CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Community-acquired pneumonia (CAP) is a term used to describe one of several diseases in which individuals who have not recently been hospitalized develop an infection of the lungs (Andrews et al., 2003). The CAP is considered to be a major medical problem and potentially life-threatening illness (Marrie, 2000). Among infectious diseases, CAP is the leading cause of death in the world and especially in elderly, children and immunocompromised patients. The disease is associated with a substantial economic burden to health (Michael et al., 2001). The major signs and symptoms of CAP are difficulty and shortness in breathing, fever, chest pain which may be pleuritic and productive cough. The later occurs because the areas of the lung which absorb oxygen (alveoli) from the atmosphere become filled with fluid and work ineffectively (Hoare and Lim, 2006; Limpar, 2011; Watkins and Lemonovish, 2011).

Complications of CAP include spreading of the infecting organism directly to extra pulmonary sites such as the pleural space giving rise to empyema or indirectly via the blood to other parts of the body (Marimon et al., 2015). Individuals with symptoms of CAP may be diagnosed clinically which may reveal fever, an increased respiratory rate (tachypnea), low blood pressure (hypotension), a fast heart rate (tachycardia) and changes in the amount of blood oxygen (Castro-Guardiola et al., 2000; Bochud et al., 2001; Flanders et al., 2004). Other diagnosis includes x rays of the chest, examination of the blood and sputum for infectious microorganisms and serology. The use of each test depends on the severity of illness, local practices and the concern for any complications resulting from the infection (Watkins and Lemonovish, 2011).

According to World Health Organization report, 3 – 4 million people die due to pneumonia, a large proportion of whom are children or elderly (Lopez et al., 2006). Pneumonia is the third most common cause of death in the world accounting for 7% of
the total mortality of 56 million people (Lopez et al., 2006). The disease is a common health problem in industrialized countries, as well as in developing countries, and may still be life threatening despite the availability of effective antibiotic therapy and supportive care in intensive care units (ICU) (Paul et al., 2002). Pneumonia ranks among the ten leading causes of death in the USA and the European countries. In the USA, pneumonia causes approximately 60,000 deaths annually, which equals 20 deaths per 100,000 population (Mokdad et al., 2000).

In adults, CAP is mainly caused by bacteria such as Streptococcus pneumonia (S. pneumoniae), Haemophilus influenza (H. influenzae) (especially Haemophilus influenzae Type B) (H. influenzae b), Klebsiella pneumoniae (K. pneumoniae), Staphylococcus aureus (S. aureus), Mycoplasma pneumoniae, Chlamydia pneumoniae and less commonly Legionella pneumophila. In children, CAP is more often viral in origin and bacterial secondary to a viral respiratory infection (Sharma et al., 2007; Ruuskanen et al., 2011). In the pre-antibiotic era, S. pneumoniae caused 95% of cases of pneumonia (Gray and Musher, 2008). Although Pneumococcus remains the most commonly identified cause of CAP, the frequency with which it is implicated has declined and it is now detected in only about 10 to 15% of inpatient cases in the United States (Restrepo et al., 2008; File et al., 2010; Musher et al., 2013; Sherwin et al., 2013). Recognized factors contributing to this decline include the widespread use of pneumococcal polysaccharide vaccine in adults, (Moberley et al., 2013) the nearly universal administration of pneumococcal conjugate vaccine in children (Griffin et al., 2013) and decreased rates of cigarette smoking (Nourt et al., 2000). In Europe and other parts of the world where Pneumococcal vaccines have been used less often and smoking rates remain high, Pneumococcus remains responsible for a higher proportion of cases of CAP (Huijts et al., 2013; Rozenbaum et al., 2013). Both capsulated and non-capsulated or non-typeable strains of H. influenza can cause pneumonia. H. influenza type b was one of the most common causes of invasive H. influenzae infection, including bacteremia, pneumonia, whereas Non typeable H.
*influenzae* was generally considered to be a major cause of chronic respiratory infections and pneumonia in adults. However, following the introduction of *H. influenza type b* conjugate vaccines, the incidence of invasive *H. influenza type b* disease including pneumonia, has declined dramatically in all countries where they are routinely used (Peltola, 2000). Prior to the introduction of the *H. influenza type b* conjugate vaccine, invasive *H. influenzae* disease was predominantly caused by *H. influenza type b* (90%) with 90% of cases occurring in young children (Marry, 2015).

*K. pneumoniae* is an important pathogen causing a wide range of infections. In Europe and North America the incidence of these infections is particularly high among alcoholics and hospitalized persons (Podschun and Ullmann, 1998). In Southeast Asia, this pathogen is frequently reported as a major cause of CAP (Liam *et al.*, 2006) and commonly found in the innate environment (Haryani *et al.*, 2007).

*K. pneumoniae* is also a commensal of the nasopharynx and intestines. The carriage prevalence in the community in Europe and North America was reported to be 1-6% in nasopharynx and 5-38% in the intestinal tract. These rates increased markedly in hospitalized patients up to 19% in pharynx and 70% in intestinal tract (Podschun and Ullmann, 1998).

### 1.2. Rationale

Community acquired pneumonia (CAP) is considered one of the most common acquiring disease in the world. The disease is not a reportable illness, thus information about its incidence is based on crude estimation. It appears that up to 5.6 million cases of CAP occur annually and as many as 1.1 million of these require hospitalization (Niederman *et al.*, 1998). In the outpatient setting, the mortality rate of CAP remains low, in the range of < 1–5%, but among patients who require hospitalization, the mortality rate is up to 12% overall (Bates *et al.*, 1998).

Compared to culture and serological methods, PCR is more sensitive in the diagnosis of CAP, allowing the detection of low number of the causative agents and also to determine the resistant of the antibiotics, it is not time consuming and it is not expensive but the problem is that it cannot differentiate between colonization and infection (Murdoch *et al.*, 2007).
2003). Also, the culture and the serological methods will not show the true picture about the carriage of the pathogens in the nasopharynx and severity and incidence of the CAP (Jin et al., 2009). In Sudan, the main problem arises from the lack of reports which reflect the number, severity, methods of diagnosis and treatment of the disease among Sudanese patients. Revising the literature, there is no any published data about CAP. This research is expected to investigate this problem thoroughly and reflect the true picture of the disease. The causative agents and its antibiograms will also be investigated.

1.3. Objectives

1.3.1. General objective

To study community-acquired bacterial pneumonia phenotypically and genotypically in Khartoum State.

1.3.2. Specific objectives

1. To detect the causative agents of community-acquired pneumonia in sputum by conventional methods.

2. To utilize PCR in detecting the causes of CAP directly in specimens.

3. To compare between phenotypic and genotypic methods applied in the investigation.

4. To detect \( SHV \) and \( TEM \) resistance genes in \( Klebsiella pneumoniae \).

5. To apply sequencing of \( Streptococcus pneumoniae \).
CHAPTER TWO

LITERATURE REVIEW

2.1. Community-acquired pneumonia

Community-acquired pneumonia (CAP) refers to pneumonia (any of several lung diseases) contracted by a person with little contact with the healthcare system. The chief difference between hospital-acquired pneumonia (HAP) and CAP is that patients with HAP live in long-term care facilities or have recently visited a hospital. CAP is commonly affecting people of all ages, and its symptoms occur as a result of oxygen-absorbing areas of the lung (alveoli) filling with fluid. This inhibits lung function, causing dyspnea, fever, chest pains and cough (Kamizono et al., 2010).

CAP, the most common type of pneumonia, is a leading cause of illness and death worldwide. Its causes include bacteria, viruses, fungi and parasites (Feldman et al., 2008). CAP is diagnosed by assessing symptoms, making a physical examination and on x-ray. Other tests, such as sputum examination, supplement chest x-rays are also applied. The use of each test depends on the severity of illness, local practices and the concern for any complications resulting from the infection (Michael et al., 2001). Patients with CAP sometimes require hospitalization, and it is treated primarily with antibiotics, antiviral, antipyretics and cough medicine (Dallaire et al., 2001).

Most commonly observed symptoms includes; shortness of breath (dyspnea), coughing which produces greenish or yellow sputum, a high fever, accompanied by sweating, chills and shivering, chest pains, rapid shallow and often-painful breathing. The less commonly observed symptoms includes, coughing up blood (hemoptysis), Headaches, including migraines, loss of appetite, excessive fatigue, bluish skin (cyanosis), nausea, vomiting, diarrhea, joint pain (arthralgia), muscle aches (myalgia), rapid heartbeat and dizziness or lightheadedness (Hoare and Lim, 2006).
According to population-based studies the annual incidence of CAP among the adult population varies between 1.6 to 9 cases per 1000 per year (Almirall et al., 2000, Woodhead, 2002). The incidence of CAP is higher in males than females (Almirall et al., 2000, Gutierrez et al., 2006). There is a seasonal variation in the incidence of CAP; the incidence is highest in winter (Woodhead et al., 1987; Almirall et al., 2000). Age is strongly associated with the incidence of CAP. Among patients aged ≥ 65 years the incidence of CAP is two to five times higher than among adult patients aged < 65 years (Almirall et al., 2000; Woodhead, 2002; Gutierrez et al., 2006). According to various studies 22 - 42% of the patients with CAP require hospitalization (Guest and Morris, 1997; Jokinen et al., 2001). Patients aged 65 years or older account for about half of all patients hospitalized for CAP (Guest and Morris, 1997; Niederman, 1998). At least partly due to the aging of the population, hospital admissions for pneumonia have been rising during the recent years (Thomsen et al., 2006; Trotter et al., 2008). Between 5% and 10% of the hospitalized patients require management in an ICU (Wilkinson and Woodhead, 2004; Thomsen et al., 2006; Woodhead et al., 2006; Trotter et al., 2008). The mortality rate among patients in open care is less than 1% and among hospitalized patients 4% to 15% (Woodhead, 2002; Colice et al., 2004; Mandell, 2004). Mortality rates are highest among elderly patients (18 %), and patients with severe disease who require treatment in an ICU (22% to more than 50%) (Fine et al., 1996; Woodhead et al., 2006).

Managing CAP has also a substantial economic impact. In the 1990s the annual cost to treat CAP was estimated to be 8.4 to 12.2 billion dollars in the USA (Niederman et al., 1998; Colice et al., 2004). Most of this cost was due to treatment of hospitalized patients with CAP; the mean treatment cost for an outpatient episode of CAP was approximately 500 dollars and for an inpatient episode 6000 to 10 000 dollars (Niederman et al., 1998; Colice et al., 2004). In the United Kingdom, the annual cost to treat CAP in the 1990s was £441 million. Hospital treatment for CAP accounted for 96% of the total costs. The average cost for outpatient treatment of CAP was £100 per episode and for hospitalized patients £1700 - £5100 per episode (Guest and Morris, 1997). More than half of
employed patients with CAP require sick leave for more than two weeks (Woodhead et al., 1987; Almirall et al., 2000). CAP affects approximately 4.5 million adults in the United States annually. About one third of these adults require hospitalization. The mortality rate among hospitalized patients with CAP varies each year and can reach 35 percent (Paul et al., 2002). Current approaches to the empirical management of CAP emphasize the type of patient ("community" or "hospital"), rather than the type of symptoms ("typical" or "atypical (Paul et al., 2002, Kristopher et al., 2004).

Over 100 microorganisms can cause CAP, most cases caused by *Streptococcus pneumoniae* (*S. pneumoniae*) in all age groups (Huijts et al., 2013). Infants can acquire lung infections before birth by breathing infected amniotic fluid or through a blood-borne infection which crossed the placenta. Infants can also inhale contaminated fluid from the vagina at birth. The most prevalent pathogen causing CAP in newborns is *Streptococcus agalactiae*. Other bacterial causes of neonatal CAP include *Listeria monocytogenes* and a variety of *Mycobacteria*. CAP-causing viruses may also be transferred from mother to child; *Herpes simplex virus* (the most common) is life-threatening, *Adenovirus, Mumps* and *Enterovirus* can also cause pneumonia (Webber et al., 1990). Another cause of CAP in this group is *Chlamydia trachomatis*, acquired at birth but not causing pneumonia until two to four weeks later; it usually presents with no fever and a characteristic, staccato cough (Zheng et al., 2015).

CAP in older infants reflects increased exposure to microorganisms, with common bacterial causes including *S. pneumoniae, H. influenzae, Escherichia coli, K. pneumoniae, Moraxella catarrhalis* and *S. aureus*. Viruses include, *Human respiratory syncytial virus* (RSV), *Adenovirus, Human para influenza viruses, Influenza and Rhinovirus*. (Sharma et al., 2007; Ruuskaenen et al., 2011; Limper, 2011).

Although children older than one month tend to be at risk for the same microorganisms as adults, children under five are much less likely to have pneumonia caused by *Mycoplasma pneumoniae* (*M. pneumoniae*), *Chlamydophila pneumoniae* (*C.
*pneumoniae* or *Legionella pneumophila* (*L. pneumonphila*). In contrast, older children and teenagers are more likely to acquire *M. pneumoniae* and *C. pneumoniae* than adults (Wubbel *et al.*, 1999 and Wattanathum *et al.*, 2003).

