

The genus *Streptococcus* is a diverse lineage belonging to the lactic acid group of bacteria. Current taxonomy places this genus, as well as the genus *Lactococcus,* within the family Streptococcaceae in the order Lactobaillaceae (Facklam, 2002). These organisms are Gram-positive, spherical, and catalase-

negative, and many are facultative anaerobes (Montes and García-Arenzana, 2007; Lal *et al*., 2011).

Genus *Streptococcus* comprises important pathogens that have a severe impact on human health (Richards *et al*., 2014).Genus *Streptococcus* was first recorded in 1683 in Van Leeuwenhoek's drawings of microscope images of the material removed from between his teeth. *Streptococcus* comes from the Greek strepto (twisted) and (spherical) (Nobbs *et al*, 2009).

The features of pathogenicity associated with the genus *Streptococcus* are also diverse: meningitis, pneumonia, endocarditis, fasciitis, and dental caries are among the better known conditions (Glazunova *et al.*, 2010). Normal human reservoirs of *Streptococcus* include different compartments of the oral cavity and skin, and the respiratory, digestive, gastric, and urinary tracts (Hardie and Whiley, 1997). However, the complete home range for most species of the genus is largely uncertain since this knowledge depends on sampling strategies that are not normally focused on revealing species habitats.

1.2.4. Pathogenicity associated with genus *Streptococcus*

1.2.4.1. Pneumonia

Pneumonia, a lower respiratory tract infection that specifically affects the lungs, is the leading cause of child deaths globally. Pneumonia illness is classified clinically either as a non-severe or a severe pneumonia illness (WHO, 2013) Childhood pneumonia deaths are often due to a severe pneumonia illness with approximately 1 in 5 of these childhood pneumonia deaths caused by *S. pneumoniae* (Black *et al.*, 2010).

Global disease burden estimates indicate there were as many as 120 million episodes of pneumonia in 2011, including 14 million episodes of severe pneumonia in children less than 5 years (Walker *et al.*, 2013).

These estimates represent widely varying regional incidence rates with reported rates, the lowest in the European region and the highest in the African and Southeast Asian regions; As many as 30 and 39% of all severe pneumonia

episodes occur during this period in the African and southeast Asian regions, respectively. The distribution pattern of childhood pneumonia deaths across world regions mirrors rates observed for pneumonia incidence. Pneumonia was responsible for approximately 935,000 deaths of children under the age of five years and that this accounted for 15% of all deaths of children under five years old globally (Liu *et al*., 2015).

1.2.4.2. Etiology of pneumonia

Pneumonia can be caused by microorganisms, irritants and unknown causes, when pneumonias are grouped this way, infectious causes are the most common type. The symptoms of infectious pneumonia are caused by the invasion of the lungs by pathogens and by the immune tissue injuries arose from the infection. Although more than one hundred strains of microorganism can cause pneumonia, only a few are responsible for most cases. The most common causes of pneumonia are bacteria and viruses, while less common causes of infectious pneumonia are fungi and parasites (Murray and Bongiorno, 2006).

1.2.4.3. Meningitis

Bacterial meningitis has been known to be associated with very high fatality, with reported annual mortality incidence in the developing countries of 98 per 100,000 in children less than 1 year (Gessner *et al* 2010). Outbreaks of epidemic meningitis are particularly frequent in the African meningitis belt, a region that extends from Gambia, in the west, to Ethiopia and Eritrea in the east (Leimkugel *et al*., 2005; Yaro *et al*., 2006). Predominant bacterial causes of meningitis include *Neisseria meningitidis, S. pneumoniae and Haemophilus influenzae* type b (Hib) in the pre-Hib vaccine era. Traditionally, the meningococcal has been known to be a leading cause of epidemic meningitis, but reports from the West African sub-region have indicated that the *pneumococcus* plays a more significant role in causing meningitis than thought previously (O'Brien *et al*., 2009, Gessner *et al.*, 2010).

1.2.4.4. Sepsis

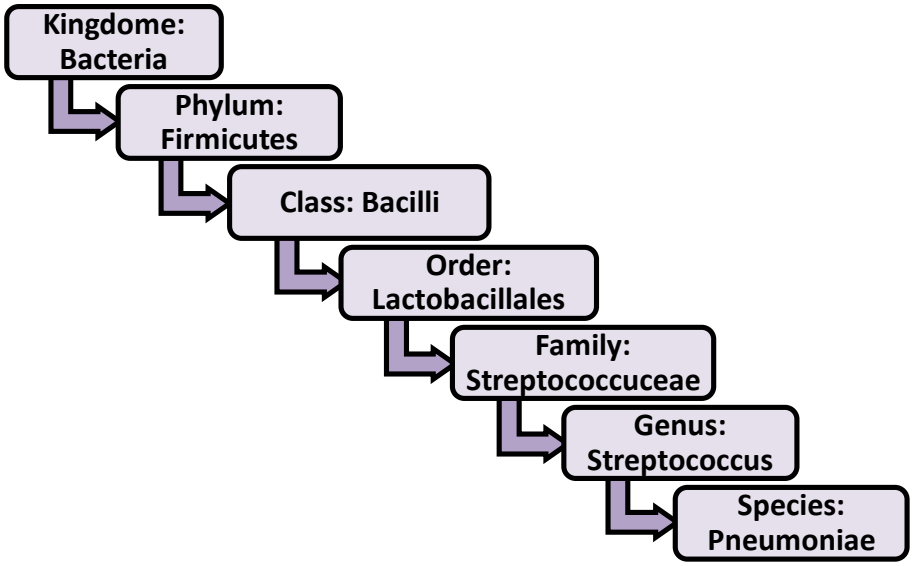
Sepsis refers to the presence of rapidly dividing bacteria in the blood stream with manifest signs of illness in the individual. Global estimates for incidence of pneumococcal sepsis is reported to be as frequent as 87 per 100,000 persons with the highest rates seen in Africa. The case fatality rates for sepsis across all age groups ranged from 22% in the Western Pacific region to 58% in Africa region with higher rates seen in very young children and the elderly (O'Brien *et al*., 2009).

1.2.4.5. Non-invasive pneumococcal disease

Pneumococci are also known to cause less severe and non-invasive forms of disease such as otitis media (middle ear infection), sinusitis and bronchitis. Pneumococci have been identified in as many as 57% of middle ear aspirates taken from cases with acute otitis media (Tamir *et al.*, 2015). Other bacteria known to cause otitis media include *H. influenzae and Moraxella catarrhalis* (Tamir *et al*., 2015).

1.2.5. *Streptococcus pneumoniae*

Scientific classification of *S.pneumoniaee* is shown below (Abachi and Rupasinghe, 2016):



Streptococcus pneumoniae is a Gram positive, alpha-hemolytic coccal-formed bacterium that is generally found in pairs, characterized as lancet-shaped diplococci, but may also occur singly or in short chains, non-motile, non-spore forming, and facultative anaerobic organism, mucoidal, and flat, other species of Streptococci which appear much smaller and with a gray color as not as *S.pneumoniae* that are surrounded by an area of greenish discoloration (Keith *et al*., 2006).

They occur in pairs or in chains, ranging from 0.5 to 1.25 µm in diameter with smooth edges. And if they left for 24-48 hours, they will be considered as older cultures that appear as "draughtsman "colonies having depressed centers and raised edge. *S. pneumoniae* is chemoorganotroph, requiring nutritionally rich media for growth and sometimes 5% CO_2. The metabolism is fermentative producing mainly lactate but no gas. They attack commonly RBCs with greenish discoloration (Ryan and Ray, 2004).

The pneumococcus has been recognized as an important human pathogen for over 100 years and continues to be a main cause of morbidity and mortality worldwide. It can asymptomatically colonize the nasopharynx and can cause a wide variety of diseases, ranging from mild infections to severe infections, as well as life-threatening invasive infections such as meningitis. It is the most common bacterial cause of acute otitis media and pneumonia and an important cause of childhood mortality. In 2010, reports estimated that *S. pneumoniae* is still responsible for approximately 1.3 million deaths yearly, especially among young children and the elderly, although the availability of vaccines and antibiotics (Walker *et al*., 2013).

Like other Gram-positive Firmicutes, *S. pneumoniae* has a thick, multilayered cell wall composed mainly of peptidoglycan (PG) and teichoic acids (TAs). The latter are either covalently attached to PG (wall teichoic acid, WTA) or anchored to the cytoplasmic membrane (lipoteichoic acid, LTA). To protect the bacterial cell from lethal components of the immune system, and

enable this pathogen to survive within the host organism and cause disease, most of the more than 90 distinct, serotype-specific capsular polysaccharides are covalently linked to the PG (Bentley *et al.*, 2006).

Even though encapsulated pneumococci typically cause invasive illness, they are known to be pathogenic and largely responsible for causing invasive pneumococcal disease (Brueggemann *et al.*, 2003). 32non-encapsulated pneumococci have also been shown to cause non-invasive diseases like conjunctivitis and otitis media and in rare instances, have been responsible for a few cases of invasive disease (Valentino *et al*., 2014; Hotomi *et al*., 2016).

Streptococcus pneumoniae strains are divided into various serotypes, based on the capsule, that are serologically and biochemically different. Presently, there are more than 95 known serotypes of pneumococci (Park *et al*., 2015;Van Tonder *et al.*, 2015).

1.2.6. Pathogenesis and virulence factors

The ability of an organism or microbe to cause harm or disease in a host it called "Pathogenicity" (Pirofski and Casadevall, 2012). Pathogenicity is thought to base on possession by the microbe of some virulence factors that intermediate the disease outcome in the host. On the other hand, Virulence is defined as the relative capability of the microbe to cause disease or harm in the host. Whilst pathogenicity is sometimes considered in terms of the presence or absence of this ability in the microbe, i.e. whether or not the microbe is pathogenic or not. Virulence is usually mentioned to the range or degree of hurt or pathology caused by the microbe to the host. It has been proposed that both pathogenicity and virulence are inherent microbial properties that are often expressed in the context of a susceptible host, thus highlighting the value of the host-pathogen interaction in the expression of these properties by the microbe (Casadevall and Pirofski, 2001).

It has many various features encoded by virulence factors. In order to achieve the access. *S. pneumoniae* has a lot of virulence factors described, the

new technologies such as signature tagged mutagenesis (STM), transposon sequencing (Tn-seq), expression assays such as microarray and deep RNA sequencing, make the numbers of bacteria increase in recent years (Van Opijnen and Camilli, 2012; Mann *et al*., 2012).

The advent of whole genome sequencing and functional characterization of these genes in animal models have also facilitated the discovery of further virulence genes. *S. pneumoniaee* may vary in their virulence properties because of virulence genes are present in almost all pneumococcal isolates while others vary from one pneumococcus to the other. This variation across pneumococcal strains is consistent with the fact that *S. pneumoniae* is highly transformable and can readily uptake exogenous DNA from closely related species.

Several virulence genes are found on the pneumococcal cell surface and others in the cytoplasm, as shown in figure (1-3).

1.2.6.1. Capsule of *Streptococcus pneumoniae*

The polysaccharide capsule is the most important virulence factor in pneumococcus. It's also the basis for the serotyping of pneumococci. (Calix et *al*., 2012).

The capsule consists of high molecular weight polymers made up of units of repeating oligosaccharides (OS), which are between two and eight monosaccharaides in range. The gene encoding synthesis of capsule is (cps2A) and the role of the capsule in the virulence of pneumococci is also well illustrated by the highly protective activity of anticapsular antibody (Snippe *et al*., 1983). The most striking features of the pneumococcal capsule locus is its huge genetic divergence, as only a few genes are conserved among the different clusters (Aanensen *et al*., 2007). The function of capsule towards pathogenicity can be summarized in disrupting many aspects of complement system and interfering with neutrophil mediated immunity leading to decrease opsonophagocytic capacity.in addition to, it is has the ability to gain access to

epithelial surfaces by avoiding entrapment in the nasal mucus (Hyams *et al*., 2010).

The capsule is also crucial for colonization as it prohibits removal by mucus, and can also restrict autolysis and decrease exposure to antibiotics (Van der Poll and Opal., 2009).

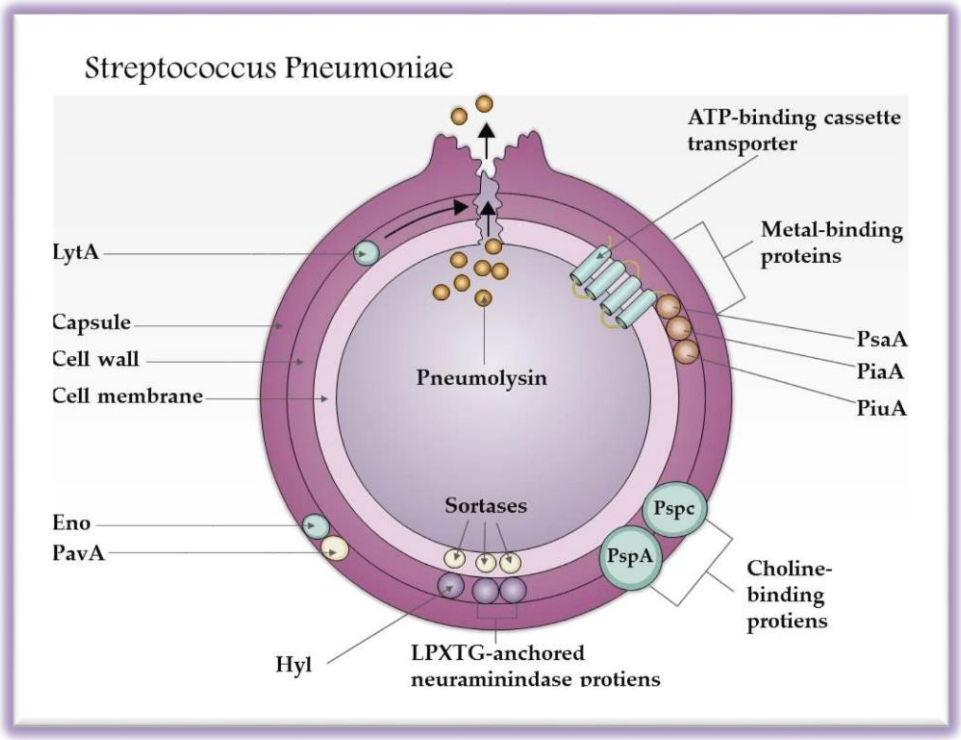


Figure (1-2) *Streptococcus pneumoniae* cell showing important virulence factors. LytA, autolysin; Eno, enolase; PavA, pneumococcal adhesion and virulence A; Hyl, hyaluronate lyase; PspA and PspC, pneumococcal surface protein A and C; PiuA, pneumococcal iron uptake A; PiaA, pneumococcal iron acquisition A; PsaA, pneumococcal surface antigen. Adapted from (Kadioglu *et al.*, 2008).

1.2.6.2. Cell Wall *Streptococcus pneumoniae*

The cell wall is defined for its ability to stimulate an inflammatory response and the presence of teichoic acid facilitates this process (Tomasz and Saukkonen, 1989). The alternative complement pathway will be activated by cell wall and by inducing the product of platelet activating factor (Cabellos *et al*., 1992). It also facilitates attachment to the host endothelial cells resulting in

loss of barrier integrity, effects that are mediated through the production of cytokines (Gratten *et al*., 1993).

1.2.6.3. Pneumolysin *Streptococcus pneumonia*

Pneumolysin, one of the most studied pneumococcal proteins, is a highly conserved pneumococcal protein on account formulation of protein vaccines, Cholesterol-dependent cytolysins.(Briles *et al.*, 2003). It is a 53 kDa cytoplasmic protein and forms part of a larger group of proteins of pathogenic Gram positive bacteria. The importance of this gene in virulence has been demonstrated in several animal infection models (Ogunniyi *et al.*, 2007; Berry *et al*., 1989b). It has been shown to be cytotoxic and inhibits ciliary movements on respiratory epithelium (Feldman *et al*., 1990). It also activates the classical complement pathway (Mitchell *et al*., 1990).

1.2.6.4. Pilli *Streptococcus pneumoniae*

Pilli, filamentous structures found on the surface of many Gram-positive bacteria,, are encoded within pathogenicity islands which are a group of mobile genetic elements obtained by bacteria through horizontal gene transfer. In 2006 Pilli were first observed on the surface of pneumococci (Barocchi *et al*., 2006). PI-1 is encoded by the RlrA accessory region and has been shown to influence colonization, virulence and the inflammatory response in mouse models. Strains containing PI-1 were more virulent, exhibited enhanced adherence to lung epithelial cells and had a competitive advantage over strains mutant strains lacking this factor (Barocchi *et al*., 2006). A second type identified, PI-2, was also involved in adherence (Bagnoli *et al.*, 2008). It was observed that PI-2 was associated with pneumococcal multilocus sequence type (MLST) belonging serotypes 1, 2, 7F, 19A and 19F. It was noted that strains belonging to clonal complex (CC) 271 were found to contain both PI-1 and P1-2 (Bagnoli *et al*., 2008).

1.2.7. *Streptococcus pneumoniae* surface proteins

There are several proteins on the surface of pneumococcal cell those proteins share in significantly to the virulence of the organism. They are marked by one of three motifs; a choline binding domain, a lipoprotein domain or the LPXTG cell wall anchor, (Bergmann and Hammerschmidt., 2006).

1.2.7.1. Choline binding proteins (Cbps)

Choline binding proteins are anchored on the cell wall by phophorylcholine on teichoic and lipoteichoic acids (Hakenbeck *et al*., 2009).Different Cbps are produced by *S. pneumoniae* that play a role in nasopharyngeal colonization. Such Cbps has also been shown to play a role in sepsis (Gosink *et al.*, 2000).

1.2.7.2. Lipoproteins PsaA

Pneumococcal surface adhesion A is a fraction of an ABC transporter operon in which PsaA is a substrate binding lipoprotein, PsaB, the ATP-binding protein and PsaC the permease likely involved in transporting manganese and zinc into the cytoplasm of pneumococcus. It is thought to function as an adhesin. In the animal model tests, Strains of the pneumococcus lacking this gene have been found to be avirulent (Sampson *et al*., 1994; Tseng *et al.*, 2002).

1.2.7.3. LPXTG-anchored surface proteins

Through a carboxyl-terminal motif the proteins covalently anchored to the cell wall, by a sortase enzyme LPXTG they could recognize. They include neuraminidases (NanA and NanB), hyaluronate lyase and serine protease. NanA and NanB, two enzymes involved in neuraminidase activity, have been shown to be essential for the colonization and infection of the upper and lower respiratory tract. The survival of *S. pneumoniae* in the respiratory tract and

blood belongs to the role of Neuraminidase that facilitates the survival of it by enabling attachment to epithelial cells (Manco, *et al*., 2006).