A full spectrum of microorganisms is responsible for CAP in adults, and patients with certain risk factors are more susceptible to infections of certain groups of microorganisms. Many less-common organisms can cause CAP in adults, and are identified from specific risk factors or treatment failure for common causes. Risk factors include poor nutritional status, low housing quality second hand smoke exposure, comorbidities such as asthma, and preceding upper respiratory tract infection (Camargo *et al.*, 2012). Alcoholism and chronic diseases, such as respiratory disease, cardiovascular disease, or kidney disease, also increase the risk for pneumonia, especially in the older population (Marrie, 2000; Müllerova *et al.*, 2012; Haessler and Schimmel, 2012). In the pediatric population, very young children are at increased risk because their immune systems have not fully developed. Diseases or medications that suppress the immune system increase the risk among all ages (Farr *et al.*, 2000).

Patients with symptoms of CAP require evaluation. Physical examination by a health provider may reveal fever, an increased respiratory rate (tachypnea), low blood pressure (hypotension), a fast heart rate (tachycardia) and changes in the amount of oxygen in the blood. Palpating the chest as it expands and tapping the chest wall (percussion) to identify dull, non-resonant areas can identify stiffness and fluid, signs of CAP. Listening to the lungs with a stethoscope (auscultation) can also reveal signs associated with CAP. A lack of normal breath sounds or the presence of crackles can indicate fluid consolidation. Increased vibration of the chest when speaking, known as tactile fremitus, and increased volume of whispered speech during auscultation can also indicate fluid (Metlay *et al.*, 1997, Kyung *et al.*, 2010).

When signs are discovered, chest X-rays, examination of the blood and sputum for infectious microorganisms and blood tests are commonly used to diagnose CAP.
Diagnostic tools depend on the severity of illness, local practices and concern about complications of the infection. All patients with CAP should have their blood oxygen monitored with pulse oximetry. In some cases, arterial blood gas analysis may be required to determine the amount of oxygen in the blood. A complete blood count (CBC) may reveal extra white blood cells, indicating infection (Michael et al., 2001).

Microscopic examination of pulmonary secretions may provide immediate information about possible causative organisms. Results of Gram staining and culture of sputum are positive in more than 60% of cases of CAP when a good quality of sputum specimen can be obtained before, or within 6 to 12 hours after, the initiation of antibiotics (Strallin et al., 2006). In addition, culture methods are time consuming and less sensitive for the isolation of the causative agents (Morozumi and Nakayama, 2006). The low sensitivity of blood and sputum cultures and the presence of a heterogeneous bacterial flora in the upper respiratory tract confuse the interpretation of the results (Forbes et al., 2007).

Polymerase chain reaction based diagnosis of CAP is used worldwide for rapid detection of the infectious agents (Murdoch, 2003). The detection of specific types of bacteria in sputum samples using PCR is a promising approach for the rapid diagnosis of the disease (Murdoch et al 2003). The method is a remarkably sensitive and type specific than other methods that used routinely for identifying respiratory pathogens (Wang et al., 2008).

Chest X-rays and X-ray computed tomography (CT) can reveal areas of opacity (seen as white), indicating consolidation. CAP does not always appear on x-rays, because the disease is in its initial stages or involves a part of the lung an x-ray does not see well. In some cases, chest CT can reveal pneumonia not seen on x-rays. X-rays can often mislead, as heart failure or other types of lung damage can mimic CAP on x-rays (Syrjälä et al., 1998).

Early initiation of antimicrobial therapy increases the likelihood of a good outcome depending in part on the diagnostic techniques that are used. Pathogen-directed therapy greatly decreases the cost of care and reducing the risk of developing complications
CAP is treated with an antibiotic that kills the offending microorganism and by managing complications. If the causative microorganism is unidentified (often the case), the laboratory identifies the most-effective antibiotic; which will take several days. Primary microorganisms are viruses, atypical bacteria, penicillin-sensitive \textit{S. pneumoniae} and \textit{H. influenzae}. Recommended drugs are macrolide antibiotics, such as azithromycin or clarithromycin, for seven to ten days (Niederman \textit{et al.}, 2001).

Health professionals consider a person's risk factors for various organisms when choosing an initial antibiotic. Additional consideration is given to the treatment setting; most patients are cured by oral medication, while others must be hospitalized for intravenous therapy or intensive care (Li \textit{et al.}, 2007, Dimopoulos \textit{et al.}, 2008).

Vaccination against \textit{H. influenzae} and \textit{S. pneumoniae} in the first year of life has reduced their role in childhood CAP. A vaccine against \textit{S. pneumoniae}, available for adults, is recommended for healthy individuals over 65 and all adults with heart failure, diabetes mellitus, cirrhosis and alcoholism. Re-vaccination may be required after five or ten years (Butler \textit{et al.}, 1993).

\section*{2.2. \textit{Streptococcus pneumoniae}}

\subsection*{2.2.1. Historical background}

The bacterium itself was discovered in 1880 by Sternberg, a US Army physician and by Louis J. Pasteur (1822–1895) independently, Pasteur named the bacteria \textit{Microbe septicemique du salive} and Sternberg named it \textit{Micrococcus pasteuri} than the first name \textit{Diplococcus pneumoniae} was given (Grabenstein and Klugman, 2012). In 1886 Fränkel identified it as the causative agent of lobar bacterial pneumonia and not so long afterwards its pathogenic role was proved also in meningitis, endocarditis, arthritis and otitis media.
In the turn of the 20th century realms of immunology and vaccinology started using *S. pneumoniae* (Pneumococcus) for fundamental researches. In 1909 Neufeld and Händel described different serotypes of Pneumococci and observed that protection by immune serum was type-specific (Neufeldand and Handel, 1909). With the help of this finding they recognized the specific antisera and its role in therapy which resulted in the first effective treatment in the pre-antibiotic era against Pneumococcal infections. This experience helped Oswald T. Avery and his colleague Michael Heidelberger in 1923 to determine the Pneumococcal antigens that induced protective immunity as carbohydrates (Kushner and Samols, 2011).

The beginnings of molecular biology could be owed to *S. pneumoniae* as well. In 1928, Griffiths proved that *S. pneumoniae* are capable of taking up large foreign DNA fragments from the surroundings (Austrian, 1981).

In 1944, Avery together with Colin MacLeod and Maclyn McCarty demonstrated their favorite experiment with the help of different virulence of R (rough, non-capsulated) and S (smooth, capsulated) colonies and proved the chemical nature of the principle of transforming substance as DNA (Avery et al., 1944). After the introduction of penicillin in 1940s, treatment of the Pneumococcal infections seemed to be solved. Nevertheless, the first penicillin resistant strains appeared in 1960s, followed by multidrug resistant (MDR) strains, which still remained a problem (Klugman, 1990).

### 2.2.2. Morphology and cultivation

*S. pneumoniae* is Gram-positive cocci, sized 0.8-1.5 μm, forming pairs or short chains (lancet shape). Chain formation is more frequent when grown on solid medium. The bacterium can have pili and are encapsulated, with a large polysaccharide capsule.

*S. pneumoniae* is a fastidious, facultative anaerobe, bacterium, growing best on media supplemented with 5% blood and requires 5% to 10% CO2 for incubation at 35-37°C. On
blood agar, it shows \( \alpha \)-hemolysis and the colonies have special umbilicus (draught man appearance) morphology, due to autolytic activity (Kilian, 2005).

### 2.2.3. Identification

As a respiratory pathogen, it can be found in the nasopharynx and in the sputum sample during infection, but sometimes the less fastidious normal microbiota members, such as the genetically related *Streptococcus viridans* (*S. viridans*) (for example *Streptococcus mitis*) can over grow the Pneumococci leading to problems in identification.

The classical diagnostic identification of Pneumococci is based on colony morphology (\( \alpha \)-haemolysis and characteristic colonies), optochin sensitivity, although optochin resistant strains were described (Pikis *et al.*, 2001) and some biochemical activities, such as the lack of catalase enzyme, bile solubility (when the surface active bile salts provide full activity for the autolytic enzymes) or inulin degradation, for separation from the *S. viridans* (Lund and Henrichsen, 1987). Antigen detection is possible from sterile body fluids (e.g. in case of meningitis) with latex agglutination. Urinary antigen tests are also available. These are rapid immune chromatographic tests which detect the C polysaccharide cell wall antigen common to all strains of *S. pneumoniae* and can be positive for weeks to several months after the infection (Domínguez *et al.*, 2001). For serotyping pneumococci, monovalent antisera and latex agglutination kits are also available (Mudd *et al.*, 1943, Sorensen, 1993). The most reliable way for identification is using molecular biological methods, such as PCR for Pneumococcal specific genes like *lytA* gene (Nagai *et al.*, 2001), *ply* gene (Matos *et al.*, 2006), *pspA* gene (Pimenta *et al.*, 2006), *cpsA* gene (Luo *et al.*, 2012) or detection of a conserved gene such as 16S r RNA (Scholz *et al.*, 2012).

### 2.2.4. Virulence factors

Properties that explain the pathogenic potential of *S. pneumoniae* include polysaccharide capsule, pili, different enzymes such as IgA1 protease, pneumolysin, autolysin and
several surface-exposed proteins that mediate contact with components of host tissues and secretions.

### 2.2.4.1. Capsule

Capsule is the major virulence factors of Pneumococci and it was the first non-protein substances shown to be antigenic in humans. Fred Neufeld described first the process to differentiate Pneumococci into serotypes with the help of type-specific antisera and he also discovered that these type-specific antigens were carbohydrates, which are the basement of the capsule. So far we know 94 different capsular polysaccharides (CPSs), which are distinguished by using a set of antisera that recognize the chemical differences in the capsules; therefore they are called serotypes (Jin et al., 2009). Expression of a capsule is important for survival in the blood and is associated with the ability of Pneumococci to cause invasive disease. CPSs are built up from repeating oligosaccharide units and antibodies against these saccharides provide protection against Pneumococcal disease. The most frequent monosaccharides in CPSs in different combinations are α/β-D-glucose, α/β-D-galactose, α/β-L-rhamnose, N-acetyl-α/β-D-glucosamine, N-acetyl-α/β-D-galactosamin, N-acetyl-β-D-mannosamine, N-acetyl-α-L-fucosamine and α/β-D-glucuronic acid (Jin et al., 2009).

There are significant differences between different serotypes. In addition to the above mentioned monosaccharides, α-L-fucose (serotype 19A), β-D-ribose (serotype 7, 19B, 19C), 2-acetamindo-4-amino-2,4,6-trideoxy-α-D-galactose (serotype 1) can be present. Comparison of the CPSs of serotype 6A and 6B shows that the only difference is the presence of 3-substituted D-ribitol in 6A and 4-substituted D-ribitol in 6B. The capsular gene (cps) locus, responsible for the expression of CPSs - with the exception of serotypes 3 and 37 (Llull et al., 1999) - is located between dex B and ali A on the Pneumococcal chromosome (Bentley et al., 2006) and the total size of alternative coding DNA at this one locus exceeds 1.8 M bp. The regulatory and processing genes wzg, wzh, wzd and wze (also known as cps ABCD) are conserved with high sequence identity in all cases
and are almost always in this gene order at the 5′ end of the cps locus (Bentley et al., 2006). In most cps clusters, the fifth gene encodes the initial glucose phosphate transferase, Wch A (also known as cps E), responsible for linkage of an activated glucose phosphate to the lipid carrier. The polysaccharide polymerase (wzy) and flippase (wzx) genes are always present downstream together with a varying set of genes for glycosyl transferases, acetyl transferases, nucleotide diphosphate sugar biosynthesis and modifying enzymes. In the regions between the cps genes and the flanking dexB and aliA genes, there is almost always evidence of mobile genetic elements. Due to the natural transformability of the Pneumococcus, horizontal recombination allows that one serotype can belong to different genotypes and a single genotype can express different capsular genes, i.e., different serotypes. This phenomenon is known as capsular switching. *S. pneumoniae* capsule can affect several aspects of complement activity. These include preventing binding of both IgG (Immunoglobulin G) and CRP (C-reactive protein) to *S. pneumoniae* and thereby inhibiting classical pathway activity, reducing alternative pathway activity through unexplained mechanisms and decreasing the degradation of C3b bound to the bacterial surface to iC3b. The effects on C3b/iC3b deposition prevent phagocytosis of encapsulated bacteria, but data also suggest that the capsule inhibits phagocytosis mediated directly by IgG and by non-opsonic phagocytic receptors. The results clarify some of the mechanism by which the *S. pneumoniae* capsule could mediate immune evasion (Hyams et al., 2010).

### 2.2.4.2. The presence of pilus

*S. pneumoniae*, like many other Gram-positive bacteria, has long filamentous pili extending on their surface through which they adhere to host cells. This may be the first of those virulence factors which are responsible for initial adhesion of the bacteria to host tissues during colonization and biofilm formation (Barocchi et al., 2006).

Pilus genes can be found in a 12 Kb pathogenicity island called Pilus islet (PI-1), encoding a positive transcriptional regulator (rlrA), the pilus-1 structural subunits (rrgA,
rrgB and rrgC) and three pili-specific sortases. RrgB encodes the major component RrgB which is strictly necessary for the pilus formation while the other two (RrgA, RrgC encoded by rrgA and rrgC) are ancillary proteins (Telford et al., 2006). PI-1 is present in about one-third of the clinical isolates (Moschioni et al., 2008) and its prevalence is higher among antibiotic non-susceptible strains (Sjöström et al., 2007). Pilus subunits are immunogenic in humans (Mora et al., 2005) and were able to elicit a protective response when tested in mouse models of infection (Rosini et al., 2006). Thus pili could be a good target to develop a new vaccine. Recent studies demonstrated that pilus can be dedicated to DNA transformation in Pneumococci (Balaban et al., 2014). According to Laurenceau the transformation pilus act as a “DNA-trap” to capture DNA in the environment (Laurenceau et al., 2013).

2.2.4.3. Surface proteins

Many of the surface proteins are virulence factors that contribute to the pathogenesis of this organism. Three main groups of them have been identified in *S. pneumoniae*: around 50 lipoproteins, up to 18 peptidoglycan binding (LPxTG) consensus sequence-carrying proteins that are covalently linked via sortase to the cell wall peptidoglycan and up to 16 choline-binding proteins (CBPs). In addition to the three main groups of surface proteins, the cell wall of Pneumococci is decorated with another cluster of proteins that lack classic leader peptide and membrane-anchoring motifs. These proteins are termed non-classical surface proteins (NCSPs) and they could also play a relevant role in subverting the physiological function of host-derived proteins (Bergman and Hammerschmidt, 2006).

2.2.4.4. Cell wall components

The cell wall of *S. pneumoniae* contains an unusually complex wall TA, which has identical repeating units as the membrane-anchored LTA. Although the structure of the Pneumococcal peptidoglycan was found to resemble that of other characterized Gram-positive bacteria, the wall TA is complex and unusual and contains choline as a structural component. Thus Pneumococcus is unique among prokaryotes due to an absolute
requirement for choline in growth, which is incorporated as phosphorylcholine into the cell wall TA and the membrane LTA (Denapaite et al., 2012).

2.2.4.5. Most important enzymes

2.2.4.5.1. Pneumolysin

A key virulence factor of Pneumococcus in colonizing the upper respiratory tract is the pneumococcal thiol-activated, membrane pore forming toxin, called pneumolysin (Hirst et al., 2004). Pneumolysin is a 53 kDa protein composed of 471 amino acids. It is common to all serotypes and it can be thought of as a multi-effective factor for virulence following pneumococcal infection. At high levels it is lytic to all cells with cholesterol in the membrane. The lytic activity of this toxin can be inhibited by pre incubation with cholesterol, consistent with the suggestion that membrane cholesterol is the receptor for this toxin (Maus et al., 2004). At lower, sublytic concentrations, which exist in the early stages of infection, the toxin also may cause a range of effects, including induction of apoptosis, activation of host complement and induce pro inflammatory reactions in immune cells. At higher lytic concentrations, which may exist in the later stage of infection, the toxin may cause widespread direct cellular and tissue damage by virtue of its membrane pore forming properties (Marriott et al., 2008).

2.2.4.5.2. Autolysin

*S. pneumoniae* has a special autolytic response that leads to the excessive lysis of cultures in vitro and causes the characteristic colony morphology called umbilicated shaped colonies. The main autolysin in the Pneumococcus is N-acetyl-muramoyl-l-alanine amidase, commonly known as LytA, which encoded by the lytA gene. LytA causes lysis by cleaving the lactyl-amide bond that links the stem peptides and the glycan strands of the peptidoglycan, resulting in hydrolysis of the cell wall. LytA orthologs are now known to be conserved throughout eubacteria and in many bacteriophages (Romero et al., 2004 and Morales et al., 2010).
The in vivo function of the suicide LytA enzyme remains controversial. One hypothesis is that LytA mediates lysis to release other virulence factors such as pneumolysin (Martner et al., 2008).

Another theory suggests that LytA is released to lyse neighbouring non-competent Pneumococcal cells. This would potentially facilitate genetic exchange between naturally competent pneumococcal populations that easily take up and incorporate DNA by homologous recombination (Eldholm et al., 2009).

A third possibility is that LytA mediates lysis to release proteins involved in immune evasion or cell wall components that may interfere with the host immune response (Martner et al., 2009).

It is also interesting that there is a highly polymorphic region in the lytA gene where two different families of alleles can be differentiated by PCR and restriction digestion. Morales et al (2010) proved that this polymorphic region arose from recombination events with homologous genes of Pneumococcal temperate phages.

2.2.4.5.3. IgA protease

Antibodies of the immunoglobulin A (IgA) class react with capsular polysaccharides of S. pneumoniae and support complement-dependent opsonophagocytosis of the organism by phagocytes. IgA may provide both local defense against mucosal infection and activity in local tissues to prevent dissemination of the infection (Fasching et al., 2007).

The IgA1 protease is one of the two to four large zinc metalloproteinase present in the Pneumococcal genome (Camilli et al., 2006). This protease is a polypeptide of about 1900 amino acids associated to the bacterium via N-terminal anchoring. The enzyme specifically cleaves the hinge regions of human IgA1, which dominates most mucosal surfaces and is the major IgA isotype in serum. This protease is expressed in all of the known pneumococcal strains and plays a major role in pathogen's resistance to the host immune response (Wani et al., 1996).
2.2.4.5.4. Other enzymes

Most of the human cell surfaces and secreted molecules are glycosylated. This glycosylation is often complex and involves a number of different sugar residues. Glycosylation serves a number of functions including recognition processes, cell-to-cell interactions and the binding and transport of positively charged molecules (Schauer, 2000). Pneumococcus expresses a variety of enzymes: surface attached exoglycosidases: neuraminidase, β-galactosidase and N-acetylglucosaminidase, which sequentially remove terminal sugars common to many human glycoconjugates. Deglycosylation of host molecules may expose cell-surface receptors, inhibit mechanisms of clearance that require these glycoproteins, or provide nutrition for the organism (Weiser, 2010).

2.2.5 Genetic background of S. pneumoniae

2.2.5.1. Evolution of S. pneumoniae

Based on Kilian et al (2008) research, it is supposed that the immediate common ancestor of S. pneumoniae, S. mitis and S. pseudo pneumoniae (pneumoniae-mitis-pseudo pneumoniae) cluster was a bacterium with resemblance to the present-day Pneumococcus with all the properties associated with virulence. One of these properties, the IgA1 protease evolved by gene duplication in response to emergence of the immunoglobulin A1 (IgA1) subclass in the common ancestor of man, chimpanzees and gorillas, which according to recent calculations existed 6 to 7 million years ago (Hobolth et al., 2007). While the Pneumococcus lineage conserved the expression of both capsule production and IgA1 protease activity to ensure their ability to colonize in the presence of IgA1 antibodies (Weiser et al., 2003), lineages evolving into a commensal life style with a more subtle relationship with the mucosal immune system and the host in general gradually lost both characters and achieved the colonization advantage of the capsule-deficient phenotype (Weiser et al., 1994). This evolutionary model proposing that the pneumoniae-mitis-pseudopneumoniae cluster arose from a Pneumococcus-like organism pathogenic to the immediate ancestor of hominoids is consistent with our inability to
isolate *S. mitis*-like bacteria from a range of mammals including old and new world monkeys, pigs, dogs, sheep, cattle, rats and mice, while there is evidence of Pneumococci causing infections in chimpanzees and other mammals (Chi *et al.*, 2007, Kilian *et al.*, 2008 and Denapaite and Hackenbeck, 2011).

### 2.2.5.2. Genotyping of Pneumococcus

For a better taxonomic resolution 16S r RNA gene sequence analysis, multilocus sequence analysis (MLSA) (Enright and Spratt, 1998), average amino acid identity (AAI) (Hanage *et al.*, 2006), Genome-to-genome distances (GGD) (Auch *et al.*, 2010) and codon usage analysis (Wright, 1990) can be rarely used (Thompson *et al.*, 2013). Currently, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are the gold standards for genotyping of Pneumococci (Lefevre *et al.*, 1993, Hall *et al.*, 1996).

Advantages of PFGE are that it has good type ability, reproducibility and resolving power. In addition, the costs for materials and equipment are relatively low and handling of the equipment is easy. However, it is laborious and time consuming and may yield ambiguous results if not performed by a well-trained technician. PFGE is quite useful for local epidemiology and it has also been used for global epidemiology once standardized (McGee *et al.*, 2001).

MLST is a DNA sequence-based method that relies on PCR amplification and sequencing of internal fragments of 7 housekeeping genes [aroE (shikimate dehydrogenase), gdh (glucose-6-phosphate dehydrogenase), gki (glucose kinase), recP (transketolase), spi (signal peptidase I), xpt (xanthine phosphoribosyltransferase), ddl (D-alanine-D-alanine ligase)] (McGee *et al.*, 2001). For allele assignment, each sequence is compared to all known alleles which are available at an online database. Different sequences are assigned different allelic numbers. The 7 assigned allele numbers form an allelic profile or sequence type (ST). MLST is expensive; therefore many laboratories cannot afford to use it routinely. However, it has the advantages of being reproducible,
unambiguous, portable allowing intra-laboratory comparisons and suited to create international databases. For *S. pneumoniae*, MLST has a good resolving power being useful for local and global epidemiology. Furthermore, in contrast to PFGE, MLST does not always require a culture and can sometimes be directly performed on samples containing bacterial DNA such as cerebrospinal fluid (Enright *et al*., 2000).

In 1992 conserved repeated sequences, named BOX elements, were identified in the genome of the Pneumococcus. The genome contains 115 and 127 BOX elements, respectively. BOX elements consist of 3 different subunits, BoxA, BoxB and BoxC. The function and origin of BOX elements are unknown; however, they may be involved in regulating the expression of virulence-associated genes (Martin *et al*., 1992). A multiple-locus variable number tandem repeat analysis (MLVA) scheme based on BOX typing was introduced in 2005. It analyses 16 BOX loci that are PCR-amplified in single PCR reactions and products are analysed by agarose gel electrophoresis. A website (www.mlva.eu) providing a database in which profiles can be compared has been created (Koeck *et al*., 2005).

### 2.2.5.3. Serotyping of Pneumococcus

*S. pneumoniae* serotyping was developed at the beginning of the 20th century using panels of specific anti-sera produced in animals and directed against polysaccharides of the pneumococcal capsule. For several decades, the traditional capsular swelling test, the Quelling reaction was the gold standard method (Siira *et al*., 2012). To perform traditional agglutination assays, growth of *S. pneumoniae* on culture media is required. However, culture is often negative if patients received antibiotics before sampling of blood, cerebrospinal fluid or other biological fluids. In recent years, immunological assays based on ELISA (Enzyme-linked immuno sorbent assay) or latex agglutination has been shown to work directly on clinical specimens (Leeming *et al*., 2005). PCR-based serotyping using primers that amplify serotype-specific sequences are also widely used method for serotyping (Azzari *et al*., 2008, Raymond *et al*., 2013).
2.2.6. Clinical aspects

*Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide, particularly in young children, individuals with chronic cardiopulmonary disease, and the elderly and immunocompromised patients. In splenectomised patients, where the encapsulated organisms are the most virulent pathogens, pneumococcus is the most important, but *Haemophilus influenzae* and *Neisseria meningitidis* are also significant (Morgan and Tomich, 2012). *S. pneumoniae* causes wide variety of infections including mucosal infections, sinusitis and otitis media, eye infections, pneumonia, arthritis, pericarditis, peritonitis and severe invasive infections such as meningitis and septicaemia. The normal habitat for pneumococci is the nasopharynx, mostly of small children. Colonization precedes pneumococcal disease and colonized individuals serve as a reservoir for horizontal spread of the bacterium in the community (Bogaert *et al*., 2004)

2.2.6.1. Carriage

The main biological niche for *S. pneumoniae* is the upper respiratory tract of humans where it colonizes the mucosal surfaces lining the nasopharynx. Disease occurs when resident organisms from the upper respiratory tract gain access to normally sterile spaces in the middle ear, lung, or bloodstream and therefore colonization is the initial step in the pathogenesis of all pneumococcal disease. The carriage rate is highest in young children, who most likely carry pneumococci in the nasopharynx at least one time and are the primary source for its spread within a community (Bogaert *et al*., 2004, Weil-Olivier *et al*., 2012).

Before the wide use of conjugate vaccines, four serogroups 6, 14, 19 and 23 were referred to as ‘pediatric serotypes’ and they were among the most commonly carried ones in children and at the same time caused the most cases of pneumococcal acute otitis media (Hicks *et al*., 2007, Tothpal *et al*., 2012).
Although conjugate vaccines against *S. pneumoniae* reduce carriage of the serotypes contained in the vaccine, there is little or no impact in most populations on the overall prevalence of pneumococcal carriage as other serotypes become more common (Brugger *et al.*, 2010). This process, known as serotype replacement is exemplified by increased carriage and disease prevalence caused by serotype 19A, observed in several populations following the introduction of seven-valent pneumococcal conjugate vaccine (PCV7) (Weinberger *et al.*, 2011).