1.2.8. Antibiotic resistance genes

The discovery of antibiotics was a revolutionary achievement for both human and veterinary medicine. Since their discovery, antimicrobials have cured humans and animals from bacterial infections (Byarugaba, 2010). Antimicrobials can be defined as any natural, synthetic or semisynthetic origin substance which kills or inhibits the growth of microorganisms (Giguère, 2013; Maartens *et al*., 2011). The antibiotics that kill bacteria are termed bactericidal while those that inhibit bacterial growth are termed bacteriostatic (Etebu and Arikekpar, 2016).

In contrast, antibiotic refers to a low molecular weight substance produced by microorganisms which act against another microorganism at low concentrations (Giguère, 2013; Maartens *et al.*, 2011). The term antibiotic has been used interchangeably with the term antimicrobial in many instances (Giguère, 2013). The first antibiotic was penicillin discovered by Alexander Fleming in 1928 and was first used therapeutically in the 1940s. However, treatment failures and bacteria resistant to penicillin were first noticed immediately after the discovery of penicillin (as reviewed in Aminov, 2010; Byarugaba, 2010; Ventola, 2015).

Antibiotic resistance is defined as the ability of microbes to resist the effects of drugs, as a result the drugs become ineffective to neither kill nor inhibit the microbes (CDC, 2015; WHO, 2014). Use of antibiotic can trigger the antimicrobial resistance by exerting selection pressure on bacterial strains (McDermott *et al.*, 2002). The emergence and rapid spread of antimicrobial resistance is now a global concerns (Laxminarayan *et al*., 2013; Ventola, 2015; WHO, 2014).

Resistance can be either intrinsic or acquired. Intrinsic resistance defines the lack of bacterial inherent structural or functional properties which is required

for the antimicrobial to act (Blair *et al*., 2015; Guardabassi and Courvalin, 2006; Mcdermott *et al.*, 2003). Intrinsic resistance is constitutive to bacterial genera or species without the requirement for the acquisition of novel genetic materials. An example of intrinsic resistance is the lipopeptide daptomycin which is effective against Gram-positive bacteria but is not effective against Gram-negative bacteria due to differences in cell membrane structure (Blair *et al*., 2015). Bacteria can acquire antimicrobial resistance either by chromosomal mutation or acquisition of resistance genes by horizontal transfer (Blair *et al*., 2015; Catry *et al*., 2003; Džidić *et al.*, 2008; Guardabassi and Courvalin, 2006; Ruppé *et al*., 2015).

Mutation in chromosomal genes can occur in a variety of ways such as spontaneous mutation, hypermutator and adaptive mutagenesis (Džidić *et al*., 2008). Horizontal gene transfer is the transfer of genetic material between individual bacteria of same species or different species. This mechanism of gene transfer is one of the important means of dissemination of antibiotic resistance genes among bacteria. Different transposable genetic elements such as plasmids, transposons, gene cassettes play an important role in carrying resistance genes and three major mechanisms by which bacteria transfer gene horizontally are conjugation, transformation and transduction (Drawz and Bonomo, 2010; Huddleston, 2014).

1.2.9. Antibiotic resistance of *S. pneumoniaee*

Antibiotic resistance is a global problem and is now gaining more attention worldwide (Laxminarayan *et al.*, 2013, Laxminarayan *et al.*, 2016). Resource poor countries, the challenge with antibiotic resistance is particularly worrying as unavailability of alternative drugs are often unaffordable by the majority of those affected. This frequently leads to delays in treatment that further worsen treatment outcomes. For *S. pneumoniae*, the problem of antibiotic resistance is of a particular concern due to the disproportionately heavy burden of pneumococcal disease and associated higher mortality rates in

developing countries in comparison to developed countries (O'Brien *et al.*, 2009).

Antibiotic therapy forms the mainstay of treatment of pneumococcal disease. Even though variations in antibiotic treatment offered across different clinical settings exist, the WHO recommends the use of penicillin as the first line antibiotic treatment (WHO, 2013). Recommendations for antibiotic treatment options in areas with high levels of proven penicillin resistance include the use of 3rd generation cephalosporins such as ceftriaxone and cefotaxime (Bradley *et al.*, 2011). A major concern with the use of antibiotics is the development of antimicrobial resistance. This is particularly worrisome with the pneumococcus, a naturally transforming organism, which has the ability to transfer and spread resistant genes between pneumococcal strains (Croucher *et al.*, 2011).

1.2.9.1. *S. pneumoniae* emergence of multidrug resistance

S. pneumoniae was first isolated in 1881 on opposite sides of the Atlantic Ocean by Louis Pasteur in France and George Sternberg in the United States in independent studies on saliva. It was originally described as Microbe septicemique de ´ la salive by Pasteur and as Micrococcus pasteuri by Sternberg (Watson *et al*., 1993). It was subsequently referred to as Pneumococcus and was renamed Diplococcus *pneumoniae* in 1920 (Winslow *et al*., 1920). It was finally given the name *S. pneumoniaee* in 1974 (Deibel and Seeley, 1974). It is the most commonly identified bacterial cause of community-acquired pneumonia, meningitis and otitis media. It is also a frequent cause of bacteraemia, and accounts for significant morbidity and mortality.

The highest incidence of pneumococcal disease is observed in children o 2 years and in adults 465 years of age. A recent review of many studies have shown that six to 11 serotypes of *S. pneumoniae* account for ≥ 70% of invasive pneumococcal disease in children (Johnson *et al.*, 2010). Treatment of these infections with antibiotics is increasingly becoming problematic due to

increased resistance to penicillin, macrolides and other antibiotics (Fuller *et al*., 2005; Jones *et al.*, 2010).

The first report of multidrug-resistant (resistant to more than three different antimicrobial classes) *S. pneumoniae* came from South Africa in 1978 (Jacobs *et al.*, 1978). Now between 15% and 30% of *S. pneumoniae* isolates are multidrug resistant (Lynch and Zhanel, 2009). Resistance too many of the drugs are due to genes present on conjugative transposons.

1.2.9.2. Mechanisms of resistance in *S. pneumoniae*

The evaluation of changes in resistance shown in table (1-1) is somewhat complicated, because the definitions of susceptibility and resistance have changed. Recently these definitions have been adjusted with respect to the site of infection (CLSI, 2015; EUCAST 2016).

1.2.10. Transposable elements

Transposable elements (TEs) shown in figure are DNA sequences that are capable of moving themselves to a new location in the host genome. There are two distinct mechanisms through which TEs can transpose.

One is the so called "copy-and-paste" mechanism, where the TE sequences in the host genome were first transcribed into RNA and then reversely transcribed to DNA and inserted back to a new genomic location in the host genome. This type of TEs is called retrotransposons. Another way through which the TE sequences jumps in the host genome is the "cut-and-paste" mechanism. The type of TEs is transposing through this mechanism is called DNA transposons (Lopez *et al.*, 2017) .

Table (1-1): Mechanisms of resistance in *S. pneumoniae*

Antibiotic resistance	Mechanism of resistance	Reference
Aminoglycosides	Low cell-wall permeability Aminoglycoside modification	(Faibis *et al*., 2003)
Beta-lactams and cephalosporins	Mosaic genes Point mutations	(Liu *et al*., 2016) (Cafini *et a*l., 2006) (Smith and Klugman., 2001)
Tetracycline	Ribosomal protection Mutations Overexpression of thiamine biosynthesis pathway	(Roberts and Mullany, 2009) (Luna and Roberts, 1988) (Lupien *et al*., 2015)
Macrolides	Ribosomal modification Efflux system Point mutation (A2062C) Point mutation (A2059G) Amino acid substitution or insertion	(Courvalin and Carlier, 1986), (Palmieri *et al*., 2012) (McDougal *et al*., 1998), (Gay and Stephens, 2001), (del Grosso *et a*l., 2007), (Santagati *et al*., 2000), Mingoia *et al*., 2014)

1.2.10.1. Nomenclature of conjugative transposons and integrative conjugative elements

The issue of nomenclature for these types of elements has been the subject of much lively debate. The originally discovered member of this family, Tn916 (Franke and Clewell, 1981), was termed a conjugative transposon and designated a Tn number according to the rules published for transposable elements (Campbell *et al*., 1977).

In 1999, Hochhut and Waldor published details of a 62-kb self-transmissible conjugative element from *Vibrio cholerae*, which encodes multiple antibiotic resistances (Hochhut and Waldor, 1999). This element, designated SXT, was called a CONSTIN, an acronym for a conjugative, self-transmissible, integrating element. This term is still used today although only in relation to the SXT element in *Vibrio sp*.

In 2008, after 2 years of discussions a consensus was generally agreed upon whereby the naming of new transposable elements of any family would be carried out using a reimplementation of the previous Tn system. The registry for Tn numbers,(Roberts *et al.*, 2008), now makes it possible to assign names in a logical way to any transposable element including those found in the increasingly large amounts of metagenomic data becoming available, for example Tn*6032* (Suenaga *et al.*, 2009).

It was also decided that the terms conjugative transposon and ICE were interchangeable (Roberts *et al.,* 2008; Wozniak and Waldor, 2010) and therefore an ICE could be assigned a number from the registry, for example ICE6013 (Smyth and Robinson, 2009). Alternatively, any transposable element that is also conjugative can be given the prefix CTn. This system now appears robust and able to cope with any newly identified transposable element for which there is not already a suitable nomenclature system in place.

1.2.10.2. Functional Roles of TEs

Functional Roles of TEs Followed by the initial discovery of TEs, the "selfish DNA" hypothesis seemed to hold when little was known about any function that these widely spread repetitive sequences encodes and how they could be involved in various basic biological processes that are necessary for the survival of their host. As genome parasites, it seems plausible that the only purpose of TEs in the host genome is simply to propagate themselves and colonize the host genome. However, accumulating evidences have been found to show that TE sequences have been widely recruited and integrated in the host genome regulatory machinery, providing functional regulatory elements **(**Feschotte., 2008).

In fact, TEs provide an abundant source of regulatory sequences in the host genomes (Feschotte, 2008; Rebollo, 2012).

1.2.11. Transposable elements in *S. pneumoniae*

Tn*1545* was initially discovered in *S. pneumoniae* strain BM4200. The element is essentially Tn*916* with an insertion of the erm(B) gene encoding macrolide, lincosamide and streptogramin (MLS) resistance and the kanamycin resistance gene aphA-3 (Cochetti *et al*., 2008). Many clinical strains of *S. pneumoniae* carry tet(M), which usually resident on Tn*916*/Tn*1545*-like elements, Eight out of the 36 pneumococcal genomes currently sequenced contain one of these elements (Santoro *et al*., 2010).

Since then, various Tn*916*/Tn*1545*-like elements have been detected and characterized in *S. pneumoniae* or found in other streptococci. Early work aimed at detecting Tn*1545*-like elements by the identification of the various resistance genes present in isolates by dot blot that showed a range of combinations of the genes present on Tn*1545* (Seral *et al*., 2001).

Additionally, 63 out of 65 *S. pneumoniae* strains showed the presence of intTn demonstrating, that elements from the Tn*916*/Tn*1545* group, shown in figure(1-3), were likely to be common in this organism (Montanari *et al.*, 2003). It is likely that this early work was in fact detecting some of the more recently characterized conjugative transposons such as Tn*6002* and Tn*6003*. Tn*6002* was initially characterized in *Streptococcus* cristatus from a clinical sample taken from a periodontal patient (Warburton *et al.*, 2007).

The element is essentially Tn*916* with an insertion in orf20. The insertion contains five genes, one of which is erm(B) conferring the MLS phenotype upon its host (Warburton *et al*., 2007). More recently, the complete sequence of the element has been published (accession number FJ11160), which shows it is >99% identical to Tn916 (Santoro *et al.*, 2010).

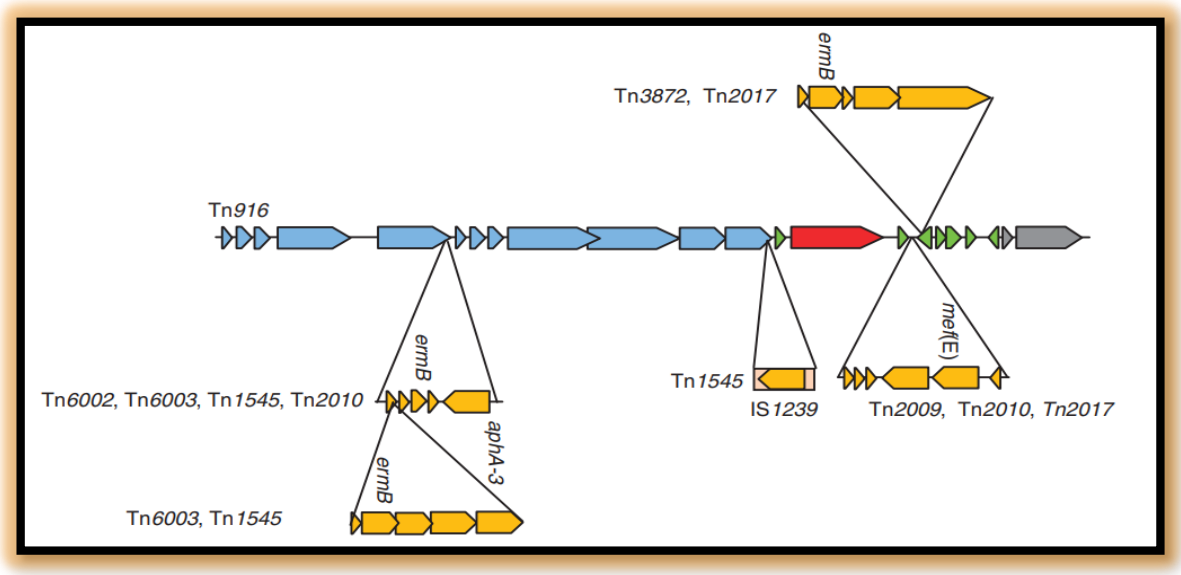


Figure (1-3): Schematic representation of the diversity of mobile elements associated with Tn916 in *S. pneumoniae* (Cochetti *et al.*, 2008).

The color coding for the functionality of the predicted proteins is as follows: blue, conjugation; green, transcriptional regulation; red, accessory genes (in the case of Tn*916* this is tetracycline resistance); grey, insertion and excision (recombination).The additional resistance genes are labelled vertically. The element in which each insertion is found are labelled near the insertion; note that some insertions are present in more than one element and some elements, for example Tn2010, contain more than one insertion. (Cochetti *et al*., 2008).

The emergence of antibiotic resistance among bacterial pathogens is a major problem in the treatment of infectious disease in both the community and in healthcare settings throughout the world. In industrialized nations, there has been a steady rise in the incidence of high-profile health care associated infections that have become resistant to one or more antibacterial agents making treatment increasingly difficult. These include, but are not limited to, methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and multidrug-resistant *S.pneumoniae* (Rossolini *et al.*, 2010).

In addition, there are pathogens, such as *Clostridium difficile*, which have risen to global prominence over the last few years and have the ability to acquire mobile genetic elements from enterococci, indicating the potential to acquire resistance to the last line of defense antibiotics, for example vancomycin and other glycopeptides (Cartman *et al.*, 2010; Jasni *et al.*, 2010). These resistances are commonly acquired on mobile genetic elements such as conjugative plasmids and conjugative transposons, which are capable of broad host range transfer between pathogens (Weigel *et al.*, 2003; Jasni *et al.*, 2010) and between commensal and pathogenic bacteria. For example, methicillin resistance, which is mediated by the product of the mecA gene and is present in MRSA strains, most likely originates from Staphylococcus fleurettii, an animal commensal (Tsubakishita *et al.*, 2010).

The Tn*916*/Tn*1545* family is responsible for a large proportion of the antibiotic resistance in these different pathogens. These conjugative elements are responsible for the dissemination of many antimicrobial resistance genes (usually resistance to tetracyclines, but also macrolides, lincosamides and streptogramins, kanamycin and mercury) to some of the most important Gram-positive pathogens.

S. pneumoniaee is the most commonly identified bacterial cause of community-acquired pneumonia, meningitis and otitis media. It is also a frequent cause of bacteremia, and accounts for significant morbidity and mortality. The highest incidence of pneumococcal disease is observed in children of 2 years and in adults 65 years of age. A recent review of many studies have shown that six to eleven serotypes of *S. pneumoniae* account for 70% of invasive pneumococcal disease in children (Johnson *et al.*, 2010).

Treatment of these infections with antibiotics is increasingly becoming problematic due to the increased resistance to penicillin, macrolides and other antibiotics (Fuller *et al.*, 2005; Jones *et al.*, 2010). The first report of multidrug-resistant (resistant to more than three different antimicrobial classes) *S.*

pneumoniae came from South Africa in 1978 (Jacobs *et al*., 1978) and now between 15% and 30% of *S. pneumoniae* isolates are multidrug resistant (Lynch and Zhanel, 2009). Resistance too many of the drugs are due to genes present on conjugative transposons.

1.2.11.1. The Tn*916*/Tn*1545* family of conjugative transposons

Originally discovered in the late 1970s in *E. faecalis*, Tn*916* was the first conjugative transposon encoding antibiotic resistance to be reported (Franke and Clewell, 1981). It is the smallest member of the Tn*916*/Tn*1545* family shown in figure (1-4), and contains 24 ORFs organized into functional modules involved in conjugal transfer, recombination (excision and insertion reactions), transcriptional regulation and accessory functions (antibiotic resistance) shown in figure (1-4a) (Roberts and Mullany, 2009).

Tn*916* has been detected in or transferred into over 35 different genera of bacteria, often in multiple species of a single genus (Clewell *et al*., 1995; Rice, 1998; Roberts and Mullany, 2009). Tn1545 is homologous to the entire Tn*916*; however, it also contains some important insertions: there is an insertion of an erm(B) and aphA-3 containing cassette within the 30 region of orf20 and a copy of IS1239 upstream of orf12 (Cochetti *et al.*, 2008).

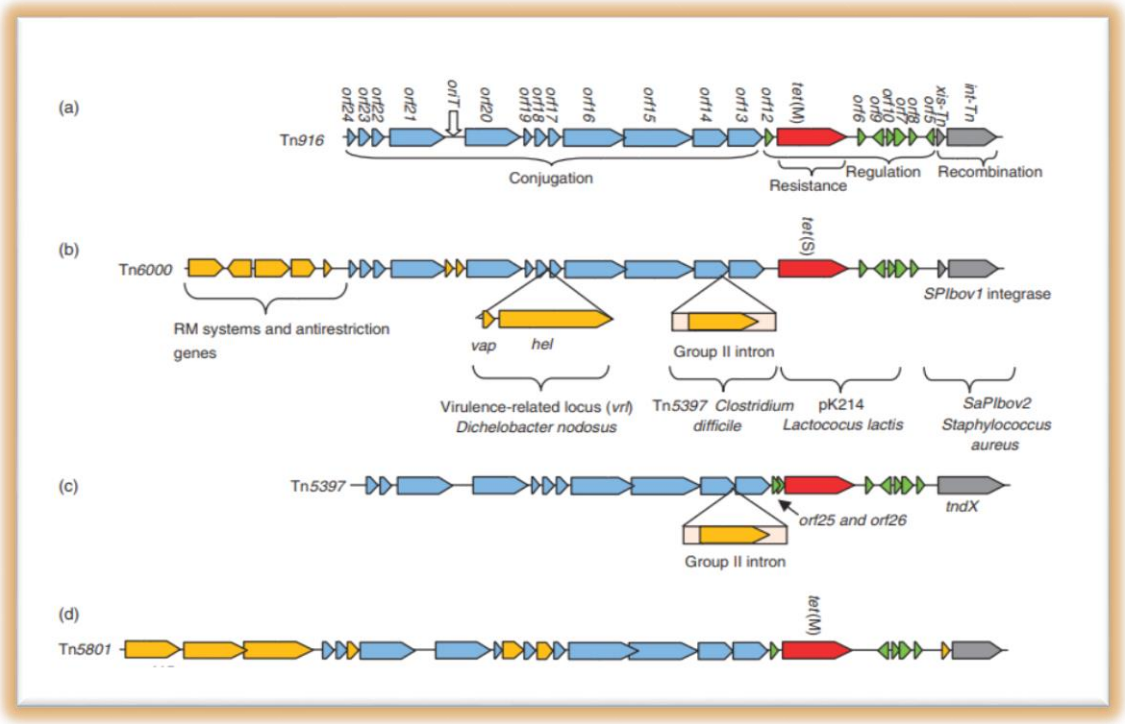


Figure (1-4):Schematic representation of Tn*916* and related elements (de Vries *et al.*, 2009) .

The arrows represent the individual orfs pointing in the probable direction of transcription and labelled above. The colour coding for the functionality of the predicted proteins is as follows: blue, conjugation; green, transcriptional regulation; red, accessory genes (in the case of Tn*916* this is tetracycline resistance); grey, insertion and excision (recombination). Genes not present in Tn916 are shown in orange on the other elements. (a) Tn*916*: the arrow points to the position of the experimentally determined oriT (Jaworski and Clewell, 1995). The modules are indicated by the brackets underneath and are labelled with the relevant function. (b) Tn*6000*: the major differences are the presence of restriction/modification genes before the conjugation module, the presence of an insertion of DNA that shares homology to the vrl of Dichelobacter nodosus and the integrase and excisionase genes that share more homology to staphylococcal pathogenicity island genes than they do to Tn*916* integrase and excisionase genes.

The closest homologues to various regions of DNA are indicated below to show the figure by brackets (adapted from Brouwer et al., 2010). (c) Tn*5397*: the major differences are the presence of tndX in Tn*5397* as opposed to xisTn and intTn in Tn*916*, the presence of a self-splicing group II intron in Tn*5397* and an 88-bp deletion in the regulatory region resulting in the removal of orf12 and the generation of orf25 and orf26. (d) Tn*5801* from Staphylococcus aureus: the major differences are the ORFs located before the conjugation module. Most of the additional genes are hypothetical; however, sav415 is predicted to encode a transposase (de Vries *et al*., 2009).

CHAPTER TWO
MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

2.1.1. Apparatus and Equipment

Apparatus and equipment with their companies and origins used in this study are listed in table (2-1).

Table (2-1): Apparatus and equipment and their companies and origins

Apparatus or Equipment	Company／ origin
Autoclave	Express／ Germany
Compound light microscope	Japan／ Olympus
Cooling centrifuge	Eppendorff／ Germany
Micro centrifuge	
Deep freeze	Japan / Sanyo
Eppendroff tube	Promega/ USA
Quantus florometer	
Electrophoresis unit	Mettle／ Switzerland
Filter papers	Whatman／England
Gel documentation system	Biorad / USA
Thermo cycler	
UV- Transilluminator	
Hood	Heraeus / Germany
Incubator	Memmert / Germany
Micropipette	Gallenkamp/ England
Oven	
Millipore filters	Sartorius Membrane/ Germany
pH- meter	Radiometer/ Denmark
PCR premix tubes	Promega / USA
Power supply	LKB /Sweden
Sensitive balance	Delta Range / Switzerland
Shaking incubator	GLF/ Germany
Water distiller	
UV-VIS- Spectrophotometer	Shimadzu / Japan
Vortex	England/Stuart scientific
VITEK-2	Biomeruex/ France
Water bath	Tafesa Hannover /Germany

2.1.2 Chemicals and Biological Materials

Chemicals and biological materials used in this study are listed in table (2-2).

Table (2-2): Chemicals and biological materials used in this study

Material	Company /Origin
Agar- Agar	Himedia / India
Agarose	Stratagene / USA
Absolute Ethanol	Romil pure chemistry / U.K.
Isopropanol	
Boric acid	BDH / England
Ethanol 96%	Local market / Iraq
Ethidium bromide	BDH
Glycerol	BDH
Gram stain	BDH
Hydrogen peroxide	Fluka /Germeny
Hydrochloric acid	Merck / Germeny
Inulin	BDH
Lactose	BDH
Manitol	BDH
Methylene blue	Himedia
Normal Saline (Sterile)	PSI / Saudi Arabia
Potassium hydroxide	Fluka / Switzerland
Raffinose	BDH
Ribose	Sigma / USA
Safranine	BDH
Sodium desoxycholate	Sigma
Sorbitol	Sigma
Sodium chloride	BDH
Tris –EDTA buffer	Sigma
Yeast extract	Himedia
Tetra-methyl-p-phenylenedihydrochloride	BDH / England

2.1.3 Antibiotics Discs

Antibiotic disks used in this study are indicated in table (2-5), these antibiotics were supplied by Bioanalyse/ Turkey**.**

Table (2-3): Antibiotic discs used in this study

Antibiotic	Symbol	Concentration (µg/ disc)	Inhibition zone		
			S	I	R
Azithromycin	AZT	15	≥18	14-17	≤13
Ciproflolxacin	Cip	5	≥22	22-30	≤30
Clindamycin	CD	2	≥19	16-18	≤15
erthromycine	E	15	≥21	16-20	≤15
Kanamycin	K	30	≥13	14-17	≤18
levofloxacine	LEV	5	≥17	14-16	≤13
Penicillin	P	10	-	-	-
Streptomycin	S	10	≥15	12-14	≤11
Tetracycline	TE	30	≥28	25-27	≤24
Trimethoprim	SXT	(1.25\23.75)	≥19	16-18	≤15

S = Sensitive, I = Intermediate, R = Resistance.

2.1.4 Ready- to- use Media

Ready to use media used in this are listed in table (2-4).

Table (2-4): Ready- to -use media

Medium	company	Origin
Blood agar base	Difco	England
Brain heart infusion agar	Oxoid	England
Brain heart infusion broth		
Muller- Hinton agar		
Nutrient agar		
Nutrient broth		

2.1.5 Kits

Kits used in this study are listed in table (2-5). They were obtained from promega ⁄USA.

Table (2-5): Kits used in this study

Kits
DNA extraction kit Wizard genomic DNA purification kit, Agarose, Ethidium bromide solution (10mg\ml), Go *Taq* green master mix, nuclease free water, TAE40X, Quantiflor DNA system.

2.1.6 Buffer solutions

2.1.6.1 Phosphate buffer solution (0.3 M)

It was prepared by dissolving 26.127g of K_2HPO_4 in 250 ml of D.W., pH was adjusted to 7.5, and then volume was completed to 500 ml with D.W. and sterilized by autoclaving.

2.1.6.2 TBE buffer solution 1X (Sambrook *et al.*, 1989)

This buffer solution was prepared by dissolving 54g of Tris-base, and 27.5 g of boric acid in 20 ml of 0.5M EDTA, pH was adjusted to 8, then volume was completed to 100 ml with D.W and sterilized by autoclaving.

2.1.6.3 TE Buffer (Sambrook *et al.*, 1989)

This buffer solutionis consisted of the following components:

Component	Concentration (M)	Volume (ml)
Tris-HCl	1	100
EDTA	0.5	20
Distilled water	_	880

pH was adjusted to 8.6 ,and sterilized by autoclaving .

2.1.7 Solutions

2.1.7.1 Normal saline solution (0.85%)

This solution was prepared according to Atlas (1995) by dissolving 8.5 g of sodium chloride in 750 ml of D.W., pH was adjusted to 7.0, then volume was completed to 1L with D.W and sterilized by autoclaving.

2.1.7.2 Carbon sources solutions (1%)

Carbon sources solutions used in fermentation tests were prepared according to Atlas (1995) by dissolving, individually, 1 g of each (Inulin, mannitol, sorbitol, trehalose and lactose) in 100 ml of D.W. All solutions were sterilized by filtration.

2.1.7.3 Lysozyme solution (Maniatis *et al.*, 1982)

Lysozyme solution was freshly prepared by dissolving 20 mg of lyophilized lysozyme in 1 ml of sterilized D.W until clear or slightly hazy colourless solution was formed, then solution was stored at -20°C until use.

2.1.7.4 Potassium hydroxide solution (40%)

It was prepared according to Brown (2005) by dissolving 40 g of potassium hydroxide in 100 ml of D.W, and stored at 4°C until use.

2.1.7.5 Bile salt solution (McFadden, 2000)

Bile salt solution (40%) was prepared by dissolving 40g of bile salt (sodium deoxycholate) in 100ml of D.W. then the solution was kept at 4°C until use.

2.1.7.6 Ethidium bromide solution (1%)

It was prepared according to Maniatis *et al.* (1982) by dissolving 1 g of ethidium bromide in 100 ml of D.W with careful stirring until complete dissolving, then solution was filtered through Whattman No.1 filter paper and stored in a dark bottle at 4°C.

2.1.8 Reagents

- **Catalase reagent:** This reagent was prepared to be consisting of 3% hydrogen peroxide.

- **Oxidase reagent (Garrity, 2001):** This reagent was prepared by dissolving 1g of tetramethyl-P-phyenylenediamine in 100 ml of D.W, and then it was kept in a dark bottle, and stored at 4°C until use.

2.2 Methods

2.2.1 Samples collection

In order to isolate *S. pneumoniae*, a total of 100 sputum samples were collected from patients suspected to be infected with pneumonia. Cerebrospinal fluid samples were also collected from patients attending different hospitals in Baghdad including Al-Yarmouk teaching hospital, Central Child Teaching Hospital and Medical City Teaching Hospital during the period from November, 2017 to April, 2018. Swabs of sputum samples were taken from each case study and kept in sterile tubes containing 2ml of normal saline; then tubes were put in a cool box and transferred to the laboratory of the college of Biotechnology within few hours after collection.

2.2.2 Preparation of Media

2.2.2.1 Ready to use media

All media mentioned in item (2.1.4) were prepared as recommended by the manufacturing companies and sterilized by autoclaving.

2.2.2.2 Laboratory- prepared media

- **Blood agar medium (Collins and Lyne, 1985)**

This medium was prepared by dissolving 33 g of blood agar base in 950 ml of D.W, pH was adjusted to 7.0 and sterilized by autoclaving. After cooling to 45°C, 50ml of sheep blood sterilized by filtration was added, mixed well and poured into sterile petri dishes.

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- **Chocolate agar**

Chocolate agar is a medium that supports the special growth requirements (Hemin and NAD^+) needed for the isolation of fastidious organisms, when incubated at 35-37 °C in a 5% CO_2 atmosphere. This medium prepared the first by heat lyse human blood by slow heating at 56°C in a water bath for 1 min . After cooling, 50 ml of lysed sheep blood was added under aseptic conditions to 950 ml of chocolate agar medium sterilized by autoclaving, mixed gently and poured in sterilized petri-dishes, and then plates were kept in plastic bags and stored at 4°C until use.

- **Sugar fermentation medium (Brown 2005)**

This medium was prepared to consisted of the following components:

Component	Weight (g)
Tryptone	10
Yeast extract	5
Bromothymol blue	2

All components were dissolved in 990 ml of distilled water and, pH was adjusted to 7.0 and sterilized by autoclaving. After cooling, 10 ml of each sugar solution (1%) was added, individually, mixed well and dispensed into sterile test tubes.

2.2.3 Isolation of *S. pneumoniae*

Sputum samples collected from each case study were cultured individually by streaking a loopfull or swab of undiluted sputum sample on blood agar medium, then subculturing was repeated several times for purifying bacterial isolates to obtain single colonies before further identification steps. Another loopfull of each sample was streaked on brain heart infusion agar to obtain more of types of microorganisms that may be presented in same specimen.

2.2.4 Identification of *S. pneumoniae*

2.2.4.1 Morphological and cultural characteristics

Bacterial isolates able to grow on different media were identified by studying their morphological characteristics, including staining characterization, under light microscope in addition to the characterization of their colonies including , size, shape, edge, color and transparency (Harley and Prescott, 1996).

Staining ability of bacterial isolates was achieved according to Macfaddin (2000).

2.2.4.2 Optochin test (Macfaddin, 2000)

Discs of filter paper containing 5µg of optochin (ethylhydrocuprein) were placed on the surface of blood agar plate inoculated (by spreading) previously with fresh culture of each bacterial isolate suspected to be *S.pneumoniae*, then plates were incubated at 37°C under 5-10 % CO_2. Growth inhibition of the suspected isolates in a zone extending radialy for at least 5 mm from the margin of the disc indicates a positive result.

2.2.4.3 Biochemical tests

The following biochemical tests were performed for the identification of suspected isolates as follows:

2.2.4.3.1 Catalase test (Atlas *et al.*, 1995)

This test was performed by adding few drops of hydrogen peroxide solution (3%) over a single colony of each bacterial isolate spread on a glass slide. Production of gaseous bubbles indicates a positive result.

2.2.4.3.2 Oxidase test (Atlas *et al.*, 1995)

This test was performed by transferring a single colony of each bacterial isolate using a sterile wooden stick applicator on a filter paper containing

oxidase reagent. Changing the colony color to dark purple within few minutes indicates a positive result.

2.2.4.3.3 Blood Hemolysis test (Forbes, 2002)

Fresh culture of bacterial isolates were streaked on blood agar medium , and incubated at 37°C for 24hs. Presence of hemolysis zones around each bacterial colony indicates a positive result.

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2.2.4.3.4 Sugar fermentation test (Brown, 2005)

This test was used to examine the ability of bacterial isolates to produce acid from sugar fermentation by inoculating test tubes containing 5ml of carbon source (inulin, mannitol, sorbitol, trehalose and lactose) individually with 100 µl of fresh culture of each bacterial isolate and incubated at 37 °C for 7 days. Presence of a yellow color indicates a positive result.

2.2.5 Identification by VITEK-2 system

VITEK-2 system (Biomeruex, 2010**)** was developed to confirm the identification of bacterial isolates. The card used in VITEK-2 was specific for Gram positive bacteria. Cell suspension of fresh culture of each bacterial isolate was centrifuged at 8000 rpm for 5 minutes, then pelleted cells were re-suspended in 0.3 ml of normal saline and the turbidity of cells suspension was adjusted to 0.50 -0.63 nm, then kits were loaded with cells suspension of bacterial isolates and placed into VITEK-2 apparatus and the results were obtained after 6 hours.

2.2.6 Sterilization methods (Atlas *et al*., 1995)

- **Autoclaving**

All culture media, solutions and reagents were sterilized by autoclaving at 121ºC (15 Ib/In^2) for 15 min.

- **Dry-heat sterilization**

Dry heat sterilization was performed by using electric oven at 180 °C for 3 hours to sterilize different glassware.

- **Filtration**

Heat sensitive solutions were sterilized by filtration using Millipore's filter unit (0.22 μm).

2.2.7 Maintenance of bacterial isolates

Maintenance of bacterial isolates was preformed according to Maniatis *et al.* (1982) as follows:

- **Short -term storage**

Bacterial isolates were maintained for a few weeks on nutrient agar plates. The plates were tightly warped with parafilm, and stored at 4 °C until use.

- **Med- term- storage**

Bacterial isolates were maintained by stabbing nutrient agar medium in a small screw- capped bottles then stored at 4 ºC until use.

2.2.8 Antibiotic susceptibility test

Susceptibility of bacterial isolates to different antibiotics was examined according to the standard disk diffusion method (CLSI, 2000) as follows:

A. A volume of five ml of sterile brain heart infusion broth was inoculated with 0.1 ml of fresh culture of each bacterial isolate and incubated at 37 °C for 4hrs in a shaker incubator (100 rpm), then serial dilutions were prepared, and 0.1 ml of the fourth dilution (10^{-4}) was spread on Muller-Hinton agar plate in different three planes by rotating the plate approximately 60° each time to obtain an even distribution of the inoculum.

B. Plates were then placed at room temperature for 30 min. to allow the absorption of excessive moisture, and then antibiotic disks were placed on

the surface of the medium (5 discs/ plate) and incubated at 37°C for 24hrs.

C. After incubation, diameters of the inhibition zones were measured and compared with the standards of the National Committee for Clinical Laboratory Standards (CLSI, 2000).

2.2.9 Amplification of antibiotic resistance genes

2.2.9.1 Primers

Antibiotic resistance genes conferred by transposable elements were amplified by using specific primers indicated in table (2-6). All Primers were provided in lyophilized form, and were dissolved in sterilized distilled water to give a final concentration of 10 picomole /µl.