### 2.2.6.2. Diseases caused by *S. pneumoniae*

#### 2.2.6.2.1. Otitis media

Otitis media (OM) is the most frequently reported paediatric bacterial infection, with approximately 80% of children experiencing an episode by the age of three years (Murphy and Parameswaran, 2009). At the beginning of the 20th century, *group A Streptococcus* (GAS) was the most common pathogen leading to complications in OM. A ‘new’ triad of OM pathogens has emerged in the last century such as *S. pneumoniae*, *H. influenzae* type *b* and *Moraxella catarrhalis* (Vergison, 2008, Murphy *et al.*, 2009).

Some Pneumococcal serotypes (i.e., serotypes 3, 5, 1, 12F, 19F, 19A) seem to have a higher OM disease potential once carriage is established, since in countries where the Pneumococcal conjugate vaccines (PCV7, and later PCV13) is widely used, non-vaccine serotypes account for a more significant proportion of this disease. Among them an increase in serotypes 6C, 22F, 23B, and 35Bwas observed after PCV13 vaccination (Van der Linden *et al.*, 2014).

#### 2.2.6.2.2. Eye infections

*S. pneumoniae* is found rarely in normal conjunctivae (0–0.3%), but obstruction of the nasolacrimal duct predisposes to ascendant colonization and infection of the lacrimal system and conjunctivae with the normal nasopharyngeal commensals including this bacterium. Therefore, Pneumococcus is typically among the top three most commonly
isolated species from cases of bacterial keratitis, an infection of the cornea of the eye (Kunimoto et al., 1998). Pneumococcal keratitis can be a sight-threatening infection if left untreated or if treatment is delayed. Corneal ulceration occurs during the course of the infection and often results in an opaque scarification of the corneal surface after the infection is cleared. The clinical picture of pneumococcal keratitis is a central yellowish or greyish white ulcer associated with infiltrates, folds in Descemet's membrane and hypopyon. Spread in an irregular fashion produces a ‘serpiginous ulcer’ (Ulcus serpens corneae) with the advancing edge of the ulcer being undermined (Parmar et al., 2003).

2.2.6.2.3. Pneumonia

*S. pneumoniae* remains the leading microbial etiology of CAP (Bartlett, 2011). It occurs most frequently in patients least well equipped to handle the effects of the disease, namely, the very young or the very old, the immunosuppressed or chronically ill. The development of Pneumococcal pneumonia results from translocation of Pneumococci from the nasopharynx to the lung through aspiration and possibly blood-borne dissemination (Obaro and Adegbola, 2002). In adult invasive and non-invasive pneumococcal CAP, a study from UK (between 2008 and 2010) showed that the most common serotypes implicated were 14, 1, 8, 3 and 19A (Bewick et al., 2012). According to CDC the use PCV7 since 2000 and PCV13 since 2010 among children in the United States has reduced Pneumococcal infections directly and indirectly among children, and indirectly among adults. Approximately, 20%–25% of IPD cases and 10% of community-acquired pneumonia cases in adults aged ≥65 years are caused by PCV13 serotypes and are potentially preventable with the use of PCV13 in this population (CDC, 2010).

2.2.6.2.4. Invasive pneumococcal diseases (meningitis, sepsis)

World Health Organization (WHO) estimates that approximately 1 million children die each year of invasive pneumococcal disease (IPD) such as meningitis and sepsis (O'Brien et al., 2009). The seven most common serotypes causing IPD in the era prior to Pneumococcal conjugate vaccine (PCV) introduction included 1, 5, 6A, 6B, 14, 19F and
23F. Serotype 14 was the most common serotype accounting for 12%–29% of IPD (Johnson et al., 2010). After the introduction of PCV7 vaccine the rate of all IPD cases dropped by almost 70%, as it happened in the US (Whitney et al., 2003). The highest incidence of IPD generally occurs among children aged 6–11 months, at the very same age when the incidence of pneumococcal acquisition is high (Simell et al., 2012).

2.2.7. Prevention of pneumococcal diseases

The first anti-pneumococcal serum appeared in the H.K. Mulford catalogue (where it remained into the 1940s) in 1895. Early studies of antiserum therapy of Pneumococcal infections came in the 1910s. The first specific record is about a whole-cell heat-treated Pneumococcal vaccine (‘Pneumo-Bacterin’) licensed in the USA in 1909, with manufacturers such as H.K. Mulford Co., Eli Lilly & Company and Parke, Davis & Co (Grabenstein et al., 2012). From 1942 to 1945, Heidelberger and MacLeod took advantage of the preceding developments in polysaccharide technology to develop and test a Pneumococcal polysaccharide vaccine at the US Army Air Force Technical School. With this finding it was proved that purified CPS can be used as active immunogens in adult humans and clinical efficacy against pneumonia was shown but the approach was abandoned with the availability of antibiotics. As the limitations of antibiotics therapy were realized, vaccination with Pneumococcal CPS was revived in the 1960s through the efforts of Robert Austrian. A 14-type mixture of the most prevalent types was selected for vaccination in 1977 and increased to 23 types in 1983. This vaccine is licensed and recommended for adults >65 y and younger subjects with conditions, such as asplenia, that predispose to Pneumococcal infection. Pneumococcal polysaccharide vaccines are poorly immunogenic in children younger than 2 years of age, who are at high risk of invasive Pneumococcal disease (Black et al., 2000).. To improve the immune response to the capsular polysaccharide in young children, third-generation vaccines in which capsular polysaccharides are conjugated to one of several different proteins were developed and tested. However, the technology limits the number of serotypes that can be
included and infections caused by non-included types are a substantial problem in some populations (Weinberger et al., 2011).

2.2.8. Antibiotic treatment

The discovery of penicillin by Alexander Fleming initiated a success story of antimicrobial compounds unmatched by any other antibiotic. This β-lactam antibiotic has long been the mainstay against Pneumococcal infections, but its efficacy is threatened by the rapid dissemination of penicillin-non susceptible clones worldwide.

2.2.8.1. Antimicrobial resistance of S. pneumoniae

2.2.8.1.1. Development of resistance

The emergence of penicillin-resistant and multidrug-resistant Pneumococcal strains has become a global concern. Since 1960s, penicillin-resistant strains have been found in various parts of the world with increasing frequency. Resistance to non-β-lactam antibiotics such as chloramphenicol, tetracycline, erythromycin, clindamycin, rifampin and trimethoprim-sulfamethoxazole has been reported (Appelbaum, 1992). Penicillin-resistant and multidrug-resistant Pneumococci are known to be restricted worldwide to a few serogroups, namely 23, 6, 19, 9 and serotype 14, which were particularly associated with carriage and disease in children in the pre-vaccination era (Corso et al., 1998). Molecular studies have shown that the penicillin-resistant and multidrug-resistant Pneumococcal populations are highly dynamic and that resistance is a combination of the spread of resistant clones, the acquisition and loss of resistance genes within those clonal lineages and the spread of resistance genes to new lineages (McGee et al., 2001).

2.2.8.1.2. Resistance mechanism

2.2.8.1.2.1. β-lactam resistance

Resistance to β-lactam antibiotics in clinical isolates of S. pneumoniae is mediated by mosaic genes encoding altered penicillin-binding proteins (PBPs; a family of enzymes
involved in peptidoglycan metabolism) with lower antibiotic binding affinities than their native versions (Fani et al., 2014). While *S. pneumoniae* contains six PBPs, variants of PBP2x, PBP2b and PBP1a are considered the most relevant for resistance and the acquisition of low-affinity PBP2x and PBP2b variants is a necessary first step for the acquisition of PBP1a variants that confer high-level resistance to β-lactams. Pneumococci have a dedicated system for the acquisition of exogenous DNA from the environment and the mosaic gene structure of low-affinity PBPs is the result of interspecies gene transfer events involving closely related streptococcal species (du Plessis et al., 2002).

The presence of other, non-PBP contributors has also been reported. For example, the cell wall of penicillin-non susceptible isolates is often highly enriched in branched-chain muropeptides, a phenomenon that has been linked to mosaic alleles of the murM gene (Filipe and Tomasz, 2000, Smith et al., 2001). Furthermore, mutations in a peptidoglycan N-acetylglucosamine (GlcNAc) deacetylase (Tait-Kamradt et al., 2009), a peptidoglycan O-acetyltransferase (Crisóstomo et al., 2006), a putative glycosyltransferase (Grebe and Hakenbeck, 1996), a serine threonine kinase (Dias et al., 2009), a histidine protein kinase that is part of a two-component signal-transducing system (Guenzi et al., 1994) and a phosphate ABC transporter (Soualhine et al., 2005) have all been implicated in resistance to β-lactams. Finally, the selection of a nonsense mutation in a putative iron permease in penicillin-resistant *S. pneumoniae* has recently been shown to decrease susceptibility to bactericidal antibiotics, including penicillin (Fani et al., 2011).

### 2.2.8.1.2.2. Macrolide resistance

Resistance to erythromycin in *S. pneumoniae* was first detected in 1967 in the United States and subsequently worldwide (Leclercq and Courvalin, 2002). Macrolide resistance is mediated by two main mechanisms: target modification due to a ribosomal methylase encoded by the *erm* (erythromycin ribosome methylase) gene, which confers high-level resistance to macrolides, lincosamides and streptogramin B (MLSB types) usually carried by transposable elements and the second one the efflux transport system associated with
the mef gene (Leclercq et al., 2002). The widely predominant erm gene is erm B gene although this is not the only representative of the erm gene class in Pneumococci. The presence of an erm A gene has been reported in some strains where it conferred cross-resistance to erythromycin and clindamycin (Syrogiannopoulos et al., 2001).

Resistance with the help of efflux pump appears expressed at moderate levels, with erythromycin MICs of between 1 and 64 μg/ml (generally between 8 and 32 μg/ml). Because the 16-membered macrolides, the lincosamides and the streptogramin B antibiotics are not substrates of the pump, these antimicrobial agents remain active, even after induction with erythromycin. Resistance to erythromycin combined with susceptibility to clindamycin, whether the cells are induced or not induced by erythromycin, defines the M resistance phenotype. The mef gene is also transferable among pneumococci (Del Grosso et al., 2002). Of the two variants of the mef gene, mefA was originally found in Streptococcus pyogenes (Clancy et al., 1996) and mef E was originally found in S. pneumoniae (Tait-Kamradt et al., 1997).

2.2.8.1.2.3. Resistance to other antibiotics

The genetic basis of sulfonamide resistance in S. pneumoniae was demonstrated to be 3- or 6-bp duplications within sulA, the chromosomal gene encoding dihydropteroate synthase. The tetracycline resistance is a result of the acquisition of one of the two genes, tetM and tetO, both of which encode ribosome protection proteins. Pneumococcal resistance to erythromycin and tetracycline is frequently associated with the insertion of the ermB gene into the transposons of the Tn916 or Tn917 family (Tn6002, Tn2010, Tn3872, Tn1545 and Tn6003) that contains the tetM gene (Zhou et al., 2012).

2.3 Haemophilus influenzae

2.3.1. History

In 1930, two major categories of H. Influenzae were defined: the unencapsulated strains and the encapsulated strains. Encapsulated strains were classified on the basis of their
distinct capsular antigens. There are six generally recognized types of encapsulated \textit{H. influenzae}: a, b, c, d, e, and f (Ryan and Ray, 2004). Genetic diversity among unencapsulated strains is greater than within the encapsulated group. Unencapsulated strains are termed nontypable (nthi) because they lack capsular serotypes; however, they can be classified by multilocus sequence typing. The pathogenesis of \textit{H. influenzae} infections is not completely understood, although the presence of the capsule in encapsulated \textit{H. influenzae type b}, a serotype causing conditions such as epiglottitis and pneumonia, is known to be a major factor in virulence. Their capsule allows them to resist phagocytosis and complement-mediated lysis in the non-immune host. The unencapsulated strains are almost always less invasive; they can, however, produce an inflammatory response in humans, which can lead to many symptoms. Vaccination with \textit{H. influenzae type b} conjugate vaccine is effective in preventing infection, but does not prevent infection with nthi strains (Peltola, 2000).

\subsection*{2.3.2 Genotyping of \textit{H. influenzae}}

\textit{H. influenzae} was the first free-living organism to have its entire genome sequenced. Completed by Craig Venter and his team, \textit{Haemophilus} was chosen because one of the project leaders, Nobel laureate Hamilton Smith, had been working on it for decades and was able to provide high-quality DNA libraries. The genome consists of 1,830,140 base pairs of DNA in a single circular chromosome that contains 1740 protein-coding genes, 2 transfer RNA genes, and 18 other RNA genes. The sequencing method used was whole-genome shotgun, which was completed and published in Science in 1995 and conducted at The Institute for Genomic Research (Fleischmann \textit{et al.}, 1995).