Table (2-6): Specific primers of amplification of antibiotic resistance genes

Primer Name	Sequence (5`→3`)	T_m (°C)	**Product Size(bp)**	**References**
J12	CCCATTGAAGACGCAGAAGT	60	801	(Olsvik *et al.*, 1995)
J11	AAAAATCCCTACCGCACT	52		
APHA1	GCCGATGTGGATTGCGAAAA	60	292	(Cochtti *et al.*, 2007)
APHA2	GCTTGATCCCCAGTAAGTCA	60		
ERMB1	GAAAAGGTACTCAACCAAATA	56	639	(Sutcliffe *et al.*, 1996)
ERMB2	AGTAACGGTACTTAAATTGTTTAC	62		
TETM2	GAACTCGAACAAGAGGAAAGC	62	740	(Jos *et al.*, 1993)
TETM3	ATGGAAGCCCAGAAAGGAT	56		
int-for	GCGTGATTGTATCTCACT	52	1046	(Doherty *et al.*, 2000)
int-rev	GACGCTCCTGTTGCTTCT	56		
xis-for	AAGCAGACTGAGATTCCTA	54	194	(Amezaga *et al.*, 2002)
xis-rev	GCGTCCAATGTATCTATAA	52		

2.2.9.2 PCR Master Mix

Polymerase chain reaction (PCR) master mix supplied by promega\USA was prepared to be consisting of the following components:

Compound	Concentration
PCR buffer (PH=8.5)	2X
$MgCl_2$	3 mM
dNTPs	400 mM
Taq DNA polymerase	5 units

2.2.9.3 Agarose Gel Electrophoresis

After PCR amplification was performed, agarose gel electrophoresis was adopted to confirm the presence of amplified products. PCR was completely dependable on the extracted DNA criteria.

- **Preparation of agarose gel**

A weight of 1 g (for 1%) agarose was dissolved in 100 ml of TAE (1X) by heating to boiling temperature in a microwave oven until complete dissolving of gel particles. After cooling to 55°C, 1 µl of ethidium bromide solution (1mg/ml) was added to agarose gel and mixed gently.

- **Casting of the horizontal gel**

Agarose gel solution was poured into the gel tray after sealing of both edges with cellophane tape, then agarose was allowed to solidify at room temperature for 30 minute. The comb was carefully removed and the gel was placed in the gel tray, then tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

2.2.9.4 Extraction of Genomic DNA

Genomic DNA was isolated from bacterial isolates according to the protocol of Wizard Genomic DNA Purification Kit, Promega as in the following steps:

- An overnight culture of each bacterial isolate was pelleted at 13000 rpm for two minutes, then supernatant was discarded.
- Pellet was suspended in 100µl of DNA rehydration solution, and then 100 µl of lysozyme solution was added and mixed by vortixing.
- Microcentrifuge tubes were incubated at 37°C for 30 min with occasional shaking.
- After incubation, tubes were centrifuged at 13000 rpm for 2 minutes, and then supernatant was discarded.
- Pellet was resuspended in 600µl of lysis solution and mixed well by pipetting.
- Microcentrifuge tubes were incubated at 80 °C for 5 minutes, and then cooled to room temperature.
- For RNA lysis, 3µl of RNase solution was added to each tube, mixed gently, and incubated at 37 °C for 15 minutes.
- Aliqoute of 200µl of protein precipitation solution was added to cell lysate in each microcentrifuge tube, mixed by vortexing and incubated at -28 for 10 min.
- DNA solution was transferred to a new microcentrifuge tube containing 600µl isopropanol, tubes were mixed gently, then centrifuged at 13000 rpm for 2 minutes.
- After centrifugation, supernatant was decanted, then 600µl of ethanol (70%) was added, mixed by vortexing and centrifuged at 13000 rpm for 2 minutes.
- After centrifugation, ethanol layer was decanted by inverting, and the pellet was rehydrated in 100µl of rehydration solution for one hour at 65°C.

2.2.9.5 Quantitation of DNA concentration

Concentration of DNA solutions was measured according to Maniatis *et al*. (1982) using quantus, by adding 2µl of DNA solution into the photocell of the apparatus, then results of DNA concentration (ng/µl) were recorded and plotted automatically.

DNA concentration (µg/ml) = O.D. 260 nm x 50x Dilution factor

Pure DNA was obtained when the ratio between absorbance at 260 nm to absorbance at 280 nm was equal to 1.8 - 2.0.

2.2.9.6 Optimization of PCR program

Optimum conditions for implication of each transposable element was described in table (2-7) and (2-8).

Table (2-7): Polymerase Chain Reaction (PCR) program for amplification of J12, APHA1, ERMB1, int, xis transposable elements

Step	Temperature (°C)	Time (min: sec)	No. of cycles
Initial Denaturation	95	5 min.	1
Denaturation	95	30 sec.	30
Annealing	55	45 sec.	
Extension	72	45 sec.	
Final extension	72	7 min.	1

Table (2-8): Polymerase Chain Reaction (PCR) program for amplification of TETM2 transposable elements

Step	Temperature (°C)	Time (min: sec)	No. of cycles
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:45	
Extension	72	00:45	
Final extension	72	07:00	1

CHAPTER THREE
RESULTS AND DISCUSSION

3- Results and Discussion

3.1 Isolation of bacterial isolates

Swab samples from pharyngitis, tonsillitis and cerebrospinal fluid samples were collected from patients suffering from respiratory tract infections who attended Al-Yarmouk Teaching Hospital, Central Child Teaching Hospital and the Baghdad Teaching Hospital in Baghdad. From these samples, a total of 100 bacterial isolates were obtained after culturing on blood agar and Chocolate agar plates. These bacterial isolates were maintained on chocolate agar medium to enhance bacterial growth when using in further, In fact, this medium comprised hemoglobin that provides hemin (X factor), and NAD (V factor) inside red blood cells which is required for growth of fastidious bacteria.

3.2 Identification of bacterial isolates

Bacterial isolates collected from patients were first grown on blood agar to detect the type of hemolysis. Among the total 100 isolate, only 15 isolates produced zones of α-hemolysis around their colonies. This type of hemolysis differentiates *S.pneumoniae* from other streptococci which give usually β-hemolysis on blood agar.

The 15 isolates were streaked on blood agar and then optochin discs were fixed in the center of each petri plate before incubation for 24 hours at 37°C in CO_2 incubator. Results showed that all the suspected isolates were sensitive to optochin with inhibition zones above 14 mm in diameter; they were regarded as *S. pneumoniae*. Furthermore, results illustrated in figure (3-1) showed that the 15 gave a positive results for the occurrence of *S. pneumoniae* according to the cultural, morphological and biochemical characteristics, According to these results, only the 15 isolates of *S.pneumoniae* were selected for using in further studies which include antibiotic susceptibility and detection of transposable elements conferring antibiotic resistance.

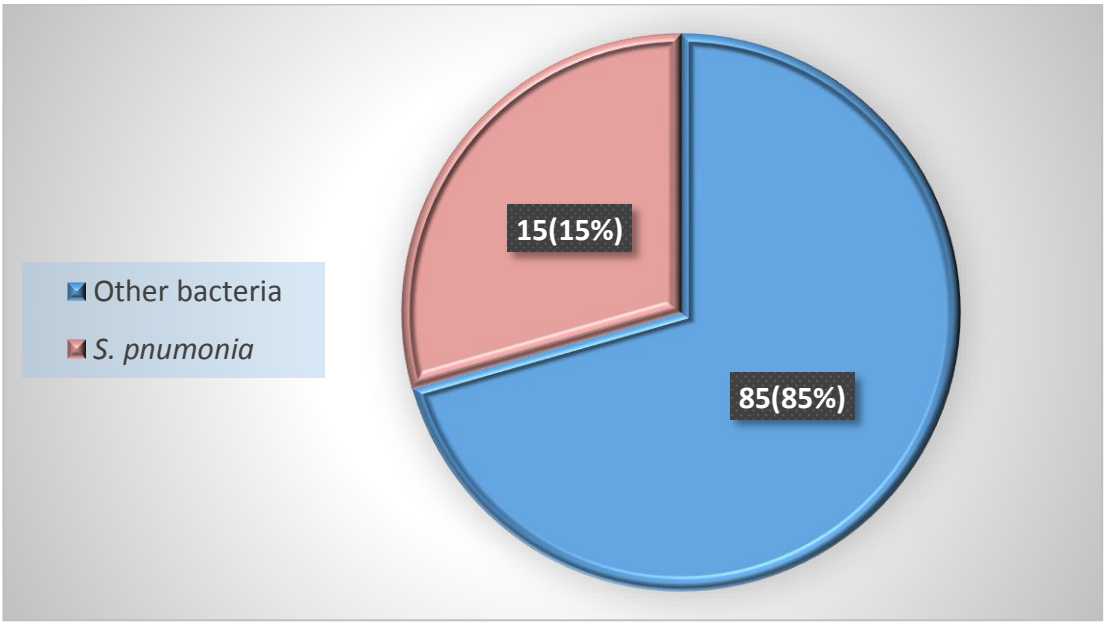

Figure (3-1): Numbers and percentages of bacterial isolates gave positive results for the occurrence of suspected *Streptococcus pneumoniae*.

3.2.1 Cultural and Microscopic characterization

Bacterial isolates able to grow on differential media were further identified by studying their cultural and morphological characteristics; Result of showed that these isolates were Gram positive lanceolate shaped diplococcic. Colonies of the suspected isolated appeared as small, grey, moist (sometime mucoidal) after culturing on blood agar medium. These results are coincident with the characterics *Streptococcus* spp according to Keith *et al.*, (2006).

3.2.2 Biochemical tests

Bacterial isolates suspected to be *Streptococcus spp* were identified according to their biochemical characteristics. Results indicated in table (3-2) showed that these isolates were negative for catalase and oxidase, able to ferment mannitol, lactose, inulin and trehalose sugars and produce alpha hemolysin on blood agar, while they not able to ferment sorbitol, According to these results, such isolates were identified as *S.pneumoniae*.

Table (3-1): Biochemical characteristics of *S. pneumoniae*

Test	Isolate symbol														
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D113	D14	D15
Oxidase	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
catalase	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
type of Hemolysis	α	α	α	α	α	α	α	α	α	α	α	α	α	α	α
Acid production from															
inulin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
mannitol	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sorbitol	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Trehalose	+	+	+	+	_	+	+	+	+	_	+	+	+	+	+

(+): Positive result (-): Negative result

3.2.3 Identification of bacterial isolates by Vitek-2:

Vitek-2 was considered the best equipment for identifying all types of bacteria cause different diseases in a short period with high accuracy. To confirm the identification of bacterial isolates as *S.pneumoniae*, biochemical characteristics of these isolates were also examined by using VITEK-2. Result indicated in appendix (1) showed that these isolates were positive for acid production from lactose, galatose,ribose, and trehalose, while they were negative for acid production from sorbitol, mannose, rafinose and salicin. They were also urease negative and sensitive to optochin. According to these results, these 15 isolates were confirmed as *S. pneumoniae*.

3.3 Antibiotics susceptibility of *S. pneumoniae*

Treatment of *S. pneumoniae* has become difficult owing to the global rise in the prevalence of antibiotic resistance, particularly against first-line antibiotics such as erythromycin and penicillin (Xu *et al.*, 2010**).**

Antibiotic susceptibility of bacterial isolates was examined on muller-hinton agar. Results illustrated in table (3-2) showed that multi-drug resistant was spread between the local isolates of *S. pneumoniae* as they gave different resistant patterns to these antibiotics. Resistance and multi resistance pattern has been reported in several studies, mostly with one or more of the following agents: penicillin, erythromycin and sulfamethoxazoletrimethopri. These result agree with Bingen., (2012) who found that *S. pneumoniae* have intrinsic resistance to a large group of antibiotics, including polypeptides, aminoglycosides and first-generation quinolones.

Results indicated in table (3-3) showed that most of the bacterial isolates (93%) were resistance to penicillin, streptomycin and trimethoprim (87%), tetracycline and trimethoprim (80%), clindamycin (73%), then to kanamycin (50%), erythromycin and azithromycin (40%), ciproflaxin and levofloxacin (20%).

Results also showed that the most resistant isolators are D1 ,D3, D5, D6, D9 and D15 as they showed resistance to all antibiotics used in this study , by D2 and D10 as they are resistant to nine antibiotics (90%), D7 (80%), D4, D8, D11 and D12 (40%), D14 (30%), then D13 (10%).

A study conducted between 2004 and 2006 in United Arab Emirates reported that the antibiotic resistance to penicillin (43 %), erythromycin (31 %), clindamycin (23 %) and tetracycline (18.6 %), among *S. pneumoniae* isolated from patients. In 2016, surveillance reports showed an increase in antibiotic resistance rates, especially to penicillin and erythromycin (Chamoun *et al.*, 2016). While in the most recent survey *S.pneumoniae* of isolates recovered from five Turkish medical centers between 2011 and 2013 showed that, 61.9 % were resistant to penicillin, 48.9 % to erythromycin and 35.1 % to clindamycin, while only 1.8 % was resistant to levofloxacin (Soyletir *et al.*, 2016).

Table (3-2): Pattern of antibiotic susceptibility of *S. pneumoniae* isolates

Isolates No.	Azi	cip	CD	E	K	LEV	P	S	T	TM
D1	R	R	R	R	R	R	R	R	R	R
D2	R	S	R	R	R	R	R	R	R	R
D3	R	R	R	R	R	R	R	R	R	R
D4	S	R	R	S	S	S	R	S	S	R
D5	R	R	R	R	R	R	R	R	R	R
D6	R	R	R	R	R	R	R	R	R	R
D7	R	S	R	R	R	S	R	R	R	R
D8	S	S	S	S	R	S	R	R	R	S
D9	R	R	R	R	R	R	R	R	R	R
D10	R	S	R	R	R	R	R	R	R	R
D11	S	S	S	S	S	S	R	R	R	R
D12	S	S	S	S	S	S	R	R	R	R
D13	S	S	R	S	S	S	S	S	S	S
D14	S	R	S	S	S	S	R	R	S	S
D15	R	R	R	R	R	R	R	R	R	R

- Resistance; S: Sensitive; AZI:azithromycin;CIP:ciproflaxin;
- CD:clindamycin;E:erythromycin;K:kanamycin;LEV:levofloxacine;
- P:pencillin;S:streptomycin;T:tetracycline;TM:Trimethoprim.

Increasing trend of antimicrobial resistance in bacteria that cause infectious diseases is a global problem, although resistance significantly varies between geographical regions. Recentelly, common bacterial pathogens can be resistant to all known antimicrobial agents (Mediavilla *et al.*, 2016; Skov and Monnet, 2016).

Table (3-3): Susceptibility pattern of the *S. pneumoniae* isolates grown on Muller Hinton agar plates and incubated at 37º C for 24 hours

Antibiotic	Susceptibility	
	Resist No. (%)	Sensitive No. (%)
Azithromycin	9 (60)	6 (40)
Ciproflaxin	8 (53)	7 (47)
Clindamycin	11(73)	4 (27)
Erythromycin	9 (60)	6 (40)
Kanamycin	10 (67)	5 (33)
Levofloxacin	8 (53)	7 (47)
Penicillin	14 (93)	1 (7)
Streptomycin	13 (87)	2 (13)
Tetracycline	9 (60)	6 (40)
Trimethoprim	13 (87)	2 (13)

Identification of antibiotic resistance genes provides valuable information; however, knowledge about their association with mobile genetic elements is crucial for the assessment of the risk for acquisition and dissemination of antimicrobial resistance.

Transposable elements can be distributed on both chromosomes and plasmids, and are able to interact by a recombination between elements and/or by transposition into other elements, forming all kinds of novel chimeric structures (Li *et al.*, 2011).

There is a variety of transposons conferring tetracycline resistance. They are most often associated with tetracycline resistance, carry the tet(M) gene and belong to the Tn*916* transposon family (Roberts and Mullany, 2011). They can mediate resistance to tetracycline by three different mechanisms: ribosomal protection, efflux and enzymatic inactivation of the active compound (Nguyen *et al.*, 2014).

Resistance to commonly prescribed antibiotics was found for azithromycin (26.3%), clindamycin (18.4%), erythromycin (21.1%), trimethoprim (78.9%) and tetracycline (15.8%), while all the isolates were sensitive to ceftriaxone in Thailand (Thummeepak *et al.*, 2015).

3.3 Genetic study

3.3.1. Isolation of genomic DNA

Genomic DNA of each bacterial isolate was extracted by using DNA extraction kit (promega\USA). Results illustrated in figure (3-2) showed that DNA bands appeared after extraction from each *S.pneumoniae* isolate, and electrophoresis on agarose gel (1%). Sharp DNA bands reflect the efficiency of the extraction procedure by using Wizard Genomic DNA Purification Kit, Promega. Results also showed that the concentration of genomic DNA was ranged between 7-20 ng/µl, as indicated in appendix (2).

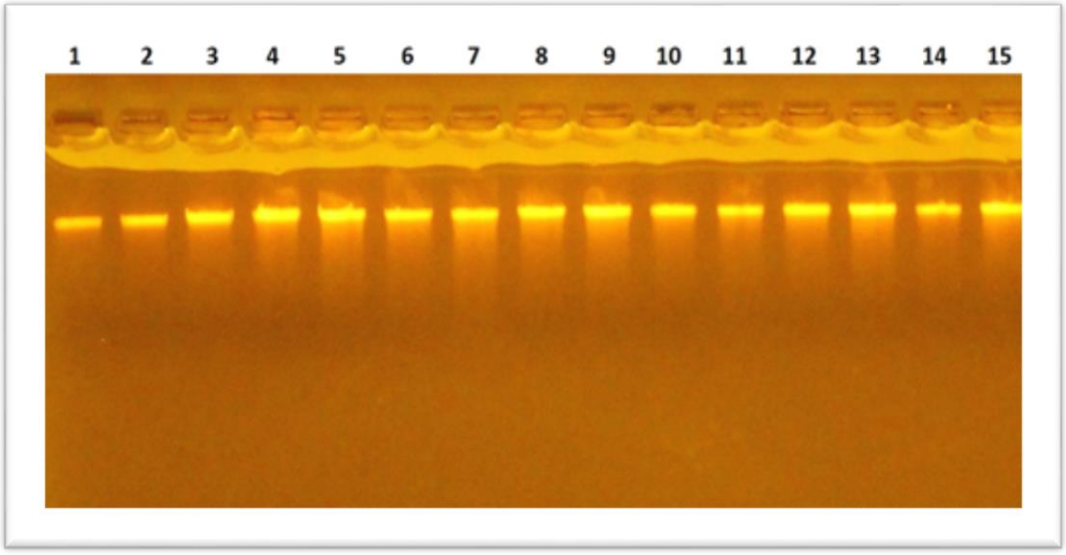

Figure (3-2): Genomic DNA of *S.pneumoniae* isolates after electrophoresis on agarose gel (1%) for 1 hour.

3.3.3. Detection of transposable elements in *S.pneumoniae* isolates

3.3.3.1. Tn*1545*

Tn*1545* is a conjugative shuttle transposon detected in multi-resistant clinical isolates of *S. pneumoniae*, and was carrying the erythromycin gene

encoding coresistance to macrolide,lincosaide and streptogramin B antibiotic (Courvalin and carlier, 1987).

Erythromycin gene of Tn*1545* was amplified by using specific primers indicated in table (2-4).Results illustrated in figure (3-3) showed that an amplified product of 740 bp appeared after electrophoresis on agarose gel (1%) represents erythromycin gene of Tn*1545*; This transposable element was detected in seven isolates of *S. pneumoniae* out of the total 15 isolate, In these isolates, Tn*1545* may be a chromosomal copy or may be located on plasmid DNA. These isolates were symbold D1, D2, D3, D6, D7, D9, and D15. All these isolates are resistant to erythromycin, and this result explain the erythromycin resistant phenotype of these seven isolates that arise from Tn*1545.*

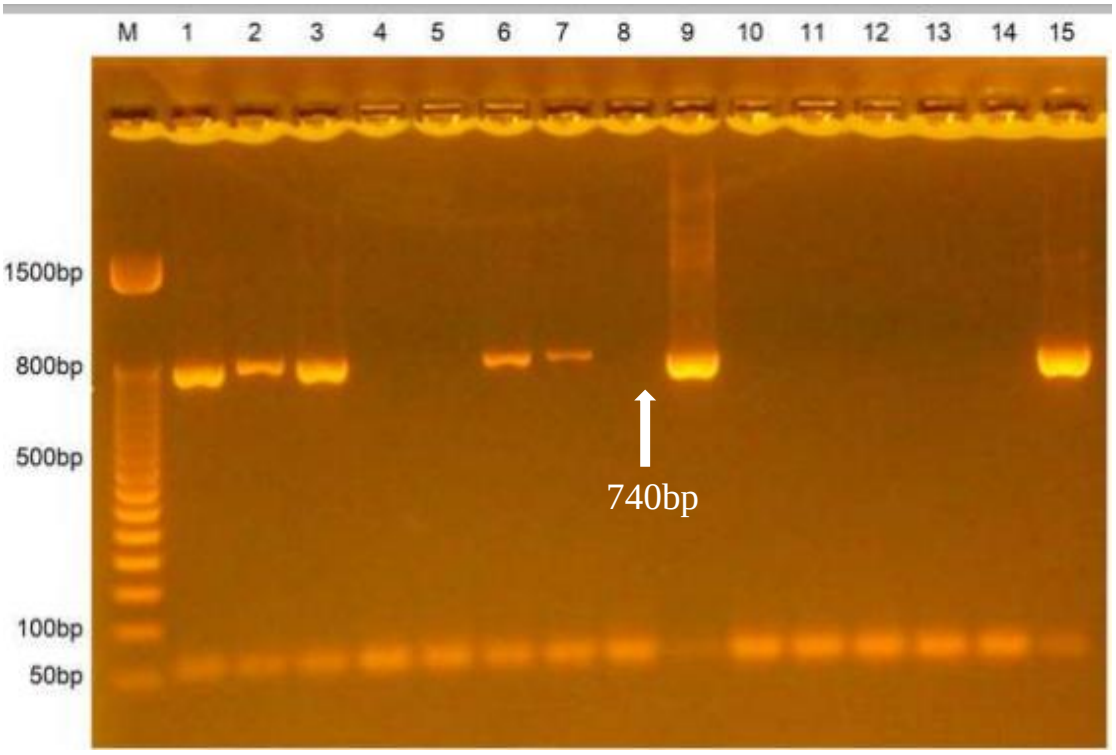

Figure (3-3): Erythromycin gene of Tn*1545* detected after amplification of genomic DNA for *S. pneumoniae* isolates and electrophoresis on agarose gel (1%) for one hour.

Lane (M): Ladder marker

Lane (1-15): Bacterial isolates of *S. pneumoniae*

Mean white, results indicated in table (3-2) show that there are other two erythromycin resistant isolates (D5 and D10) that possess a chromosomal or plasmid copy of erythromycin resistance gene, or may be due to other structural erm gene carried by other type of transposable elements presents in these bacterial isolates. Furthermore, there are six isolates of *S. pneumoniae* (D4, D8, D11, D12, D13, and D14) were sensitive to erythromycin among the total resistant isolates (9 isolates) and were unable to grow on enrichment medium containing this antibiotic.

Nucleotide sequence of erythromycin gene in Tn*1545* was illustrated in figure (3-4). Alignment of these sequences specified for *S.pneumoniae* isolates (Query) with transposon sequences of *S.pneumoniae* standard strains recorded in NCBI (subject) are illustrated in figure (3-5).Results of alignment showed that erm gene sequence of Tn*1545* in these seven bacterial isolates was identical (100% identity) with chromosomal erythromycin gene sequences in different standard strains of *S. pneumoniae* and with genomic erythromycin resistance gene in other standard strains of this bacterium .which supports the results concluded in this study that the seven isolates of *S.pneumoniae* are harboring chromosomal copy of Tn*1545* conferring erythromycin resistance.

High-level resistance to aminoglycoside can also occur through the acquisition of the mobile genetic elements Tn*1545* or Tn*6003*, which were identified in the chromosome of a pneumococcal strain in 1986 (Courvalin and Carlier ,1986 ; Cochetti *et al* ., 2007),respectively. Tn*1545* was shown to carry resistance determinants for three different antimicrobial families: 3'-aminoglycoside phosphotranferase type III (aphA-3), which was reported for the first time in 1984 (Collatz *et al.*, 1984), erythromycin ribosome methylase B (*erm*B) and the tetracycline resistance protein (*tet*M), which confers a high level of resistance to aminoglycosides, macrolides and tetracycline, respectively (Courvalin and Carlier, 1986).

1	CATCAACACATCGAGGTCAGTCTGAACTTTGCGGAAAAG	40
41	TTTTCAAAATTGAGTATTCGGAAAAAAGACAGCGTC TTG	80
81	CATATATA CGTCTTTATAGTGGCGTACTGCATTTGC GAG	120
121	ATTCGGTTAGAATATCGGAAAAGGAAAAAATAAAAATTA	160
161	CAGAAATGTATACTTCAATAAATGGTGAATTATGTAA AA	200
201	TCGATAAGGCTTATTCCGGGGAAATTGTTATTTTGCA G A	240
241	ATGAGTTTTTGAAGTTAAATAGTGT TCTTGGAGATAC AA	280
281	AGCTATTGCCACAGAGAGAGAGAATTGAAAATCCC C T C	320
321	CTCTGCTGCAAACGACTGTTGAACCGAGCAAACCTCAAC	360
361	AAAGGGAAATGTTACTTGATGCACTTTTAGAAATCTC CG	400
401	ACAGTGACCCGCTTCTGCGATATTATGTGGATTCTGC G A	440
441	CAC ATGAAATCATACTTTCTTTCTTAGGGAAA GTACAAA	480
481	TGGAAGTGACTTGTGCTCTGCTGCAAGAAAAGT ATC ATG	520
521	TGGAGATAGAAATAAAAGAGCCTACAGTCATTTAT ATG G	560
561	GGAGTAAAAGACATTTTACTAGAGCTATTCAATCGC A TT	600
601	ATTGGTGCTTAAATAAAACCGTTCTTTTGTGGA ATATA A	640
641	GTGGTTTTCTTATGTTCCGCAAAGGAATGGTACACCA A A	680
681	CGAA ATAAAAGAGCCTACAGTCATTTATATGGAAAGACC	720
721	GTTAAAAAAAGCAGAGTATACCATTCACATCGAAGTTC C	760
761	ACCGAATCCT 771	

Figure (3-4): Nucleotide sequence of erythromycin gene of Tn*1545* carried by *S.pneumoniae* isolates.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae strain M23734 chromosome, complete genome	1112	1112	100%	0.0	100%	CP031247.1
Streptococcus pneumoniae strain PMEN32 ICE element containing genomic region	1112	1112	100%	0.0	100%	MH283017.1
Streptococcus pneumoniae strain GPS_US_PATH6887 ICE element containing genomic region	1112	1112	100%	0.0	100%	MH283015.1
Streptococcus pneumoniae strain GPS_ZA1599 ICE element containing genomic region	1112	1112	100%	0.0	100%	MH283013.1
Streptococcus pneumoniae strain 335 chromosome, complete genome	1112	1112	100%	0.0	100%	CP026670.1
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK1157	1112	1112	100%	0.0	100%	AP018044.1
Streptococcus pneumoniae strain Hu15 genome	1112	1112	100%	0.0	100%	CP020551.1
Streptococcus pneumoniae DNA, complete genome, strain: KK0981	1112	1112	100%	0.0	100%	AP017971.1
Streptococcus pneumoniae strain SWU02, complete genome	1112	1112	100%	0.0	100%	CP018347.1
Streptococcus pneumoniae 9409 tet(M) gene for tetracycline resistance ribosomal protection protein Tet(M), complete CDS	1112	1112	100%	0.0	100%	NG_048253.1
Streptococcus pneumoniae tet(M) gene for tetracycline resistance ribosomal protection protein Tet(M), complete CDS	1112	1112	100%	0.0	100%	NG_048217.1
Streptococcus pneumoniae ST556, complete genome	1112	1112	100%	0.0	100%	CP003357.2
Streptococcus pneumoniae A026 genome	1112	1112	100%	0.0	100%	CP006844.1
Streptococcus pneumoniae Tn916-type integrative and conjugative element, strain 9409	1112	1112	100%	0.0	100%	FR671418.1
Streptococcus pneumoniae Tn916-type integrative and conjugative element, strain H034800032	1112	1112	100%	0.0	100%	FR671414.1
Streptococcus pneumoniae strain DP1322 conjugative transposon Tn5253, complete sequence	1112	1112	100%	0.0	100%	EU351020.1
Streptococcus pneumoniae transposon TN5251, complete sequence	1112	1112	100%	0.0	100%	FJ711160.1
Streptococcus pneumoniae Taiwan19F-14, complete genome	1112	1112	100%	0.0	100%	CP000921.1
Streptococcus pneumoniae P1031, complete genome	1112	1112	100%	0.0	100%	CP000920.1

Figure (3-5): Alignment of erythromycin gene of Tn*1545* carried by *S. pneumoniae* isolates with erythromycin gene carried by standard strains of the same bacteria recorded in NCBI.

3.3.3.2. Tn*917*

Tn*917* is a nonconjugative transposon which is responsible for the spread of erythromycin resistance (Cochetti *et al.*, 2008). Tn*917* was found to be inserted into a number of different Tn*916*-like elements.

Erythromycin gene of Tn*917* was amplified by using specific primers indicated in table (2-6). Results illustrated in figure (3-6) showed an amplified product of 292 bp that appeared after electrophoresis on agarose gel (1%) to represent erythromycin gene of Tn*917*, This transposable element was detected in four isolates of *S. pneumoniae* out of the total isolates (15 isolate), These isolates are D1, D5, D6 and D7. All these isolates are resistant to erythromycin and this result explains the erythromycin resistant phenotype in these four isolates that arise from the presence of Tn*917*.

Mean white, results indicated in table (3-2) showed that there are another five erythromycin resistant isolates (D2, D3, D9, D10, and D15), due to the presence of erythromycin resistance gene located in Tn*1545* possessed by these isolates as mentioned previously in item (3.3.3.1) that these isolates of *S.pneumoniae* harboring Tn*1545* conferring erythromycin resistance trait. These results indicate that those isolates harboring two types of transposons (Tn*1545* and Tn*917*), and this is familiar in bacteria. This result agree with (Palmieri *et al.*, 2012**)** who observed the presence of genetic determinants of resistance to both tetracycline's and MLS antibiotics was also described for other large transposons, such as Tn*2009*, Tn*2010*, Tn*2017*, Tn*3872*, Tn*5253*, Tn*6058*, Tn*5385*, Tn*6002* and Tn*6003* that arose as a combination of smaller transposons. Results also showed that there are six isolates of *S.pneumoniae* (D4, D8, D11, D12, D13, and D14) sensitive to erythromycin among the total resistant isolates (9 isolates) and were unable to grow on enrichment medium containing this antibiotic.

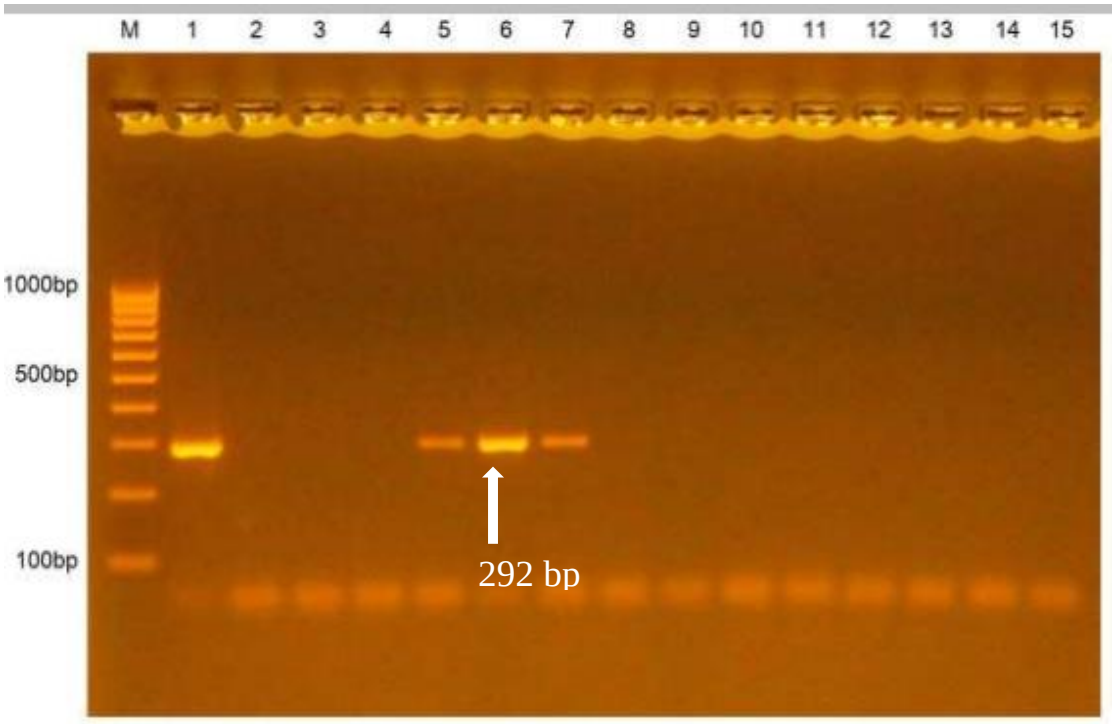

Figure (3-6): Erythromycin gene of Tn*917* detected after amplification of genomic DNA for *S.pneumoniae* isolates and electrophoresis on agarose gel (1%) for one hour.

Lane (M): Ladder marker

Lane (1-15): Bacterial isolates of *S.pneumoniae*

Nucleotide sequence of erythromycin gene in Tn*917* was illustrated in figure (3-7). Alignment of these sequences specified for *S.pneumoniae* isolates (Query) with transposon sequences of *S.pneumoniae* standard strains recorded in NCBI (subjct) are illustrated in figure (3-8). results of alignment showed that erythromycin gene sequence of Tn*917* in four bacterial isolates was identical (100% identity) with chromosomal erythromycin gene sequences in different standard strains of *S. pneumoniae* and with genomic erythromycin resistance gene in other strains of this bacterium which supports the results concluded in this study that the four isolates of *S. pneumoniae* are harboring chromosomal or plasmid copy of Tn*917* conferring erythromycin resistance.

```
  1  TATGATTTTTTAAAGACGGACCCGAAGAGGAACTT G TCT   40
 41 TTTCCCACGGCGACCTGGGAGACAGCAACATCT TTG TGA   80
 81  AAGATGGCA AAGTAAGTGG CTTTATTGATCTTGGGAGAA  120
121  GCGGCAGGGCGGACAAGTGGTATGACATTGCCTTC T GCG  160
161  TCCGGTCGATCAGGGAGGAT ATCGGGGAAGAACAGTATG  200
```

Figure (3-7): Nucleotide sequence of erythromycin gene of Tn*917* carried by *S.pneumoniae* isolates.

In *S. pneumoniaee*, methylation is erm(B) mediated in almost all cases (Weisblum *et al.*, 1995). Worldwide, the predominant mechanism responsible for *S. pneumoniaee* is the Erm(B) methylase (Farrell *el al.*, 2002). One possible explanation for the presence of genes at different samples is that it has erm gene, The Erm(B) methylase gene erm(B) is most likely located on Tn*917*.

In North Lebanon (El Ashkar *el al*., 2017), PCR analysis of the 45 macrolide-resistant *S. pneumoniae* isolates showed that the erm(B) was the prevailing gene present in 37.8% of all strains. In another study, the erm(B) gene was reported to be predominant in several regions, such as Belgium (91.5%), France (90%), Spain (88.3%), Serbia (82.4%), Hungary (82.4%), Poland (80.8%), China (76.5%), Japan (58%) and Italy (55.8%) (Felmingham *el al*., 2000).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae Xen35, complete genome	409	409	100%	2e-113	100%	CP025256.1
Streptococcus pneumoniae strain 19F chromosome, complete genome	409	409	100%	2e-113	100%	CP025076.1
Streptococcus pneumoniae strain NT_110_58, complete genome	409	409	100%	2e-113	100%	CP007593.1
Streptococcus pneumoniae integrative and conjugative element ICESpnSPN8332, isolate SPN8332	409	409	100%	2e-113	100%	HG799498.1
Streptococcus pneumoniae Tn916-type integrative and conjugative element, strain 9409	409	409	100%	2e-113	100%	FR671418.1
Streptococcus pneumoniae integrative and conjugative element ICESpn11876, strain 11876	409	409	100%	2e-113	100%	FR671404.1
Streptococcus pneumoniae transposon Tn1311, strain SpnF21	409	409	100%	2e-113	100%	FN667862.2
Streptococcus pneumoniae CGSP14, complete genome	409	409	100%	2e-113	100%	CP001033.1
Streptococcus pneumoniae partial transposon Tn1545	409	409	100%	2e-113	100%	AM903082.1
Streptococcus pneumoniae transposon Tn6003, strain Ar4	409	409	100%	2e-113	100%	AM410044.5
Streptococcus pneumoniae Ri3 SpnRi3erm(B) element	409	409	100%	2e-113	100%	AM490850.1
Streptococcus pneumoniae strain BLS147 capsular gene locus, partial sequence	403	403	100%	9e-112	99%	KY750636.1
Streptococcus pneumoniae strain KAG1015 cps gene cluster, complete sequence; Glf (glf), aminoglycoside phosphotransferase (kanR), and RpsL (rpsL) genes, complete cds; and AliA (aliA) gene, partial cd	403	403	100%	9e-112	99%	KX470741.1
Mutant Streptococcus pneumoniae strain MBO15 glutamine synthetase type I (SP_0502) gene, partial cds; hypothetical protein (SP_0503) and hypothetical protein (SP_0504) genes, complete cds; Janus ca	398	398	100%	4e-110	99%	MF927926.1
Mutant Streptococcus pneumoniae strain KAG1014 cps gene locus, complete sequence; and putative oligopeptide-binding protein (aliA) gene, partial cds	398	398	100%	4e-110	99%	KX096820.1
Mutant Streptococcus pneumoniae strain JC02 cps gene locus, partial sequence	398	398	100%	4e-110	99%	JF301958.1
Streptococcus pneumoniae strain MNZ786 cps gene locus, partial sequence	398	398	100%	4e-110	99%	GU074961.1
Streptococcus pneumoniae strain BLS143 capsular gene locus, partial sequence	392	392	100%	2e-108	99%	KY750635.1
Streptococcus pneumoniae strain BLS140 cps gene locus, partial sequence	392	392	100%	2e-108	99%	KX840355.1

Figure (3-8): Alignment of erythromycin gene of Tn917 carried by *S. pneumoniae* isolates erythromycin gene carried by standard strains of the same bacterial recorded in NCBI.