\subsection*{2.3.3. Diseases}

Most strains of \textit{H. influenzae} are opportunistic pathogens; that is, they usually live in their host without causing disease, but cause problems only when other factors (such as a viral infection, reduced immune function or chronically inflamed tissues, e.g. From allergies) create an opportunity. They infect the host by sticking to the host cell using
trimeric auto transporter adhesins. Naturally acquired disease caused by *H. influenzae* seems to occur in humans only. In infants and young children, *H. influenzae type B* causes bacteremia, pneumonia (CAP), epiglottitis and acute bacterial meningitis. On occasions, it causes cellulitis, osteomyelitis, and infectious arthritis. Due to routine use of the *H. influenzae type B* conjugate vaccine in the U.S. since 1990, the incidence of invasive *H. influenzae* disease has decreased to 1.3/100,000 in children. However, *H. influenzae type B* remains a major cause of lower respiratory tract infections in infants and children in developing countries where the vaccine is not widely used. *Unencapsulated H. influenzae* strains are unaffected by the *H. influenzae type B* vaccine and cause ear infections (otitis media), eye infections (conjunctivitis), and sinusitis in children, and are associated with pneumonia (Behrman *et al.*, 2004).

### 2.3.4. Diagnosis

Clinical features may include initial symptoms of an upper respiratory tract infection mimicking a viral infection, usually associated with fevers, often low-grade. This may progress to the lower respiratory tract in a few days, with features often resembling those of a wheezy bronchitis. Sputum may be difficult to expectorate and is often grey or creamy in color. The cough may persist for weeks without appropriate treatment. Many cases are diagnosed after presenting chest infections do not respond to penicillins or first-generation cephalosporins (Slack, 1998).

Clinical diagnosis of *H. influenzae* is typically performed by bacterial culture or latex particle agglutinations. Diagnosis is considered confirmed when the organism is isolated from a sterile body site. In this respect, *H. influenzae* cultured from the nasopharyngeal cavity or sputum would not indicate *H. influenzae* disease, because these sites are colonized in disease-free individuals. However, *H. influenzae* isolated from cerebrospinal fluid or blood would indicate *H. influenzae* infection (Chang *et al.*, 2010).
2.3.4.1. Culture

Bacterial culture of *H. influenzae* is performed on agar plates, the preferable one being chocolate agar, with added X (hemin) and V (nicotinamide adenine dinucleotide) factors at 37 °C in a CO2-enriched incubator. Blood agar growth is only achieved as a satellite phenomenon around other bacteria. Colonies of *H. influenzae* appear as convex, smooth, pale, grey or transparent colonies. Gram-stained and microscopic observation of a specimen of *H. influenzae* will show Gram-negative, rod shapes with no specific arrangement. The cultured organism can be further characterized using catalase and oxidase tests, both of which should be positive. Further serological testing is necessary to distinguish the capsular polysaccharide and differentiate between *H. influenzae b* and non-encapsulated species. Although highly specific bacterial culture of *H. influenzae* lacks sensitivity. Use of antibiotics prior to sample collection greatly reduces the isolation rate by killing the bacteria before identification is possible. Beyond this, *H. influenzae* is a finicky bacterium to culture, and any modification of culture procedures can greatly reduce isolation rates. Poor quality of laboratories in developing countries has resulted in poor isolation rates of *H. influenzae* (Puri et al., 1999). If bacteraemic pneumonia is suspected, blood cultures should be taken. However, the sensitivity of blood cultures is generally low, especially when antimicrobial chemotherapy has already been initiated and the yield of significant pathogens from blood cultures ranges from 13–27% in children with complicated CAP, but is less than 5% in those with mild or moderate CAP. *H. influenzae* is a highly fastidious organism and the recovery of this bacterium is highly dependent on both the blood culture broth and the media that are used for sub culturing any potentially positive blood cultures (Esposito and Pnincipi, 2012). Delay in incubating the blood cultures and the presence of antimicrobials in the blood will considerably reduce the chance of recovering *H. influenzae*. Commercial blood culture systems provide quality assured blood culture broths that do support the growth of *H. influenzae*, but it is also important to use appropriate media that contain adequate concentrations of X- and V-factors when subculturing any bottles that signal “positive”. Blood cultures
may therefore significantly underestimate the presence of pathogens, including *H. influenzae*, in the lower respiratory tract (Obaro and Madhi, 2006).

*H. influenzae* will grow in the hemolytic zone of *S. aureus* on blood agar plates; the hemolysis of cells by *S. aureus* releases factor V which is needed for its growth. *H. influenzae* will not grow outside the hemolytic zone of *S. aureus* due to the lack of nutrients such as factor V in these areas. Fildes agar is best for isolation. In Levinthal medium, capsulated strains show distinctive iridescence (Levine *et al.*, 1997).

### 2.3.4.2 Latex particle agglutination

The latex particle agglutination test (LAT) is a more sensitive method to detect *H. influenzae* than culture. Because the method relies on antigen rather than viable bacteria, the results are not disrupted by prior antibiotic use. It also has the added benefit of being much quicker than culture methods. However, antibiotic sensitivity testing is not possible with LAT alone, so a parallel culture is necessary (Kennedy *et al.*, 2007).

### 2.3.4.3. Molecular methods

Polymerase chain reaction (PCR) assays have been proven to be more sensitive than either LAT or culture tests, and highly specific. However, PCR assays have not yet become routine in clinical settings. Countercurrent immune electrophoresis has been shown to be an effective research diagnostic method, but has been largely supplanted by PCR (Garcha *et al.*, 2012). The most reliable way for identification is using molecular biological methods, such as pcr for *H. influenzae* specific genes like *cap* gene (Luo *et al.*, 2012), *glpq* gene (Lai *et al.*, 2012), bex A gene (Shereen *et al.*, 2001).

### 2.3.5. Interaction with *S. pneumoniae*

Both *H. influenzae* and *S. pneumoniae* can be found in the upper respiratory system of humans. In an in vitro study of competition, *S. pneumoniae* always overpowered *H. influenzae* by attacking it with hydrogen peroxide and stripping off the surface molecules...
H. influenzae needs for survival. When both bacteria are placed together into a nasal cavity, within 2 weeks, only H. influenzae survives. When either is placed separately into a nasal cavity, each one survives. Upon examining the upper respiratory tissue from mice exposed to both bacteria species, an extraordinarily large number of neutrophils (immune cells) were found. In mice exposed to only one bacterium, the cells were not present.

Lab tests showed neutrophils exposed to dead H. influenzae were more aggressive in attacking S. pneumoniae than unexposed neutrophils. Exposure to dead H. influenzae had no effect on live one. Two scenarios may be responsible for this response: When H. influenzae is attacked by S. pneumoniae, it signals the immune system to attack the S. pneumonia, and the combination of the two species triggers an immune system response that is not set off by either species individually (Pericone et al., 2000). Carriage is a dynamic process and different patterns of colonization are seen in the first two years of life, ranging from short-term colonization with a single strain, to prolonged colonization with one strain, to recurrent colonization with different or multiple strains of H. influenzae (Watson et al., 2006).

2.3.6. Treatment

H. influenzae produces beta-lactamases, and it is also able to modify its penicillin-binding proteins, so it has gained resistance to the penicillin family of antibiotics. In severe cases, cefotaxime and ceftriaxone delivered directly into the bloodstream are the elected antibiotics, and, for the less severe cases, an association of ampicillin and sulbactam, cephalosporins of the second and third generation, or Fluoroquinolones are preferred (Fluoroquinolone-resistant H. influenzae has been observed) (Chang et al., 2011). Macrolide antibiotics (e.g., clarithromycin) may be used in patients with a history of allergy to beta-lactam antibiotics. Macrolide resistance has also been observed (Roberts et al., 2011).
2.3.7. Prevention

The effective vaccines for *H. influenza* Type *b* was available since the early 1990s, and is recommended for children under age 5 and asplenic patients. The World Health Organization recommends a pentavalent vaccine, combining vaccines against diphtheria, tetanus, pertussis, hepatitis B and *H. influenzae* Type *B*. There is not yet sufficient evidence on how effective this pentavalent vaccine is in relation to the individual vaccines (Baron *et al.*, 2012).

*H. influenzae* Type *b* vaccines cost about seven times the total cost of vaccines against measles, polio, tuberculosis, diphtheria, tetanus, and pertussis. Consequently, whereas 92% of the populations of developed countries were vaccinated against *H. influenza* type *b* as of 2003, vaccination coverage was 42% for developing countries, and only 8% for least-developed countries (Ladhani *et al.*, 2009).

2.4. *Klebsiella pneumoniae*

2.4.1. History

Danish scientist Hans Christian Gram (1853–1938) developed the technique now known as Gram staining in 1884 to discriminate between *K. pneumoniae* and *S. pneumoniae*. The genus *Klebsiella* was named after the German bacteriologist Edwin Klebs (1834–1913). Also known as Friedlander's bacillum in honor of Carl Friedlander, a German pathologist, who proposed that this bacterium was the etiological factor for the pneumonia seen specially in immunocompromised individuals such as patients suffering of chronic diseases or alcoholics (Alves *et al.*, 2006).

*K. pneumoniae* is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. Although found in the normal flora of the mouth, skin, and intestines (Ryan and Ray, 2004), it can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum. In the clinical setting, it is the most significant member of the
*Klebsiella* genus of Enterobacteriaceae. *Klebsiella oxytoca* (*K. oxytoca*) and *Klebsiella rhinoscleromatis* (*K. rhinoscleromatis*) have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* species have become important pathogens in nosocomial infections. It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions (Postgate, 1998). As a free-living diazotroph, its nitrogen fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions. Members of the *Klebsiella* genus typically express two types of antigens on their cell surfaces. The first, O antigen is a component of the lipopolysaccharide (LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for sero grouping. It is closely related to *K. Oxytoca* from which it is distinguished by being indole-negative and by its ability to grow on melezitose but not 3-hydroxybutyrate (Riggs *et al*., 2001).

### 2.4.2. Clinical significance

*K. pneumoniae* can cause destructive changes to human lungs via inflammation and hemorrhage with cell death (necrosis) that sometimes produces a thick, bloody, mucoid sputum (currant jelly sputum). These bacteria gain access typically after a person aspirates colonizing oropharyngeal microbes into the lower respiratory tract.

As a general rule, *K. pneumoniae* infections are seen mostly in people with a weakened immune system. Most often, illness affects middle-aged and older men with debilitating diseases. This patient population is believed to have impaired respiratory host defenses, including persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary diseases, glucocorticoid therapy, renal failure, and certain occupational exposures (such as papermill workers) (Riggs *et al*., 2001). Many of these infections are obtained when a person is in the hospital for some other reason (a nosocomial infection). Feces are the most significant source of patient infection, followed by contact with contaminated instruments. The most common condition caused by *K.*
outside the hospital is pneumonia (CAP), typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a death rate of about 50%, even with antimicrobial therapy. The mortality rate can be nearly 100% for people with alcoholism and bacteremia. In addition to pneumonia, K. pneumoniae can also cause infections in the urinary tract, lower biliary tract, and surgical wound sites. The range of clinical diseases includes pneumonia, thrombophlebitis, urinary tract infection, Cholecystitis, diarrhea, upper respiratory tract infection, wound infection, Osteomyelitis, meningitis, and bacteremia and septicemia. For patients with an invasive device in their bodies, contamination of the device becomes a risk; for example, neonatal ward devices, respiratory support equipment, and urinary catheters put patients at increased risk. Also, the use of antibiotics can be a factor that increases the risk of nosocomial infection with the bacteria. Sepsis and septic shock can follow entry of the bacteria into the blood (Podschun and Ullmann, 1998).

Since the first identification of K. pneumoniae as a cause of pneumonia by pathologist Karl Friedländer in 1882, capsular polysaccharide has been established as the species’ most distinguishing characteristic and most studied virulence factor (Eisenstadt and Crane, 1994). Previous studies have led to a greater understanding of the potential functions capsule may be performing for this species during infection. In vitro, the presence of capsule significantly inhibits the deposition of complement components onto the bacterium, and has been shown to measurably reduce phagocytosis of the bacterium by macrophages (Favre-Bonte et al., 1999; Cortes et al., 2002). The production of capsule also appears to inhibit the proper assembly of type 1 fimbriae on the bacterial surface, and may lead to a transcriptional inhibition in the production of another adhesion. Accordingly, isogenic capsule-negative strains show higher levels of adherence to, and invasion of, a variety of cultured cells when compared with wild-type strains (Sahly et al., 2000). The importance of capsule as a virulence factor for K. pneumonia has also been examined using in vivo models of colonization and pathogenesis. Using a catheter
inoculation method, a capsule-negative derivative failed to colonize mouse bladder tissue to the same density as wild type (Struve and Krogfelt, 2003). Sahly et al (2000) demonstrated that an isogenic capsule mutant is less successful at colonizing the lungs of infected mice than a wild-type strain and fails to disseminate to the spleen following pulmonary inoculation.