3.3.3.3. Tn*5397*

Tn*5397* is a conjugative transposon carrying the tetracycline gene tet(M) (Roberts *et al.,* 2001).Tetracycline (M) gene of Tn*5397* was amplified by using specific primers indicated in table (2-6). Results illustrated in figure (3-9) showed an amplified product of 801bp to be appear after electrophoresis on agarose gel (1%) that represents tetracycline gene of Tn*5397*, This transposable elements was detected in five isolates of *S. pneumoniae* out of the total isolates (15 isolate). These isolates are D1, D2, D3, D6 and D9. All these isolates are resistant to tetracycline and this result explain the tetracycline resistant phenotype in these five isolates that arise from the presence of Tn*5397*.

Meanwhile, results indicated in table (3-2) showed that there are other eight tetracycline resistant isolates (D5, D7, D8, D10, D11, D112, and D15) possessing a chromosomal or plasmid copy of tetracycline resistance gene, this may be due to other structural tetracycline genes carried by other type of transposable elements present in these bacterial isolates. Furthermore there are three isolates of *S.pneumoniae* (D4, D13 and D14) were sensitive to tetracycline among the total resistant isolates (12 isolates) and were unable to grow on enrichment medium containing this antibiotic.

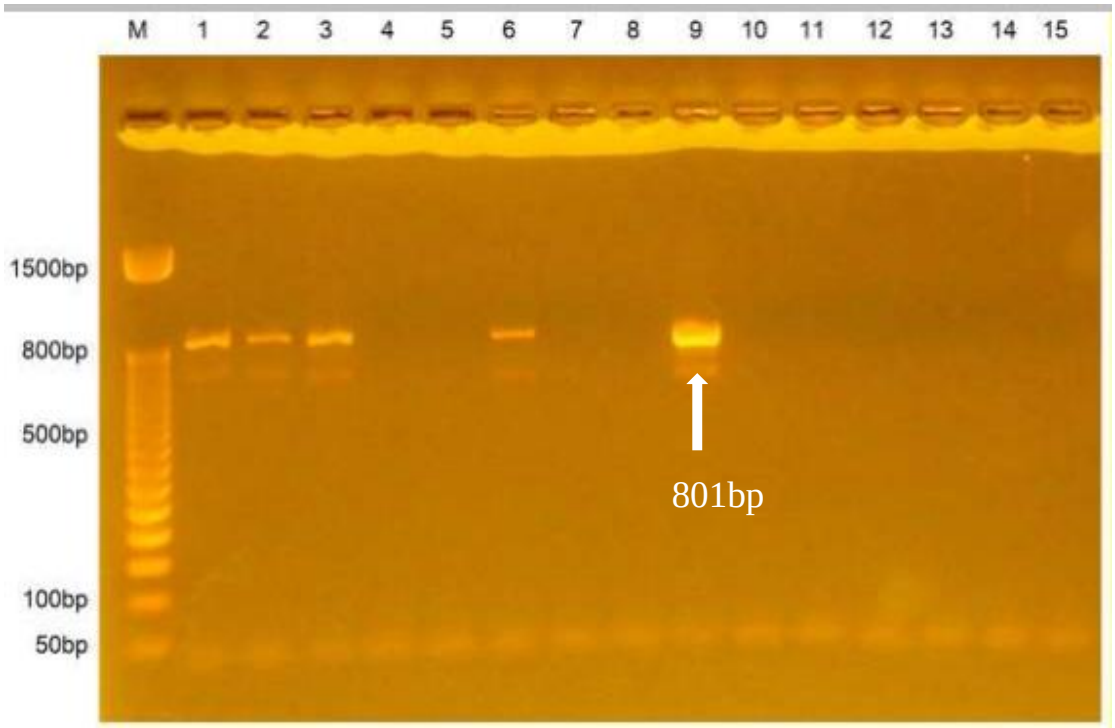

Figure (3-9): Tetracycline gene of Tn*5397* detected after amplification of genomic DNA for *S.pneumoniae* isolates and electrophoresis on agarose gel (1%) for one hour.

Lane (M): Ladder marker

Lane (1-15): Bacterial isolates of *S.pneumoniae*

Nucleotide sequence of tetracycline gene of tetracycline gene in Tn*5397* was illustrated in figure (3-10). Alignment of these sequences specified for *S.pneumoniae* isolates (Query) with transposon sequences of *S.pneumoniae* standard strains recorded in NCBI (subjct) are illustrated in figure (3-11). The results of alignment showed that tetracycline gene sequence was identical (100% identity) with chromosomal tetracycline gene sequences in different standard strains of *S.pneumoniae* and with genomic tetracycline resistance gene in other strains of this bacterium which supports the results concluded in this study that the four isolates of *S. pneumoniae* harbor the chromosomal or plasmid copy of *Tn5397* conferring tetracycline resistance.

1	TACTCGTCTATGAC AATCCAG AGCATACC GCCTTTAAAA	40
41	TTATCAATCGGTATATCCGT TTTGTAGATAAAGACG ATT	80
81	CCAAACCTCGTTCTGATTGGAAACTGAATGAAGAAT GGG	120
121	CTTGGTTTATTGGGAACAATCGTGAACGATTAAAAC TAA	160
161	CCACAAAAC CAGAGCCTTACTCC CTTCCAAAGGACGCTG	200
201	AACTGGCTATCTCATCAAGTTGCCCCGACCTTAAA GG TT	240
241	GCGATTAAACTTGATGAAATCAACCAGA CGCAGGTTGTA	280
281	AAAGACATTCTCGACCATGCGAAA CTGACAGA CCGACAC	320
321	AAGCAGATTTTGAAGCAACAGTCAGTAA AAGAACAGGAC	360
361	GTGATAACAACAAAAAAATAACTCAAATACAA ATTCATT	400
401	GAATATAGAGAGGAGAACATTTTTATGAATTTTGG A CAA	440
441	AACCTTTATAACTGGTTTCTATCAAACG CTCAAT C AC TG	480
481	GTGCTTTTAGCAATCGTTGTGAT TGG CTTGTAT CTT GGC	520
521	TTCAAGCGTGAGTTTAGCAAACTG ATT GGCTTT TT AATT	360
361	ATTGCGATTATTGCGGTTGGC TTAGTCTTC AA CG CTGCT	400
401	GGAGTAAAAGACATTTTACTA GAGCTATTCAAT CGCATT	440
441	ATTGGTGCTTAAATAAAA CCGTTCTTTTGTGGAAT ATA A	480
481	GTGGTTTTCTTATGTTCCG CAAAGGAATGGTAC ACCAAA	520
521	CGAA	525

Figure (3-10): Nucleotide sequence of tetracycline gene of Tn*5397* carried by *S.pneumoniae* isolates.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae HU-OH DNA, complete genome	1303	1303	100%	0.0	100%	AP018937.1
Streptococcus pneumoniae strain M26365 chromosome, complete genome	1303	1303	100%	0.0	100%	CP031248.1
Streptococcus pneumoniae strain M23734 chromosome, complete genome	1303	1303	100%	0.0	100%	CP031247.1
Streptococcus pneumoniae strain PMEN32 ICE element containing genomic region	1303	1303	100%	0.0	100%	MH283017.1
Streptococcus pneumoniae strain GPS_US_PATH396 ICE element containing genomic region	1303	1303	100%	0.0	100%	MH283016.1
Streptococcus pneumoniae strain GPS_US_PATH6887 ICE element containing genomic region	1303	1303	100%	0.0	100%	MH283015.1
Streptococcus pneumoniae strain GPS_BR_0186_12 ICE element containing genomic region	1303	1303	100%	0.0	100%	MH283014.1
Streptococcus pneumoniae strain GPS_IN33 ICE element containing genomic region	1303	1303	100%	0.0	100%	MH283011.1
Streptococcus pneumoniae strain 4041STDY6583227 genome assembly, chromosome: 1	1303	1303	100%	0.0	100%	LS483450.1
Streptococcus pneumoniae strain 335 chromosome, complete genome	1303	1303	100%	0.0	100%	CP026670.1
Streptococcus pneumoniae MDRSPN001 DNA, complete genome	1303	1303	100%	0.0	100%	AP018391.1
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK1157	1303	1303	100%	0.0	100%	AP018044.1
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK0381	1303	1303	100%	0.0	100%	AP018043.1
Streptococcus pneumoniae ST556, complete genome	1303	1303	100%	0.0	100%	CP003357.2
Streptococcus pneumoniae integrative and conjugative element ICE6BST90, isolate IC161	1303	1303	100%	0.0	100%	HG799499.1
Streptococcus pneumoniae integrative and conjugative element ICESpnIC1, isolate 9611+04103	1303	1303	100%	0.0	100%	HG799494.1
Streptococcus pneumoniae putatative integrative and conjugative element sequence, isolate 0FQ8K	1303	1303	100%	0.0	100%	LK020697.1
Streptococcus pneumoniae integrative and conjugative element ICESpnPT814, isolate PT814	1303	1303	100%	0.0	100%	HG799502.1
Streptococcus pneumoniae integrative and conjugative element ICESpn6027, isolate 6027	1303	1303	100%	0.0	100%	HG799501.1

Figure (3-11): Alignment of tetracycline gene of Tn*5397* carried by *S. pneumoniae* isolates with tetracycline gene carried by standard strains of the same bacterial recorded in NCBI.

3.3.3.4. Tn*3872*

Tn*3872* is a composite element resulting from the insertion of the erm(B) containing Tn*917* transposon into orf9 of Tn*916* (Del Grosso., 2004).

Erythromycin (B) gene of Tn*3872* was amplified in *S.pneumoniae* isolates by using specific primers indicated in table (2-6). Results illustrated in figure (3-12) showed an amplified product of 1096 bp appearing after electrophoresis on agarose gel (1%) represents the erythromycin gene of Tn*3872*, This transposable element was detected in eight isolates of *S.pneumoniae* out of the total isolates (15 isolate), these isolates are D1, D2, D3, D5, D6, D9.D10, and D15. All these isolates are resistant to erythromycin and this result explains the erythromycin resistant phenotype in these eight isolates that arise from the presence of Tn*3872*.

Meanwhile, results indicated in table (3-2) showed that there is another one erythromycin resistant isolate (D7) due to the presence of erythromycin resistance gene carried by Tn*917* possessed by this isolate as mentioned previously in item (3.3.3.2.) that *S.pneumoniae* D7 harbor Tn*917* conferring erythromycin resistance trait. These results indicates that those isolates harbor two types of transposons (Tn*3872* and Tn*917*) that were detected in *S.pneumoniae* isolates , and the coexistence of these transposons in the same bacteria is familiar in kingdom bacteria. The mef(E)/mel-containing genetic element Mega is found in at least six distinct chromosomal sites within the pneumococcal genome (Chancey *et al*., 2015a) ,This result agree with Taha *et al*., (2012) who observed that all the strains belonging to the MLSB phenotype harbored the erm(B) gene, while all the strains with M phenotype had the mef(A/E) gene. The presence of both resistance genes was confirmed in 14 strains with MLSB phenotype. In the same context, two recent studies conducted in Lebanon

and Iran described the same distribution with predominance of macrolide resistant *S.pneumoniae* isolates harboring erm(B) gene followed by both erm(B and mef(A/E) genes and mef(A/E) gene, respectively Furthermore, there are six isolates of *S.pneumoniae* (D4, D8, D11, D12, D13,and D14) which were sensitive to erythromycin among the total resistant isolates (9 isolates) and were unable to grow on enrichment medium containing this antibiotic.

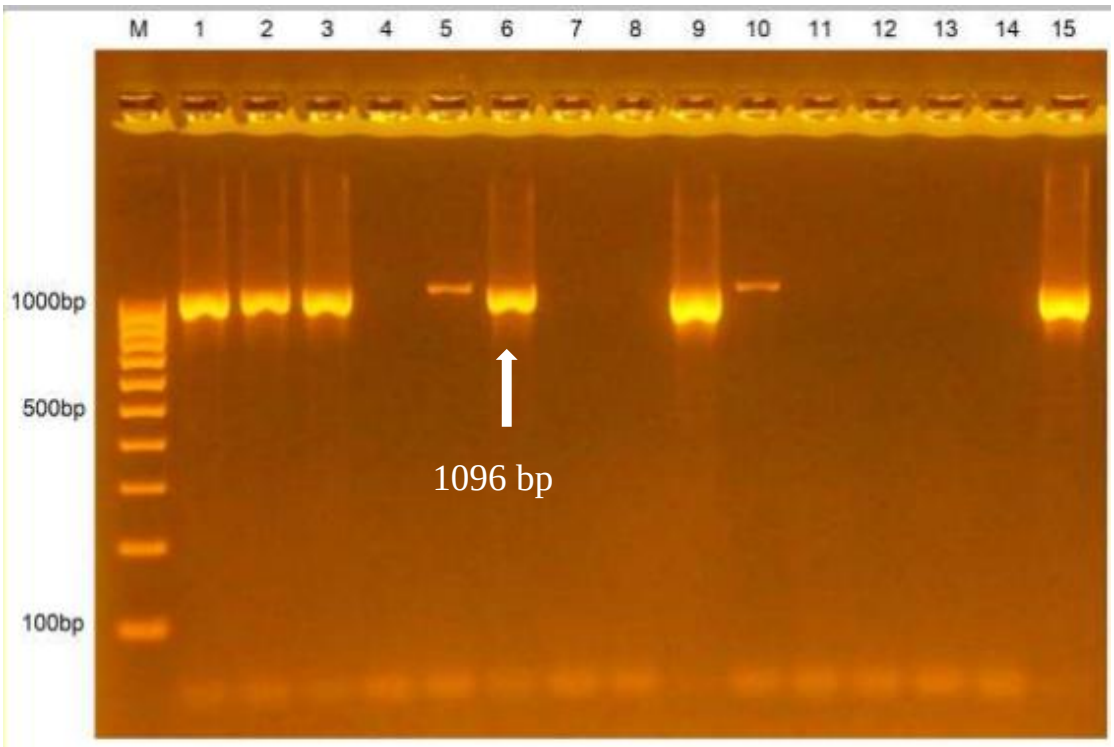

Figure (3-12): Erythromycin gene of Tn*3872* detected after amplification of genomic DNA for *S.pneumoniae* isolates and electrophoresis on agarose gel (1%)
Lane (M): Ladder marker
Lane (1-15): Bacterial isolates of *S. pneumoniae*

Nucleotide sequence of erythromycin gene in Tn*3872* was illustrated in figure (3-13). Alignment of these sequences specified for *S. pneumoniae* isolates (Query) with transposon sequences of *S. pneumoniae* standard strains recorded in NCBI (subjct) are illustrated in figure (3-14).Results

of alignment showed that erythromycin gene sequence of Tn*3872* in those eight bacterial isolates was identical (100% identity) with chromosomal erythromycin gene sequences in different standard strains of *S. pneumoniae* and with genomic erythromycin resistance gene in other strains of this bacterium. This supports the results concluded in this study that the four isolates of *S. pneumoniae* harbor a chromosomal or plasmid copy of Tn*3872* conferring erythromycin resistance.

1	GAAGACATTCATGA TGGTATT GATGTTGTAGGAAA GAAA	40
41	ATGACACTCTGCCAGCT TTACGCAAAACAGAACGCTCA A	80
81	AGACCAAAGGTTAGAAAAAACACTGAAA CTGGACGCAA A	120
121	TATCTTATGGATATTTTGAAGAAAG AC AAG TTAG GTG TA	160
161	AGAAGTATTGACAGTATTAAGCCATCAGACGCTAA AG AA	200
201	TGGGCTATTAGAATGAGTGAAAATGG TTAT GCTTA TC AA	240
241	ACCATCAATAACTACAAAC GTTCT TTA AAG GCTTCAT TC	280
281	TATATTGCTATACAAGATGATTGTGTTCGGAAGAATCC AT	320
321	TTGACTTTCAACTGAAAGCAGTT CTT GATGA TGAT A C TG	360
361	TCCCTAAGACCGTACTAACAGAAGAACAGGAAGA A AAAC	400
401	TGTTAGCCTTTGCAAAAGCTGATAAA ACCTAC AGCA A AA	440
441	ATTATGATGAAATTCTGATACTCTTA AAA A CA GGTCTTC	480
481	GTATTTCAGAGTTTGGTGGTT TGA CACT TC C AGAT TTAG	520
521	ATTTTGAGAATC GTCTTGTCA AT AT AGACCATCAGCTAT	560
561	TGAGAGATACTGAAATTGGGTACTACATTGAAACACCAAA	600
601	GACCAAAAGTGGCGAACGTCAAGT TCCT ATGGTTG AAGA	640
641	AGCCTATCAAGCATTTAAG CGAG TGTT AGCGAATCGAAA	680
681	GAATGATAAGC GTG TTGA GATTG ATGGATATAGTGATTT	720
721	CCTCTTTCTTAATAG AAAGA ACTATC CAAAAG TGGC AAG	760
761	TGATTACAACGGCATGATGAAAGGTCTTGTT AAGAAATAC	800
801	AATAAGTATAACGAGGATAAATTGCCACACATCACTCC AC	840
841	ATAGTTTGCGACA TAC ATT CTGTAC CAACTATG CAA ATG	880
881	CAGGAATGAATCCAAAG GCAT TACAGTAC ATTATGGGAC	920
921	ATGCTAATATAGCCAT GACGCTGAA CTATTA CGC ACATG	960
961	CAACATTCGATTCTGCAATGGCAGAA ATGAA ACG CTT GA	1000
1001	ATAAGAGA	1009

Figure (3-13): Nucleotide sequence of erythromycin gene of Tn*3872* carried by *S.pneumoniae* isolates.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae HU-OH DNA, complete genome	1816	1816	99%	0.0	99%	AP018937.1
Streptococcus pneumoniae strain SPN XDR SMC1710-32 chromosome, complete genome	1816	1816	99%	0.0	99%	CP025838.1
Streptococcus pneumoniae strain M26365 chromosome, complete genome	1816	1816	99%	0.0	99%	CP031248.1
Streptococcus pneumoniae strain PMEN32 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283017.1
Streptococcus pneumoniae strain GPS_US_PATH396 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283016.1
Streptococcus pneumoniae strain GPS_US_PATH6887 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283015.1
Streptococcus pneumoniae strain GPS_BR_0186_12 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283014.1
Streptococcus pneumoniae strain GPS_ZA1599 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283013.1
Streptococcus pneumoniae strain GPS_ZA808 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283012.1
Streptococcus pneumoniae strain GPS_IN33 ICE element containing genomic region	1816	1816	99%	0.0	99%	MH283011.1
Streptococcus pneumoniae strain 11A chromosome, complete genome	1816	1816	99%	0.0	99%	CP018838.1
Streptococcus pneumoniae MDRSPN001 DNA, complete genome	1816	1816	99%	0.0	99%	AP018391.1
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK0381	1816	1816	99%	0.0	99%	AP018043.1
Streptococcus pneumoniae strain Hu15 genome	1816	1816	99%	0.0	99%	CP020551.1
Streptococcus pneumoniae strain Hu17 genome	1816	1816	99%	0.0	99%	CP020549.1
Streptococcus pneumoniae strain SWU02, complete genome	1816	1816	99%	0.0	99%	CP018347.1
Streptococcus pneumoniae strain SP64, complete genome	1816	1816	99%	0.0	99%	CP018138.1
Streptococcus pneumoniae strain SP61, complete genome	1816	1816	99%	0.0	99%	CP018137.1
Streptococcus pneumoniae ST556, complete genome	1816	1816	99%	0.0	99%	CP003357.2

Figure (3-14): Alignment of erythromycin gene Tn*3872* carried by *S. pneumoniae* isolates with erythromycin gene carried by standard strains of the same bacteria recorded in NCBI.