Lipopolysaccharide (LPS) is major component of Gram-negative bacteria cell walls and possesses potent immune modulatory properties (Strieter et al., 1990). However, in the context of wild-type capsule production the significance of LPS as a virulence factor for K. pneumonia is unclear. Three studies have examined the importance of LPS during pulmonary infection, and their conclusions vary significantly. Two studies find no attenuation of disease due to LPS deficiency (Cortes et al., 2002; Izquierdo et al., 2003), while the other study observes that an LPS mutant is unable to disseminate beyond the respiratory tract (Shankar-Sinha et al., 2004). Confounding these data is the observation that some studies on LPS mutants of K. pneumonia have not verified wild-type capsule production in these strains; conversely, evaluations of capsule mutants have not always determined whether the mutants in question produce wild type levels of LPS. With the potential overlap among polysaccharide biosynthetic pathways, in addition to the known interactions between these structures in the bacterial cell surface, it is not unreasonable to assume that insertional mutations which affect the production of one of these molecules would impact the production of both (Izquierdo et al., 2003).

There are few other K. pneumonia components which have been implicated to be necessary during infection. A chromosomal region associated with allantoin metabolism is also suspected to play a role in vivo as a potential contributing factor to invasive liver infections, a common complication during K. pneumonia systemic infection (Chou et al., 2004). A number of adhesions have been suggested as potential virulence factors, including type 3fimbriae and the adhesion CF29K; however, their importance has yet to be demonstrated in vivo (Di Martino et al., 2003).
2.4.3. Treatment

As with many bacteria, the recommended treatment has changed as the organism has developed resistances. The choice of a specific antimicrobial agent or agents depends on local susceptibility patterns and on the part of the body infected. For patients with severe infections, a prudent approach is the use of an initial short course (48–72 h) of combination therapy, followed by a switch to a specific mono therapy once the susceptibility pattern is known for the specific patient. If the specific *K. pneumoniae* in a particular patient does not show antibiotic resistance, then the antibiotics used to treat such susceptible isolates include ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanate, ceftazidime, cefepime, levofloxacin, norfloxacin, gatifloxacin, moxifloxacin, meropenem, and ertapenem. Some experts recommend the use of meropenem for patients with ESBL producing *Klebsiella*. The claim is that meropenem produces the best bacterial clearing. The use of antibiotics is usually not enough. Surgical clearing (frequently done as interventional radiology drainage) is often needed after the patient is started on antimicrobial agents (Nathisuwan *et al*., 2001; Corey *et al*., 2014).

2.5. Beta-lactamase

The β-lactamases are enzymes produced by some bacteria that are responsible for their resistance to β-lactam antibiotics like penicillins, cephalosporins monobactams and carbapnems. The enzymes cleave the β-lactam ring, thus rendering the antibiotic inactive. Over 470 β-lactamases are known that are grouped into four distinct functional classes (classes A, B, C, and D) (Jooyoung *et al*., 2008).

The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against β-lactamase-mediated bacterial resistance to antibiotics. These cephalosporins had been developed in response to the increased prevalence of β-lactamases in certain organisms and the spread of these β-lactamases into new hosts, (Paterson and Bonomo., 2005). Many genera of Gram-negative bacteria have adapted to broad-spectrum β-lactam antibiotics by modifying the
substrate spectrum of common plasmid-mediated β-lactamases and by mobilizing resistance-promoting chromosomal β-lactamase genes into plasmids, allowing their spread to new hosts (Paterson and Bonomo., 2005). These Gram-negative bacteria were found to be resistant to all extended spectrum of penicillins, cephalosporins (e.g. ceftazidime, cefotaxime, and ceftarixone) and monobactams (e.g. Aztreonam). These are referred to as extended spectrum beta-lactamase (ESBL), (George., 1983).

2.5.1. Extended spectrum β lactamase (ESBL)

Members of the family Enterobacteriaceae commonly express plasmid-encoded β-lactamases (e.g., TEM-1, TEM-2, and SHV-1) which confer resistance to penicillins but not to expanded-spectrum cephalosporins. In the mid-1980s a new group of enzymes, the extended-spectrum β-lactamases (ESBLs), was detected in Germany in 1983 (Knothe et al., 1983).

ESBLs are enzymes that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime (CTX), ceftriaxone (CRO), and ceftazidime (CAZ), aztreonam (ATM), and related oxyimino-β- lactams. Typically, they derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases. This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. An increasing number of ESBLs not of TEM or SHV lineage have recently been described (Knothe et al., 1983).

The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (for example, amino glycosides). Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited. Carbapenems are the treatment of choice for serious infections due to ESBL-producing organisms, yet carbapenem-resistant isolates have recently been reported. ESBL-producing organisms may appear susceptible to some extended-spectrum cephalosporins. However, treatment with such antibiotics has been associated with high failure rates (George., 1983, Paterson and Bonomo., 2005).
There is no consensus of the precise definition of ESBLs. A commonly used working definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first, second, and third-generation cephalosporins, and aztreonam (but not the cephemycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid. (Paterson and Bonomo, 2005). These enzymes produce mainly by *K. pneumoniae* and *E. coli*.

### 2.5.2. TEM β-Lactamase

The *TEM*-type ESBLs are derivatives of *TEM*-1 and *TEM*-2. *TEM*-1 was first reported in 1965 from an *E. coli* isolate from a patient in Athens, Greece, named Temoneira (hence the designation *TEM*) (Patricia and Bradford, 2001). Currently 144 variants of the TEM enzymes are recognized (Jooyoung *et al.*, 2008). *TEM*-1 is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. This enzyme is also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae*. *TEM*-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine, (Patricia and Bradford, 2001). It is inhibited by clavulanic acid. *TEM*-2 has the same hydrolytic profile as *TEM*-1; *TEM*-13 also has a similar hydrolytic profile to *TEM*-1 and *TEM*-2 (Stratton., 2000). TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other species of Gram-negative bacteria with increasing frequency (Joumana and George., 2003).

Amino acid substitutions that occur within the *TEM* enzyme occur at a limited number of positions. The combinations of these amino acid changes result in various subtle alterations in the ESBL phenotypes, such as the ability to hydrolyze specific oxyimino-cephalosporins such as ceftazidime and cefotaxime, or a change in their isoelectric points, which can range from 5.2 to 6.5. A number of amino acid residues are especially important for producing the ESBL phenotype when substitutions occur at that position. They include glutamate to lysine at position 104, arginine to either serine or histidine at
position 164, glycine to serine at position 238, and glutamate to lysine at position 240 (Patricia and Bradford, 2001).

2.5.3. SHV β-Lactamase

Most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Patricia and Bradford, 2001). The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs. *SHV* refers to sulf hydryl variable. The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs. *SHV*- shares 68% of its amino acid with *TEM*-1 (Patricia and Bradford, 2001, Joumana and George, 2003). Recently 72 of the *SHV* β-lactamases variant are known. The majority of *SHV* variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to *SHV*-5 also have a substitution of lysine for glutamate at position 240. It is interesting that both the Gly 238 Ser and Glu 240 Lys amino acid substitutions mirror those seen in *TEM*-type ESBLs. The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime (Patricia and Bradford, 2001).
CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Design

3.1.1. Type of the Study

This is a descriptive cross sectional study.

3.1.2. Study Area

Greater Khartoum is located between latitudes 15º26 and 15º45 N and longitudes 32º25 and 32º40 E, at an altitude of 405.6 m above sea level. Khartoum State lies at the junction of the two rivers, the White and the Blue Niles in the North Eastern part of central Sudan. A total area is about 20,736 km2. The Khartoum State consists of three localities with a total population 5274321 peoples were distributed as fellows; Omdurman Locality (2215330), Al-Khartoum Locality (1582027) and Bahri Locality (1247745). The people residence in Khartoum is from different ethnic groups. From the total population of Khartoum State about 61% of the population is aged 15–64 years indicating that, the more productive groups are migrating to Khartoum. Omdurman is the most populous part of the city, housing 43.5 per cent of total population (Central Statistical Organization: http://www.cbs.gov.sd/en/files.php?id=7#&panel11-2).

The specimens for this study were collected from three major Localities in Khartoum State including Al-Khartoum Locality, Bahri Locality and Omdurman Locality. Culture and molecular parts were carried out in the Research Laboratory, Sudan University of Science and Technology (SUST). Gene sequencing was done by Macrogen, Inc. Korea.
3.1.3. Study population

Patients suspected to have signs and symptoms of pneumonia (CAP) and presented to the chest units of Omdurman Teaching Hospital, Abu Anja Teaching Hospital, Bahry Teaching Hospital and Al-Shaab Teaching Hospital were enrolled. The age groups of the target population were divided into three categories as follows; young adult; 13-30 years old, middle age; 31-60 years old and elderly more than 60 years old (Salama et al., 2012).

3.1.4. Study duration

This study was carried during the period December 2013- November 2015

3.2. Inclusion Criteria

Only patients presenting with symptoms of chest infection regardless sex and age were including in this study.

3.3. Exclusion Criteria

Any patient without symptoms of chest infection or refuses to sign consent was excluded from this study.

3.4. Sampling

3.4.1. Sample frame

Individual sampling.

3.4.2. Sample size

The sample size was determined according to the following formula (Wood and Mark, 1999),
\[ N = Z^2 \times \delta^2 / Se^2 \]

Where:

\( N \) = sample size

\( Z^2 \) = tabulated level of confidence limited medical research 95\% = 1.96\(^2\) = 3.84

\( \delta^2 \) = the expected population value. In this study it was equal to 50\%, because there is no previous study.

\( Se^2 \) = standard error chance in medical research, which can be accepted in the result, =5\%

\[ N = [(1.96) \times (50/100)]^2 / (0.05)^2 = 380. \]

**3.5. Data collection**

Full information of each patient was collected using a predesigned questionnaire (Appendix I) including age, sex, hospital, residence and date of collection.

**3.6. Ethical considerations**

The proposal of the study was approved by the College Ethical Committee, College of Medical Laboratory Science, SUST. The information regarding risk factors (if any) was explained to all patients under the study. Maintaining confidentiality of information obtained from patients. Consent to collect the specimens and sociodemographic data were obtained from patients and hospitals administration participated in this study (Appendix II).

**3.7. Target pathogenic bacteria**

*S. pneumoniae, H. influenzae* and *K. pneumonia* were considered as target pathogenic bacteria.
3.8. Sampling technique

Expectorated sputum specimens were collected in sterile containers with screw caps. All specimens were transported to the reasearch laboratory within 24 hrs of collection according to (CLSI).

3.9. Detection of community acquired pneumonia

3.9.1. Phenotypic detection

3.9.1.1. Culture media

The following culture media were used for the isolation of the *S. pneumoiae*, *H. influenzae* and *K. pneumoniae* including Blood agar base, and MacConkey agar were obtained from Oxoid Ltd, UK and HiMedia, India. They were prepared according to the manufacturer's instruction.

3.9.1.2. Sterilization

All culture media were sterilized in autoclave at 121 °C in 15 lbs pressure for 15 min. All glass wares were sterilized in hot air oven at 160°C for 60 min.

3.9.1.3. Bacteriological Techniques

All bacteriological techniques were done under aseptic condition in the cabinet under laminar flow near Bunsen burner.

3.9.1.4. Cultivation of specimens

Sputum specimens were inoculated under aseptic condition on Chocolate blood Agar, blood agar, and MacConkey agar media using sterile standard bacteriological wire loop. The inoculated culture media were incubated aerobically at 37°C over-night and examined for growth.
3.9.1.5. Colonial morphology

The colonial morphology was used for primary identification depending on size, color, edges, side views and fermentation of lactose on MacConkey Agar, and hemolysis on Chocolate Agar and Blood Agar.

3.9.1.6. Microscopic examination

Smears from the growth of each isolate were prepared. The smear were dried by air and fixed by flame. Gram’s stain was performed for each slide as follows; slide was covered with crystal violet stain for 30-60 sec, then washed with water and covered with logol’s iodine for 30-60 sec, washed, decolorized with alcohol for few seconds, washed with water immediately and covered with saffranin for 2 minutes then washed with water and examined microscopically by oil immersion lens (X 100).

3.9.1.7. Identification of *S. pneumoniae* by Optochin disc

For each strain, a 5-μg optochin disc (Oxoid) was placed on purity plate. Optochin susceptibility is determined by the zone of inhibition around the disc (O'Brien et al., 2003). After overnight incubation, the strain is considered optochin-susceptible if a zone of inhibition exceeds 14 mm, optochin-nonsusceptible if zone of inhibition is 7-14 mm and optochin-resistant if the zone of inhibition is ≤ 7 mm (O'Brien et al., 2003).

3.9.1.8. Identification of *H. influenzae* by X & V factors

*Haemophilus* species have varying requirements for X and V growth factors. Consequently, the significant differences in growth factor requirements of *Haemophilus species* allows for their differentiation. Differentiation is based on the presence or absence of growth around and/or between disks impregnated with factors X, V and XV. Each X-Factor Disk is impregnated with hemin. Each V-Factor Disk is impregnated with NAD (nicotinamide adenine dinucleotide). Each XV-Factor Disk is impregnated with a
combination of hemin and NAD. *H. influenza* requires the growth of each factor (Murray *et al.*, 2007).

### 3.9.1.9. Identification of *K. pneumoniae* by API 20E system

One of the diagnostic tests most frequently employed is the API 20E test strip manufactured by Biomerieux; Inc. It is used to diagnose Gram negative rod shaped bacterium. The API 20E system consists of a plastic strip of 20 individual, miniaturized tests tubes (cupules) each containing a different reagent used to determine the metabolic capabilities of the genus and species of enteric bacteria in the family Enterobacteraceae (Willey *et al.*, 2008).

### 3.9.1.10. Loading the cupules

A saline suspension of a pure bacterial culture was inoculated in each cupules with a rehydrating dried reagent in each tube. Some of the tubes were completely filled (CIT, VP and GEL tests), whereas others are topped off with mineral oil so that anaerobic reactions can be carried out (ADH, LDC, ODC, H₂S, URE tests) (Willey *et al.*, 2008).

### 3.9.1.11. Incubating the API 20E strip

The strip was then incubated in a small, plastic humidity chamber for 18-24 hours at 37°C. The reagents in the cupules were specifically designed to test the presence of products of bacterial metabolism specific to certain kinds of bacteria (Willey *et al.*, 2008).

### 3.9.1.12. Reading API 20E Results

At the end of the incubation period, each tube was examined for a specific color change indicating the presence of a metabolic reaction that sheds light on the microbes' identity. Some of the cupules contents change color due to pH differences, others contain end products that have to be identified using additional reagents (Willey *et al.*, 2008).
3.9.1.13. The API 20E interpretation

The interpretation of the 20 reactions, in addition to the oxidase reaction (which is done separately), was converted to a seven-digit code. Then the code looked up in a huge manual that had the names of bacterial species associated with each seven-digit string of numbers (Willey et al., 2008)

3.9.2. Molecular techniques

3.9.2.1. DNA extraction

The DNA extraction was done by bacterial DNA Preparation Kit, (Jena Bioscience, Germany), following the manufacturer’s instructions. The procedure was carried out as follows.

a) 1 ml of sputum was transferred into a 1.5 ml micro tube.

b) The sputum was centrifuged at 15,000 rpm for 1 min then the supernatant was discarded.

c) 300 µl of Cell Lyses Solution was added to resuspend the pellet.

d) 1.5 µl of RNase Solution was added and mixed well.

e) Incubation was done at 37 °C for 15-30 min.

f) 100 µl of Protein Precipitation Solution was added and vortexed vigorously for 20-30 sec, then the mixture was centrifuged at 15,000 rpm for 5 min.

g) The supernatant was transferred to a clean 1.5 ml micro tube containing 300 µl Isopropanol >99 %, mixed by inverting gently for 1 min, then mixed and centrifuged at 15,000 rpm for 1 min. At the end of this step, the DNA was a visible as a small white pellet.
h) The supernatant was discarded and the tube was drained carefully on clean absorbent paper, then 500 µl washing buffer was added and the tube was inverted several times to wash the DNA pellet, centrifuged at 15,000 rpm for 1 min and the ethanol was discarded, and air dried at room temperature for 10-15 min.

i) A moment of 50-100 µl of DNA hydration solution was added to the dried DNA pellet and then hydrated by incubating at 65 °C for 60 min.

j) The obtained DNA was stored at -20°C until used.

3.9.2.2. Electrophoresis of the extracted DNA in agarose gel

The extracted DNA was checked by electrophoresis on agarose gel and stained with Ethidium bromide, fluorescence was excited by ultra-violet radiation when it complexes with nucleic acids. The gel was prepared by mixing 1.5 gm agarose, 100 ml 1X TBE buffer and 4 µl of Ethidium bromide (10 mg/ml), and then 2-3µl of the extracted DNA was mixed with 2-3 µl of loading dye. 4µl of DNA was loaded on the gel. Gel was run in 1X TBE running buffer and electrophoresis was carried out at 100 to 145 volts for 10-20 min. Then the gel was viewed under UV light and photographed.

3.9.2.3. Spectrophotometrically determination of DNA concentration and purity

The DNA yield is determined spectrophotometrically by measuring the absorbance at 260 and 280nm.

The DNA was diluted 1: 50 with distilled water (10 µl DNA+ 490 µl H₂O).

The reading of DNA concentration will be done at 260 and 280nm using spectrophotometer,

Distilled water is used as a blank.

1A260 double-stranded DNA = 50 µg/mL
1\text{A260 single stranded DNA} = 37 \mu g/mL

\text{DNA concentration} = \text{A260} \times \text{dilution factor} \times \text{conversion factor}

\textbf{DNA Purity A260/A280}

An A260/A280 ratio greater than 1.8 indicates highly purified preparations of DNA and RNA respectively. Contaminants that absorb at 280 nm (e.g. a protein) will lower the ratio (Clark and K. Christopher, 2000).

\textbf{3.9.2.4. Polymerase chain reaction (PCR)}

\textbf{3.9.2.4.1. Primers}

The following primers were used for detection of \textit{S. pneumoniae, H. influenzae and K. pneumonia}
Table 1. Primers of *S. pneumoniae*, *H. influenza* and *K. pneumoniae*

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Amplicon size (bp)</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>CpsA</td>
<td>653</td>
<td>F 5'- AGTGGTAACTGCGTTAGTCCT-3</td>
<td>(Luo et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R 5'- GTGGCGTTGTTGGTCAAGAG-3</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>Cap</td>
<td>177</td>
<td>F 5'- ATGTTAGATCGTGCGGATACTC-3</td>
<td>(Luo et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R 5'- GCGAGGAACAGAACCATCAG-3</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>RpoB</td>
<td>108</td>
<td>F 5'- CAA CGG TGT GGT TAC TGA CG-3</td>
<td>(Chander et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R 5'- TCT ACG AAG TGG CCG TTT TC-3</td>
<td></td>
</tr>
</tbody>
</table>

3.9.2.4.2. Standard PCR reaction

The experiment consists of the experimental DNA, positive control and a negative control, 2 µl added to PCR tube and the following solutions were placed in a total volume of 20 µl:

- 10X Taq buffer (final concentration 1X).
- 2.5 mM 4dNTP stock (final concentration 200 µmol).
- 10 pmol/µl primer F.
- 10 pmol/µl primer R.
- 100 ng of genomic DNA template.
- MgCl2 (final concentration 1.5 µm).
H₂O (up to the total volume of 20 µl).

2.5 uu Taq Polymerase.

3.9.2.4.3. PCR Programming

3.9.2.4.3.1. Protocol used for amplification of Cps A gene S. pneumoniae

Initialization: This step consists of heating the reaction to a temperature of 94 °C which is held for 5 minutes.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94°C for 30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases

Annealing step: The reaction temperature is lowered to 55 °C for 30 seconds allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72 °C for 30 seconds.

Final elongation: This single step is occasionally performed at a temperature of 72 °C for 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

3.9.2.4.3.2. Protocol used for amplification of Cap gene H. influenzae

Initialization: This step consists of heating the reaction to a temperature of 94 °C which is held for 5 minutes.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94°C for 1 minute. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases
Annealing step: The reaction temperature is lowered to 55 °C for 1 minutes allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72 °C for 1 minutes.

Final elongation: This single step is occasionally performed at a temperature of 72 °C for 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

3.9.2.4.3.3. **Protocol used for amplification of Rpo gene K. pneumoniae**

Initialization: This step consists of heating the reaction to a temperature of 94 °C which is held for 5 minutes.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94°C for 45 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases

Annealing step: The reaction temperature is lowered to 54 °C for 45 seconds allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72 °C for 45 seconds.

Final elongation: This single step is occasionally performed at a temperature of 72 °C for 7 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
3.9.2.4.3.4. Protocol used for amplification of *SHV* and *TEM* genes *K. pneumoniae*

Initialization: This step consists of heating the reaction to a temperature of 94 °C which is held for 5 minutes.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94°C for 45 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases.

Annealing step: The reaction temperature is lowered to 54 °C for 45 seconds allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72 °C for 45 seconds.

Final elongation: This single step is occasionally performed at a temperature of 72 °C for 7 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

3.9.2.4.3.5. Checking the PCR products

To confirm the presence of amplifiable DNA in the samples, the specificity of PCR is typically analyzed by evaluating the production of the target fragment by gel electrophoresis of 8µl PCR products on 1.5% Agarose gel stained with Ethidium bromide.

3.10. Sequence similarities and phylogenetic analysis

The PCR products were obtained and sent to Macrogen (Korea) for standard sequence DNA analysis. The same primers as above and an automated sequencer were used for this purpose. The sequence was compared for similarity level with the references of *S. pneumoniae* in genomic database banks, using the NCBI Blast available at the ncbi.nlm-
nih.gov Web site. The obtained results were viewed using BioEdit v7.0.9 software (www.mobioncsu.edu/bioedit/bioedit.html). The software Clustal W2 (http://www.ebi.ac.uk/Tools/Msa/ClustalW2) was used to calculate the identities, similarities and differences among queries sequenced.

To construct the phylogenetic tree of the queries sequenced the results were submitted to the NCBI data bank.

3.12. Data analysis

The collected data were recorded and then analyzed using statistical package of social science (SPSS, version 11) program and chi-square test. P. values of < 0.05 were considered statistically significant.
CHAPTER FOUR

RESULTS

In this study a total of 360 patients with chest infection were enrolled. Among them 218 (60.6%) were males and 142 (39.4%) were females (Fig1). The highest frequency (54.4%) of patient's age group was 31-60 years followed by age group 13-30 years (31.1%) while the lowest frequency was age group 61-90 years (14.4%) (Fig2). Specimens collected from patients attended to different hospitals in Khartoum State were distributed as shown in Fig (3).

Conventional culture methods revealed that 122 (33.8%) of specimens were positive for bacterial growth, while 238 (66.2%) were negative. The isolates were identified as 64 (17.8%) S. pneumoniae, followed by 39 (10.8%) K. pneumoniae, 19 (5.3%) H. influenzae (Table2).

DNA extracted for all sputa. Analysis of the extracted genomic DNA in 1.0% agarose gel with 1X TBE buffer to check the purity revealed that all genomic DNA were pure (Fig 4). PCR technique was done for all genomic DNA, specific primers for CAP showed 157 (43.6%) positive, while 203 (56.4%), were negative. These were 81 (22.5%) positive for Cps gene of S. pneumoniae, while 279 (77.5%) were negative followed by 43 (11.9%) positive for Rpo gene of K. pneumoniae, while 317 (88.1%) were negative followed by 33 (9.2%) positive for Cap gene of H. influenzae, while 327 (90.8%) were negative (Table 3). All K. pneumoniae contain SHV gene while all of the isolates were negative for the presence of the TEM gene. The relationship between Hospitals and S. pneumoniae were significant (P-value = 0.004) and for K. pneumoniae and H. influenzae were insignificant, (P-value = 0.299), (P-value = 0.127) respectively (Tables 4, 5 and 6).

The relationship between genders and S. pneumoniae, K. pneumoniae and H. influenzae were insignificant were the (P- value = 0.990), (P- value = 0.498) and (P- value = 0.133) respectively (Table 7).
The relationship between age groups and *S. pneumoniae*, *K. pneumoniae* and *H. influenzae* were significant were the (*P*- value = 0.727), (*P*- value = 0.041) and (*P*- value = 0.724) respectively (Tables 8, 9 and 10).

The probability values between *S. pneumoniae* PCR, *K. pneumoniae* PCR and *H. influenzae* PCR with culture were significant were the (*P*-value = 0.000) (Table 11).

Sequencing test done for *S. pneumoniae* PCR products, the results showed identity range and query cover range by Blast analysis of *S. pneumoniae*, the identity range between 99% to 100% were the query cover range between 97% to 100%.

Multiple Sequence alignment and Phylogenetic tree done for *S. pneumoniae* (Figures 9, 10, 11).
Fig 1. Distribution of patients according to gender
Fig 2. Distribution of patients according to age group

Fig 3. Distribution specimens according to the Hospitals
Table 2. Conventional culture methods for detection of CAP among enrolled patients (n=360)

<table>
<thead>
<tr>
<th>Result</th>
<th>Phenotypic detection of</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>S. pneumoniae</strong></td>
<td><strong>K. pneumoniae</strong></td>
<td><strong>H. influenzae</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>No (%)</strong></td>
<td><strong>No (%)</strong></td>
<td><strong>No (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>64(17.8)</td>
<td>39(10.8)</td>
<td>19(5.3)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>296(82.2)</td>
<td>321(89.2)</td>
<td>341(94.7)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Molecular detection of CAP among enrolled patients (n=360)

<table>
<thead>
<tr>
<th>Result</th>
<th>Molecular detection of</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>S. pneumoniae</strong></td>
<td><strong>K. pneumoniae</strong></td>
<td><strong>H. influenzae</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>No (%)</strong></td>
<td><strong>No (%)</strong></td>
<td><strong>No (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>81(22.5)</td>
<td>43(11.9)</td>
<td>33(9.2)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>279(77.5)</td>
<td>317(88.1)</td>
<td>327(90.8)</td>
<td></td>
</tr>
</tbody>
</table>
Fig4. PCR product for CpsA gene of S. pneumoniae 653 bp PCR product:
1.5 % agarose gel electrophoresis of S. pneumoniae by PCR and they have land one and eleven M. Mw 100 – 1000 bp fragments – lane two control Positive. The pictorial showed all (7) isolates (4, 5, 6, 7, 8, 9, 10), with a band typical in size (653bp) which are positive for Cps gene, (3) negative control.