3.3.3.5. Tn*6002*

Tn*6002* is one of the most extensively investigated erm(B)- carrying Tn*916*-related streptococcal elements in recent years (Varaldo *et al.*, 2009).

Erythromycin (B) gene of Tn*6002* was amplified by using specific primers indicated in table (2-6). Results illustrated in figure (3-15) showed an amplified product of 194 bp, appear after electrophoresis on agarose gel (1%) represents erythromycin gene of this transposable element detected in eight isolates of *S.pneumoniae* out of the total isolates (15 isolate), In these isolates, Tn*6002* may be a chromosomal copy or may be located on plasmid DNA. These isolates are D1, D2, D3, D5, D6, D9.D10, and D15. All these isolates are resistant to erythromycin and this result explains the erythromycin resistant phenotype in these eight isolates that arise from the presence of Tn*6002*.

Meanwhile, The results indicated in table (3-2) showed that there is another erythromycin resistant isolate (D7), due to the presence of erythromycin resistance gene located in Tn*1545* and Tn*917* carried by this isolate as mentioned previously in items (3.3.3.1)and (3.3.3.2) refers that those isolates of *S.pneumoniae* harbors Tn*1545* and Tn*917* conferring erythromycin resistance genes ,from these results it was mentioned that isolate (D7) harboring three types of transposons (Tn*1545*, Tn*917*, Tn*6002*) these transposons were detected in *S.pneumoniae* isolates , which occurs familiar in kingdom bacteria. The mef(E)/mel-containing genetic element Mega is found in at least six distinct chromosomal sites within the pneumococcal genome (Chancey *et al*., 2015a), These results agree with Azadegan *et al*., (2015**)** who observed that all the strains belonging to the MLSB phenotype harbored the erm(B) gene, while all the strains with M phenotype had the mef(A/E)

gene. The presence of both resistance genes was confirmed in 14 strains with MLSB phenotype. In the same context, two other studies showed the same distribution with predominance of macrolide resistant *S. pneumoniae* isolates harboring erm(B) gene followed by both erm(B) and mef(A/E) genes and mef(A/E) gene, respectively; (El Ashkar *el al.*, 2017) and also agree with Del Grosso *et al.*, (2006) who observed the presence of genetic determinants of resistance to both tetracycline's and MLS antibiotics in addition to other large transposons such as Tn*2009*, Tn*2010*, Tn*2017*, Tn*3872*, Tn*5253*, Tn*6058*, Tn*5385*, Tn*6002* and Tn*6003* that arose as a combination of smaller transposons. Furthermore, results showed that there are six isolates of *S.pneumoniae* (D4, D8, D11, D12, D13, and D14) sensitive to erythromycin among the total resistant isolates (9 isolates) and were unable to grow on enrichment medium containing this antibiotic.

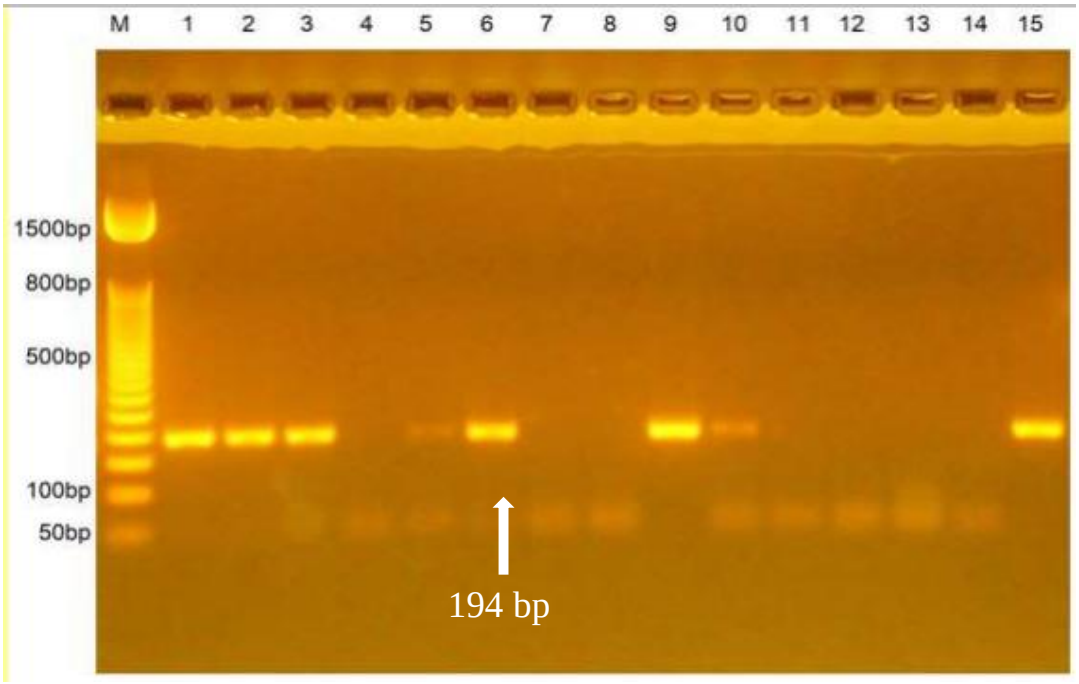

Figure (3-15): Erythromycin gene Tn*6002* detected after amplification of genomic DNA for *S. pneumoniae* isolates and electrophoresis on agarose gel (1%) for one hour.

Lane (M): Ladder marker

Lane (1-15): Bacterial isolates of *S. pneumoniae*

Nucleotide sequence of erythromycin gene in Tn*6002* was illustrated in figure (3-16). Alignments of these sequences specified for *S.pneumoniae* isolates (Query) with transposon sequences of *S.pneumoniae* standard strains recorded in NCBI (subjct) are illustrated in figure (3-17). Results of alignment showed that erythromycin gene sequence of Tn*6002* in those eight bacterial isolates *was* identical (100% identity) with erythromycin gene sequences in different standard strains of *S. pneumoniae*, This finding supports the results concluded in this study that the four isolates of *S. pneumoniae* are harboring chromosomal or plasmid copy of Tn*6002* conferring erythromycin resistance.

Multiple resistance of pneumococci especially resistance to macrolides and tetracycline is generally associated with their unique recombination-mediated genetic plasticity and possessing the mobile genetic elements (Roberts and Mullany, 2011; Shiojima *et al*., 2005). Two major mechanisms of macrolide resistance in *S. pneumoniae* are noted. The first one is the target site modification by a ribosome methylase, encoded by *erm(B)* gene and related to high-level resistance to macrolide, lincosamides, and streptogramin B (MLS_Bphenotype). *Erm*(B) resistance can be expressed by pneumococci either constitutively ($cMLS_B$ phenotype) or inducibly ($iMLS_B$ phenotype) (Montanari *et al*., 2001).The majority of macrolide-resistant *S. pneumoniae* strains are also resistant to tetracycline. This association is due to the insertion of *erm*(B) into conjugative and composite transposons of the Tn*916* family that harbor's *tet(M)* gene, encoding ribosome protection proteins. Members of this family, which carry *erm*(B), include Tn*6002* (Brenciani *et al*., 2007).

1	GCGTCAAAATATTTTCGTATTGGCGAA AACAAG CT ACGA	40
41	CGCTTGGCAGAGGAAAATAAA AATGCAAA TTGGCTGATT	80
81	ATGAATGGCAATCGTATTC AGATTAAACGAAAACAATT T	120
121	GAAAAAATTATAGATACATTGGA	144

Figure (3-16): Nucleotide sequence of erythromycin gene of Tn*6002* carried by *S.pneumoniae* isolates.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae HU-OH DNA, complete genome	259	259	100%	1e-68	100%	AP018937.1
Streptococcus pneumoniae strain SPN XDR SMC1710-32 chromosome, complete genome	259	259	100%	1e-68	100%	CP025838.1
Streptococcus pneumoniae strain M23734 chromosome, complete genome	259	259	100%	1e-68	100%	CP031247.1
Streptococcus pneumoniae strain PMEN32 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283017.1
Streptococcus pneumoniae strain GPS_US_PATH396 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283016.1
Streptococcus pneumoniae strain GPS_US_PATH6887 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283015.1
Streptococcus pneumoniae strain GPS_BR_0186_12 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283014.1
Streptococcus pneumoniae strain GPS_ZA1599 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283013.1
Streptococcus pneumoniae strain GPS_ZA808 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283012.1
Streptococcus pneumoniae strain GPS_IN33 ICE element containing genomic region	259	259	100%	1e-68	100%	MH283011.1
Streptococcus pneumoniae strain 335 chromosome, complete genome	259	259	100%	1e-68	100%	CP026670.1
Streptococcus pneumoniae strain 11A chromosome, complete genome	259	259	100%	1e-68	100%	CP018838.1
Streptococcus pneumoniae MDRSPN001 DNA, complete genome	259	259	100%	1e-68	100%	AP018391.1
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK1157	259	259	100%	1e-68	100%	AP018044.1
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK0381	259	259	100%	1e-68	100%	AP018043.1
Streptococcus pneumoniae strain Hu15 genome	259	259	100%	1e-68	100%	CP020551.1
Streptococcus pneumoniae strain Hu17 genome	259	259	100%	1e-68	100%	CP020549.1
Streptococcus pneumoniae DNA, complete genome, strain: KK0981	259	259	100%	1e-68	100%	AP017971.1
Streptococcus pneumoniae strain SWU02, complete genome	259	259	100%	1e-68	100%	CP018347.1

Figure (3-17): Alignment of erythromycin gene of Tn*6002 carried by S. pneumoniae* isolates with erythromycin gene carried by standard strains of the same bacteria recorded in NCBI.

3.3.3.6. Tn*916*

Tn*916* was the first conjugative transposon to be identified, and it is the prototype of a closely related family of conjugative transposons widespread in gram-positive cocci (Clewell *et al.*, 1995). The Tn*916* transposon family has a broad host range and transfer readily to a wide variety of Gram-positive and Gram-negative bacteria (Roberts and Mullany, 2011). Tn*916* is a conjugative transposon that carries the tetracycline gene tet(M).

Tetracycline (M) gene of Tn*916* was amplified by using specific primers indicated in table (2-6). Results illustrated in figure (3-18) showed an amplified product of 639 bp appeared after electrophoresis on agarose gel (1%) that represents tetracycline gene of Tn*916*, this transposable element was detected in four isolates of *S.pneumoniae* out of the total isolates (15 isolate), In these isolates. These isolates are D5, D6, D9 and D15. All these isolates are resistant to tetracycline and this result explains the tetracycline resistant phenotype in these isolates that arise from the presence of Tn*916*.

On the other hand, results indicated in table (3-2) showed that there are other eight tetracycline resistant isolates (D1, D2, D3, D7, D8, D10, D11, and D12) that possess a chromosomal or plasmid copy of tetracycline resistance gene. Due to the presence of tetracycline gene located in Tn*5397* carried by D1, D2 and D3 isolates as mentioned previously in item (3.3.3.3) which mentioned that these isolates harboring Tn*5397* conferring tetracycline resistance trait. According to these results those four isolates harboring transposons were detected in *S. pneumoniae* isolates, and the coexistence of these transposons in the same bacteria is familiar in kingdom bacteria. The mef(E)/mel-containing genetic element Mega is found in at least six distinct chromosomal sites within the pneumococcal genome (Chancey *et al*., 2015a), These results agree with

(Warburton *et al.*, 2007) that observed the presence of genetic determinants of resistance to both tetracycline's and MLS antibiotics was also described for other large transposons, such as Tn*2009*, Tn*2010*, Tn*2017*, Tn*3872*, Tn*5253*, Tn*6058*, Tn*5385*, Tn*6002* and Tn*6003* that arose as a combination of smaller transposons Furthermore, there are three isolates of *S. pneumoniae* (D4 ,D13, and D14) that were sensitive to tetracycline among the total resistant isolates (7 isolates) and were unable to grow on enrichment medium containing this antibiotic.

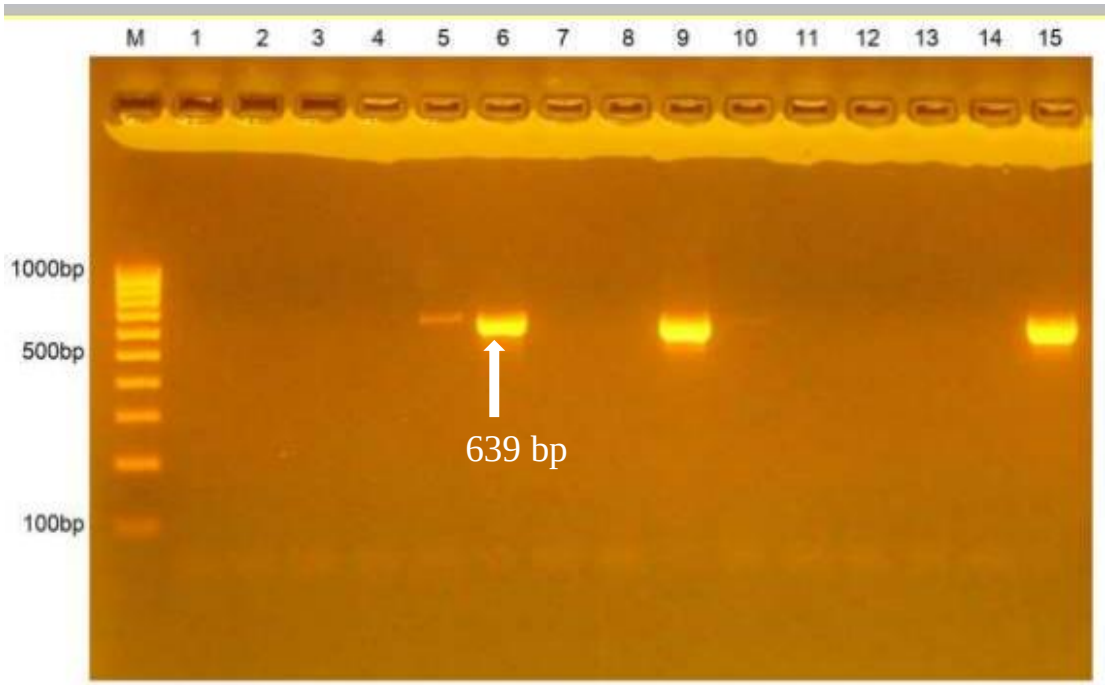

Figure (3-18): Tetracycline gene of Tn*916* detected after amplification of genomic DNA for *S. pneumoniae* isolates and electrophoresis on agarose gel (1%) for one hour.

Lane (M): Ladder marker

Lane (1-15): Bacterial isolates of *S. pneumoniae*

Nucleotide sequence of part of tetracycline gene in Tn*916* was illustrated in figure (3-19). Alignment of these sequences specified for *S.pneumoniae* isolates (Query) with transposon sequences of *S.pneumoniae* standard strains recorded in NCBI (subjct) are illustrated in

figure (3-20). Results of alignment showed that tetracycline gene sequence of Tn*916* in those four bacterial isolates was identical (100% identity) with chromosomal tetracycline gene sequences in different standard strains of *S. pneumonia*. It was also indicated with genomic tetracycline resistance gene in other strains of this bacterium which supports the results concluded in this study that the four isolates of *S. pneumoniae* are harbouring chromosomal or plasmid copy of Tn*916* conferring erythromycin resistance.

In multidrug-resistant *Streptococcus pneumonia*, these resistances are usually acquired on mobile genetic elements such as conjugative plasmids and conjugative transposons, which are capable of broad hosting the range transfer between pathogens and commensal and pathogenic bacteria. For example, tetra resistance, which is mediated by the product of the TET (M) gene, is found in *S. pneumoniae* strains. The Tn*916*/Tn*1545*/Tn*2009*/ family is responsible for a large proportion of the antibiotic resistance in this bacteria. These conjugative elements are responsible for the dissemination of many antimicrobial resistance genes (usually resistance to tetracyclines, but also macrolides, lincosamides and streptogramins, kanamycin and mercury) (Tsubakishita *et al.,* 2010).

Tetracycline resistance is usually associated with the acquisition of tet genes such as tetA, tetB, tetC and tet31, coding for efflux proteins, and tetT, tetW, tetM and tetO, encoding for proteins involved in ribosomal protection (Chopra and Roberts, 2001).

1	TTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAA	40
41	AATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCA	80
81	TCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATAC	120
121	TCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCA	160
161	ATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGTAT	200
201	TCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGT	240
241	TTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGA	280
281	AGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAAC	320
321	ACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCA	360
361	ATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACC	400
401	AAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATAC	440
441	CACAGATGTTCCAGATAAATATTGGAAGCTATATACGTA	480
481	CTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACT	520
521	GTTTACTAAAAATCAGTTTCATCAAGCAATGAAACACGC	360
361	CAAAGTAAACAATT	375

Figure (3-19): Nucleotide sequence of tetracycline gene of Tn*916* carried by *S.pneumoniae* isolates.

However, numerous studies reported tetracycline susceptibility among tetM-harbouring strains (Varaldo *et al.,* 2009). In 2012, framing errors or frameshift mutations in seven tetracycline-susceptible *S.pneumoniaee* strains, caused by either deletion or insertion, were identified as the reasons behind a low transcription level of tetM and therefore the susceptible profile (Grohs *et al.,* 2012). Resistant strains presented truncated tetM promoter that resulted in an increase in the gene transcription, thus causing a higher level of resistance.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae strain SPN XDR SMC1710-32 chromosome, complete genome	1037	2057	100%	0.0	100%	CP025838.1
Streptococcus pneumoniae strain GPS_ZA1599 ICE element containing genomic region	1037	1037	100%	0.0	100%	MH283013.1
Streptococcus pneumoniae strain GPS_ZA808 ICE element containing genomic region	1037	1037	100%	0.0	100%	MH283012.1
Streptococcus pneumoniae strain 11A chromosome, complete genome	1037	2057	100%	0.0	100%	CP018838.1
Streptococcus pneumoniae strain Hu15 genome	1037	1037	100%	0.0	100%	CP020551.1
Streptococcus pneumoniae strain Hu17 genome	1037	1037	100%	0.0	100%	CP020549.1
Streptococcus pneumoniae strain SWU02, complete genome	1037	1037	100%	0.0	100%	CP018347.1
Streptococcus pneumoniae strain SP64, complete genome	1037	1037	100%	0.0	100%	CP018138.1
Streptococcus pneumoniae strain SP61, complete genome	1037	1037	100%	0.0	100%	CP018137.1
Streptococcus pneumoniae strain NT_110_58, complete genome	1037	2035	100%	0.0	100%	CP007593.1
Streptococcus pneumoniae putatative integrative and conjugative element sequence, isolate NFPTS	1037	1037	100%	0.0	100%	LK020698.1
Streptococcus pneumoniae putatative integrative and conjugative element sequence, isolate 397079	1037	1037	100%	0.0	100%	LK020692.1
Streptococcus pneumoniae putatative integrative and conjugative element sequence, isolate R34-3225	1037	1037	100%	0.0	100%	LK020687.1
Streptococcus pneumoniae integrative and conjugative element ICESpnSPN28652, isolate SPN28652	1037	1037	100%	0.0	100%	HG799503.1
Streptococcus pneumoniae integrative and conjugative element ICESpnSPN8332, isolate SPN8332	1037	1037	100%	0.0	100%	HG799498.1
Streptococcus pneumoniae integrative and conjugative element ICESpnDCC1902, isolate DCC1902	1037	1037	100%	0.0	100%	HG799491.1
Streptococcus pneumoniae integrative and conjugative element ICESpn22664, isolate 22664	1037	1037	100%	0.0	100%	HG799489.1
Streptococcus pneumoniae A026 genome	1037	1037	100%	0.0	100%	CP006844.1

Figure (3-20): Alignment of tetracycline gene Tn*916* carried by *S.pneumoniae* isolates with tetracycline gene carried by standard strains of the same bacteria recorded in NCBI.

Results of the current study showed that the 15 isolates of *S. pneumoniae* were diverted in harboring transposons conferring antibiotic resistance as indicated in table (3-5). Among them D4, D8, D11, D12, D13, and D14 *S.pneumoniae* isolates did not contain any type of transposons detected in other isolates collected in this study. However, these isolates were resistant to different antibiotics which may lead to a conclusion that the antibiotic resistance genes in these isolates were chromosomally located or may be carried by a bacterial plasmid rather than carried by transposable element.

Furthermore, the results indicated in table (3-5) showed that two *S.pneumoniae* isolates (D7 and D10) harboring two transposons, while the isolates D2, D4, D5, and D15 harboring four transposons, and the isolates D1, D8, and D9 carrying five different transposons, while isolate D6 carries six transposons. The presence of different types of transposons in a single bacterial isolate is a familiar case in *S. pneumoniae* and other bacterial species as mentioned by table (3-4).

Table (3-4): Pattern of antibiotic resistance gene conferred by transposable elements in local isolates of *S. pneumoniae.*

Isolate	Transposon					
	Tn*1545*	Tn*917*	Tn*5397*	Tn*3872*	Tn*6002*	Tn*916*
D1	+	+	+	+	+	-
D2	+	-	+	+	+	-
D3	+	-	+	+	+	-
D4	-	-	-	-	-	-
D5	-	+	-	+	+	+
D6	+	+	+	+	+	+
D7	+	+	-	-	-	-
D8	-	-	-	-	-	-
D9	+	-	+	+	+	+
D10	-	-	-	+	+	-
D11	-	-	-	-	-	-
D12	-	-	-	-	-	-
D13	-	-	-	-	-	-
D14	-	-	-	-	-	-
D15	+	-	-	+	+	+

CONCLUSIONS AND RECOMMENDATIONS

4. Conclusions and recommendations

4.1. Conclusions

1- Respiratory tracts and cerebrospinal fluid is an important source for isolation of *Streptococcus pneumoniae* in case of pharyngitis and tonsillitis infections.

2- *S. pneumoniae* isolates of this study were multidrug resistance.

3- Tn*1545*, Tn*917*, Tn3872, Tn*5397*, Tn*917* and Tn*6002* were detected in clinical isolates of *S. pneumoniae*.

4- Tn*1545*, Tn*917*, Tn3872, and Tn*6002* carrying erythromycin resistance genes were detected in different local isolates of *S. pneumoniae*.

5- Tn*5397* and Tn*916* carrying tetracycline resistance genes were detected in different *S. pneumoniae* isolates.

6- Two of *S. pneumoniae* isolates harbored two types of transposons, Four of *S. pneumoniae* isolates harbored four different types of transposons, Three isolates of *S. pneumoniae* harbored five different types of transposons, One isolate of *S. pneumoniae* harbored six different types of transposons.

7- Not all the antibiotics resistance phenotype of bacterial isolates the related to the presence of transposons, but a chromosomal or plasmid copy of the antibiotic resistance gene.

4.2. Recommendations

1- Studying the gene expression of antibiotic resistance genes located on different transposable elements under antibiotic stress on growth of *Streptococcus pneumoniae* isolates.

2- Detecting transposable elements in other pathogenic bacteria in relation with antibiotic resistance pattern.

3- Studying the conjugative ability of transposons under study with other isolates of *S. pneumoniae* sensitive to the related antibiotics.

4- Studying the shuttle phenotype of transposons under study with other bacterial species sensitive to the related antibiotics.

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APPENDICES

Appendix (1): Results of Biochemical Tests for Identification of *S.pneumoniae* by Vitek-2

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): Concentration of genomic DNA extracted from *S.pneumoniae* isolates

Isolate NO.	Concentration(ng/µl)
01	20
02	11
03	15
04	9
05	7
06	8
07	20
08	18
09	20
10	11
11	8
12	18
13	15
14	11
15	15

Appendix (3): Nucleotide sequence of tetracycline gene carried by Tn*5397* in *S.pneumoniae* isolates

FASTA sequence

J12 primer

Sample3

TACTCGTCTATGACAATCCAGAGCATACCGCCTTTAAAATTATCAATCGGTATATCCGTTTTGTAGATAAAGACGATTCCAAACCTCG
TTCTGATTGGAAACTGAATGAAGAATGGGCTTGGTTTATTGGGAACAATCGTGAACGATTAAAACTAACCACAAAACCAGAGCCTT
ACTCCTTCCAAAGGACGCTGAACTGGCTATCTCATCAAGTTGCCCCGACCTTAAAGGTTGCGATTAAACTTGATGAAATCAACCAGA
CGCAGGTTGTAAAAGACATTCTCGACCATGCGAAACTGACAGACCGACACAAGCAGATTTTGAAGCAACAGTCAGTAAAAGAACA
GGACGTGATAACAACAAAAAAATAACTCAAATACAAATTCATTGAATATAGAGAGGAGAACATTTTTATGAATTTTGGACAAAACC
TTTATAACTGGTTTCTATCAAACGCTCAATCACTGGTGCTTTTAGCAATCGTTGTGATTGGCTTGTATCTTGGCTTCAAGCGTGAGTT
TAGCAAACTGATTGGCTTTTTAATTATTGCGATTATTGCGGTTGGCTTAGTCTTCAACGCTGCTGGAGTAAAAGACATTTTACTAGA
GCTATTCAATCGCATTATTGGTGCTTAAATAAAACCGTTCTTTTGTGGAATATAAGTGGTTTTCTTATGTTCCGCAAAGGAATGGTAC
ACCAAACGAA

Appendix (4): Nucleotide sequence of erythromycin gene carried by Tn*917* in *S.pneumoniae* isolates

APHA1 primer

Sample1

TATGATTTTTTAAAGACGGAAAAGCCCGAAGAGGAACTTGTCTTTTCCCACGGCGACCTGGGAGACAGCAACATCTTTGTGAAAGA
TGGCAAAGTAAGTGGCTTTATTGATCTTGGGAGAAGCGGCAGGGCGGACAAGTGGTATGACATTGCCTTCTGCGTCCGGTCGATC
AGGGAGGATATCGGGGAAGAACAGTATGTCGAGCTATTTTTTGACTTACT

Appendix (5): Nucleotide sequence of tetracycline gene carried by Tn*916* in *S.pneumoniae* isolates

ERMB1 primer

Sample6

TTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTC
AACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGA
GGTATAAAATTGTTGGGAGTATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACATCT
ATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTC
AGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACAG
ATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATC
AGTTTCATCAAGCAATGAAACACGCCAAAGTAAACAATT

Appendix (6): Nucleotide sequence of erythromycin gene carried by Tn*1545* in *S.pneumoniae* isolates

TETM2 primer

Sample1

CATCAACACATCGAGGTCAGTCTGAACTTTGCGGAAAAGTTTTCAAAATTGAGTATTCGGAAAAAAGACAGCGTCTTGCATATATA
CGTCTTTATAGTGGCGTACTGCATTTGCGAGATTCGGTTAGAATATCGGAAAAGGAAAAAATAAAAATTACAGAAATGTATACTTC
AATAAATGGTGAATTATGTAAAATCGATAAGGCTTATTCCGGGGAAATTGTTATTTTGCAGAATGAGTTTTTGAAGTTAAATAGTGT
TCTTGGAGATACAAAGCTATTGCCACAGAGAGAGAGAATTGAAAATCCCCTCCCTCTGCTGCAAACGACTGTTGAACCGAGCAAAC
CTCAACAAAGGGAAATGTTACTTGATGCACTTTTAGAAATCTCCGACAGTGACCCGCTTCTGCGATATTATGTGGATTCTGCGACAC
ATGAAATCATACTTTCTTTCTTAGGGAAAGTACAAATGGAAGTGACTTGTGCTCTGCTGCAAGAAAAGTATCATGTGGAGATAGAA
ATAAAAGAGCCTACAGTCATTTATATGGAAAGACCGTTAAAAAAAGCAGAGTATACCATTCACATCGAAGTTCCACCGAATCCT

Appendix (6): Nucleotide sequence of erythromycin gene carried by Tn*3872* in *S.pneumoniae* isolates

Int primer

Sample3

GAAGACATTCATGATGGTATTGATGTTGTAGGAAAGAAAATGACACTCTGCCAGCTTTACGCAAAACAGAACGCTCAAAGACCAAA
GGTTAGAAAAAACACTGAAACTGGACGCAAATATCTTATGGATATTTTGAAGAAAGACAAGTTAGGTGTAAGAAGTATTGACAGT
ATTAAGCCATCAGACGCTAAAGAATGGGCTATTAGAATGAGTGAAAATGGTTATGCTTATCAAACCATCAATAACTACAAACGTTCT
TTAAAGGCTTCATTCTATATTGCTATACAAGATGATTGTGTTCGGAAGAATCCATTTGACTTTCAACTGAAAGCAGTTCTTGATGATG
ATACTGTCCCTAAGACCGTACTAACAGAAGAACAGGAAGAAAAACTGTTAGCCTTTGCAAAAGCTGATAAAACCTACAGCAAAAAT
TATGATGAAATTCTGATACTCTTAAAAACAGGTCTTCGTATTTCAGAGTTTGGTGGTTTGACACTTCCAGATTTAGATTTTGAGAATC
GTCTTGTCAATATAGACCATCAGCTATTGAGAGATACTGAAATTGGGTACTACATTGAAACACCAAAGACCAAAAGTGGCGAACGT
CAAGTTCCTATGGTTGAAGAAGCCTATCAAGCATTTAAGCGAGTGTTAGCGAATCGAAAGAATGATAAGCGTGTTGAGATTGATG
GATATAGTGATTTCCTCTTTCTTAATAGAAAGAACTATCCAAAAGTGGCAAGTGATTACAACGGCATGATGAAAGGTCTTGTTAAG
AAATACAATAAGTATAACGAGGATAAATTGCCACACATCACTCCACATAGTTTGCGACATACATTCTGTACCAACTATGCAAATGCA
GGAATGAATCCAAAGGCATTACAGTACATTATGGGACATGCTAATATAGCCATGACGCTGAACTATTACGCACATGCAACATTCGA
TTCTGCAATGGCAGAAATGAAACGCTTGAATAAGAGA

Appendix (7): Nucleotide sequence of erythromycin gene carried by Tn*6002* in *S.pneumoniae* isolates

Xis primer

Sample2

GCGTCAAAATATTTTCGTATTGGCGAAAACAAGCTACGACGCTTGGCAGAGGAAAATAAAAATGCAAATTGGCTGATTATGAATG
GCAATCGTATTCAGATTAAACGAAAACAATTTGAAAAAATTATAGATACATTGGA

صفة المقاومة للتراسايكلين. من ناحية اخرى فقد تم تحديد التعاقب النكليوتيدي لكل جين من جينات المقاومة لمضادات الحياة المحمولة على هذه العناصر المنتقلة ومقارنتها بطريق التراصف النكليوتيدي (Nucleotide alignment) مع نضيراتها من جينات المقاومة لنفس العناصر المنتقلة الموجودة في السلالات القياسية لبكتريا *S.pneumoniae* المبينه في قواعد البيانات العالمية NCBI واشارت نتائج التراصف النكليوتيدي الى وجود تطابق تام 100% بين التعاقب النكليوتيدي لجينات المقاومة لمضادات الحياة المحمولة على العناصر المنتقلة للعزلات المحلية مع التعاقب النكليوتيدي لنفس الجينات المحمولة على نفس العناصر المنتقلة في السلالات القياسية لبكتريا *S.pneumoniae* .

الخـــلاصـــة

جمعت عينات سريرية من نهايات البلعوم واللوزتين فضلا عن عينات من سائل النخاع الشوكي من مرضى قنوات النهايات التنفسية المراجعين لمستشفيات مختلفة من محافظة بغداد. وتم الحصول على 100 عزلة بكتيرية من تلك العينات بعد التنمية على وسائط اكار الدم والشو كليت المتصلبة بالاكار شخصت 51 عزلة من بين العدد الكلي للعزلات مبدئيا على انها *Streptococcus spp*، واظهرت النتائج 15 عزلة على انها *Streptococcus pneumoniae* لقابليتها على تحلل الدم نوع الفا والحساسية الى Optochin. واظهرت نتائج التشخيص المظهري والكيموحيوي لهذه العزلات على انها *S.pneumoniae* حيث كانت موجبة لصبغة غرام، سالبة لاختباري الاوكسيديز والكاتليز، مخمرة للأينولين والتريهالوز واللاكتوز في حين كانت غير مخمرة للسكريات الكحولية المانيتول والسوبريول وقد تم تاكيد تشخيص العزلات البكتيريه باستخدام نظام التشخيص vitek.2. اختبار حساسية العزلات البكتيرية لمضادات الحياة المختلفة واشارت النتائج الى تباين تلك العزلات في نمط المقاومة. حيث بلغت مقاومتها للبنسلين بنسبة 93% ثم للستربتومايسين 87% ثم للتتراسايكلين والترايميثوبريم 80% ثم للكلنداميسين 50% ثم للكاناميسين 50% ثم للارثرومايسين والازثرومايسين 40% ثم للسبروفلاكسين والليفوفلوكاين 20%. استخلص الدنا المجني من عزلات بكتريا *S.pneumoniae* باستخدام العدة التشخيصية المجهزة من شركة promega\USA للتحري عن وجود العناصر المنتقلة الترانسبوزون المشفرة لصدمة المقاومة لمضادات الحياة باستخدام بادئات نكليوتيدية متخصصة لتضخيم جينات المقاومة للمضادات المحمولة على تلك العناصر المنتقلة. و اشارت النتائج الى احتواء7 من العزلات البكتيرية على الترانسبوزون Tn*1545* الذي اكسبها صفة المقاومة للارثرومايسين و4 عزلات حاوية على الترانسبوزون Tn*917* اكسبها صفة المقاومة للارثرومايسين ايضا و5 عزلات حاوية على الترانسبوزون Tn*5397* اكسبها صفة المقاومة للتراسايكلين، و8 عزلات حاوية على الترانسبوزون Tn*3872* اكسبها صفة المقاومة للارثرومايسين , و8 عزلات حاوية على الترانسبوزون Tn*6002* اكسبها صفة المقاومة للارثرومايسين و4 عزلات بكتيرية حاوية على الترانسبوزون Tn*916* اكسبها

بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

نَرْفَعُ دَرَجَاتٍ مَّن نَّشَاءُ وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ ۞

صَدَقَ اللّٰهُ العَظيم

سورة يوسف

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية التقنيات الاحيائية

التحري الجزيئي عن العناصر المنتقلة المشفرة للمقاومة لمضادات الحياة في عزلات سريرية لبكتريا *Streptococcus pneumoniae*

رسالة

مقدمة الى مجلس كلية التقنيات الأحيائية، جامعة النهرين وهي جزء من متطلبات نيل درجة الماجستير في التقنيات الاحيائية

من قبل

دعاء حمود أدعير آل منشد

بكالوريوس تقانة احيائية ،كلية العلوم، جامعة النهرين، 2015

تحت أشراف

الأستاذ الدكتور

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ربيع الأول **كانون أول**

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