**Key:** First lane, Marker; second lane, Positive control, Lanes (4, 5, 6, 7, 8, 9, 10) were Cps gene, lane (3) negative control, lane (11) marker.
Fig5. PCR product for *Rpo* gene of *K. pneumoniae* 108bp PCR product: 

1.5 % agarose gel electrophoresis of *K. pneumoniae* by PCR and they have land one and eleven M. Mw 100 – 1000 bp fragments – lane two control Positive. The pictorial showed all (7) isolates (4, 5, 6, 7, 8, 9, 10), with a band typical in size (108bp) which are positive for *Rpo* gene, (3) negative control.

**Key:** First lane, Marker; second lane, Positive control, Lanes (4, 5, 6, 7, 8, 9, 10) were *Rpo* gene, lane (3) negative control, lane (11) marker.
Fig 6. PCR product for Cap gene of *H. influenzae* 177bp PCR product:

1.5 % agarose gel electrophoresis of *H. influenzae* by PCR and they have land one M. Mw 100 – 1000 bp fragments – lane three control Positive. The pictorial showed all (3) isolates (4, 16, 17), with a band typical in size (177bp) which are positive for Cap gene, lane (5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20) were negative for Cap gene, (2) negative control.

**Key:** First lane, Marker; second lane, negative control, Lanes (4, 16, 17) were Cap gene, lane (3) positive control.
Fig 7. PCR product for *SHV* gene of *K. pneumoniae* 768bp PCR product

1.5% agarose gel electrophoresis of *K. pneumoniae* by PCR and they have lane one and nine M. Mw 100 – 1000 bp fragments – lane two control Positive. The pictorial showed all (5) isolates (4, 5, 6, 7, 8), with a band typical in size (768bp) which are positive for *SHV* gene, (3) negative control.

**Key:** First lane, Marker; second lane, Positive control, Lanes (4, 5, 6, 7, 8) were *SHV* gene, lane (3) negative control, lane (9) marker.
Table 4. Relationship between hospitals and *S. pneumoniae*

<table>
<thead>
<tr>
<th>Hospital</th>
<th><em>S. pneumoniae</em></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Omdurman Teaching Hospital</td>
<td>19(5.3%)</td>
<td>71(19.7%)</td>
</tr>
<tr>
<td>Abu anja Teaching Hospital</td>
<td>9(2.5%)</td>
<td>81(22.5%)</td>
</tr>
<tr>
<td>Al shaab Teaching Hospital</td>
<td>27(7.5%)</td>
<td>63(17.5%)</td>
</tr>
<tr>
<td>Bahry Teaching Hospital</td>
<td>26(7.2%)</td>
<td>64(17.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>81(22.5%)</td>
<td>279(77.5%)</td>
</tr>
</tbody>
</table>

*P* = 0.004
Table 5. Relationship between hospitals and *K. pneumoniae*

<table>
<thead>
<tr>
<th>Hospital</th>
<th><em>K. pneumoniae</em></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Omdurman Teaching Hospital</td>
<td>12(3.3%)</td>
<td>78(21.7%)</td>
</tr>
<tr>
<td>Abu anja Teaching Hospital</td>
<td>6(1.7%)</td>
<td>(23.3%)</td>
</tr>
<tr>
<td>Al shaab Teaching Hospital</td>
<td>11(3.1%)</td>
<td>63(21.9%)</td>
</tr>
<tr>
<td>Bahry Teaching Hospital</td>
<td>14(3.9%)</td>
<td>76(21.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>43(11.9%)</td>
<td>317(88.1%)</td>
</tr>
</tbody>
</table>

*P= 0.299*

Table 6. Relationship between hospitals and *H. influenzae*

<table>
<thead>
<tr>
<th>Hospital</th>
<th><em>H. influenzae</em></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Omdurman Teaching Hospital</td>
<td>9(2.5%)</td>
<td>81(22.5%)</td>
</tr>
<tr>
<td>Abu anja Teaching Hospital</td>
<td>3(0.8%)</td>
<td>87(24.2%)</td>
</tr>
<tr>
<td>Al shaab Teaching Hospital</td>
<td>12(3.3%)</td>
<td>78(21.7%)</td>
</tr>
<tr>
<td>Bahry Teaching Hospital</td>
<td>9(2.5%)</td>
<td>81(22.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>33(9.2%)</td>
<td>327(90.8%)</td>
</tr>
</tbody>
</table>

*P= 0.127*
Table 7. Correlation between CAP and gender

<table>
<thead>
<tr>
<th>Agent</th>
<th>Gender</th>
<th></th>
<th>P −Value&lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (%)</td>
<td>F (%)</td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>49(13.6%)</td>
<td>32(8.9%)</td>
<td>0.990</td>
</tr>
<tr>
<td>K. Pneumoniae</td>
<td>24(6.7%)</td>
<td>19(5.3%)</td>
<td>0.498</td>
</tr>
<tr>
<td>H. influenzae</td>
<td>24(6.7%)</td>
<td>9(2.5%)</td>
<td>0.133</td>
</tr>
<tr>
<td>Total</td>
<td>97(26.9%)</td>
<td>60(16.6%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Relationship between age group and S. pneumoniae

<table>
<thead>
<tr>
<th>Age group (year)</th>
<th>S. pneumoniae</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>1-30</td>
<td>24(6.7%)</td>
<td>88(24.4%)</td>
</tr>
<tr>
<td>31-60</td>
<td>47(13.1%)</td>
<td>149(41.4%)</td>
</tr>
<tr>
<td>61-91</td>
<td>10(2.8%)</td>
<td>42(11.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>81(22.5%)</td>
<td>279(77.5%)</td>
</tr>
</tbody>
</table>

P= 0.727

Table 9. Relationship between age group and K. pneumoniae

<table>
<thead>
<tr>
<th>Age group (year)</th>
<th>K. pneumoniae</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>1-30</td>
<td>7(1.9%)</td>
<td>105(29.2%)</td>
</tr>
<tr>
<td>31-60</td>
<td>26(7.2%)</td>
<td>170(47.2%)</td>
</tr>
<tr>
<td>61-91</td>
<td>10(2.8%)</td>
<td>42(11.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>43(11.9%)</td>
<td>317(88.1%)</td>
</tr>
</tbody>
</table>

P= 0.041
Table 10. Relationship between age groups and *H. influenzae*

<table>
<thead>
<tr>
<th>Age group (year)</th>
<th><em>H. influenzae</em></th>
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<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>1-30</td>
<td>11(3.1%)</td>
<td>101(28.0%)</td>
<td>112(31.1%)</td>
</tr>
<tr>
<td>31-60</td>
<td>16(4.4%)</td>
<td>180(50.0%)</td>
<td>196(54.4%)</td>
</tr>
<tr>
<td>61-91</td>
<td>6(1.7%)</td>
<td>46(12.7%)</td>
<td>52(14.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>43(11.9%)</td>
<td>317(88.1%)</td>
<td>360(100.0%)</td>
</tr>
</tbody>
</table>

*P= 0.724*

Table 11. Comparison between conventional culture method and molecular techniques in detection of CAP

<table>
<thead>
<tr>
<th>Agent</th>
<th>Culture</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No.  (%)</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>64(17.8%)</td>
<td>81(22.5%)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>39(10.8%)</td>
<td>43(11.9%)</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>19(5.3%)</td>
<td>33(9.2%)</td>
</tr>
</tbody>
</table>

*P= 0.000*
Fig 8. Blast analysis of *S. pneuomoiae* 653bp showed (100%) identity to nucleotide sequence of *Streptococcus* 9/30 & 9/29 partial capsule biosynthesis
CLUSTAL O(1.2.1) multiple sequence alignment

**Fig 9. Multiple sequence alignment of S. pneumoniae**
Fig 10. Phylogenetic tree result of *S. pneumoniae*
CHAPTER FIVE

DISCUSSION

5.1. Discussion

The present study investigated 218 (60.6%) males and 142 (39.4%) females. This showed insignificant relationship between gender and CAP the P-value between gender and S. pneumoniae was (0.990), K. pneumoniae (P=0.498) and H. influenza (P=0.133) (Table 7). The prevalence of S. pneumoniae in males was 13.6% and 8.9% in females and for H. influenzae in males was 24(6.7%) and 9(2.5%) in females. But there was no statistical significant (P-value 0.990) (P-value 0.133) association found between gender and S. pneumonia and H. influenzae positively respectively. Yasemin et al., (2010) in their study found 10 out of 54(18.5%) males and 3 out of 23(13.0%) females to be infected with S. pneumoniae and 3 out of 54(5.5%) males and 1 out of 23(4.3%) females to be infected with H. influenzae. Stralin et al., (2006) reported 32 out of 103(31.0%) males and 23 out of 103(22.0%) females to have S. pneumoniae infection. No statistical significance between sex and S. pneumoniae infections were found in the above studies, which concords the current findings. The results showed that age group 31 – 60 were most affected but statistically insignificant relationship between age group and S. pneumoniae, H. influenzae (P= 0.724, 0.724 respectively) but significant with K. pneumoniae (P = 0.041).This may be due random selection of patients.

Our study is in agreement with Stralin et al., (2006) who showed that 55and 24 out of 103 with the age range 18 – 70 to be infected with S. pneumoniae and H. influenzae respectively. Niclas et al., (2010) showed that 70(38%) and 9(5%) out of 184 with the age group 18 – 70 to have S. pneumoniae and H. influenzae respectively. In both studies there was no statistical significant between the age group and causative agents which will agree with our study.

The present study showed significant relationship between S. pneumoniae and hospitals (P= 0.004). This result may be due to different factors such as host, environment, and
bacterial virulence factors and also due to hospitals for example Al shaab hospital specialized in chest and cardiology. Patients attended to this hospital more than other and from different area of Khartoum State and insignificant relationship between *H. influenzae* and *K. pneumoniae* (P-value 0.127, 0.299 respectively) with hospitals. This may be due to random selection of patients from different hospitals.

Cultivation of specimens revealed that 122 (33.8%) of the sputa were found to be positive for bacterial growth and 238 (66.2%) were negative. The isolates were *S. pneumonia* 64 (17.8%), *K. pneumonia* 39 (6.6%) and *H. influenzae* 19 (5.3). The genotypic investigations revealed that 157 (43.6%) of the specimens were positive for CAP agents. These were 81 (22.5%) *S. pneumonia*, 43 (11.9%) *K. pneumonia* and 33 (9.2%) *H. influenzae*. *S. pneumonia* was found to be the most common causative organism. This result is in consistent with other studies carried out elsewhere (Luna *et al.*, 2000). Youning Liu *et al.*, (2009) reported that the most common pathogen isolated from patients with CAP was *S. pneumonia*. Niclas *et al.*, (2010) from Sweden showed that *S. pneumonia* 38%, *H. influenza* (5%). Our result confirmed their finding. The PCR method was found more sensitive than cultures. This is in agreement with Kristoffer *et al.*, (2006) from Denmark who showed that the sensitivity of culture was 50% and PCR was 76%. *K. pneumonia* was the most common etiology of CAP in Southeast Asia mostly 76% as a single pathogen. This fact was reported in 10 studies (Hara *et al.*, 2011).

In the present study *K. pneumonia* account only 43 (11.9%) of the positive specimens. This finding disagrees with Ryota *et al.*, (2015) from Japan and Yi (2015) from Taiwan whom reported *K. pneumonia* as 46% and 51% respectively. This may be due to different environmental conditions such as temperature and humidity and may be due to differences in food quality. On the other hand, PCR confirmed that 203 (56.4 %) of the samples were negative for bacterial infection. This might be of viral infections, other bacterial cause or non-infected (Nolte, 2008, Johanson *et al.*, 2010).

The study results showed that *S. pneumonia*, *H. influenzae* and *K. pneumoniae* were the major causative bacterial agents for CAP in Sudanese population. The PCR technique is a rapid test for the detection and differentiation of community acquired bacterial
pneumonia in sputum samples. The results can be available within 24 hours of specimen collection. PCR is more sensitive and specific than the conventional sputum culture and it is more useful for fastidious microorganisms. PCR for detection of bacteria in respiratory samples is also problematic. In most instances, bacteria that cause pneumonia reach the lungs after colonizing the upper airways, so a positive PCR result may reflect colonization or infection (Stralin et al., 2008). Further studies are recommended for the detection of other bacterial species in sputum samples coupled with viral detection using PCR, especially for bacterial PCR-negative samples. Studies addressing the assessment of antibiograms and resistance genes of the bacteria causing community acquired pneumonia are also needed.
Conculsion

The study concluded that:

- The prevalence of community- acquired bacterial pneumonia is relatively high.
- *S. pneumoniae* is the major cause of community acquired bacterial pneumonia among enrolled patients.
- PCR technique is more accurate in detection of respiratory pathogens but it cannot differentiate between colonization and infection.
- PCR is very useful in detection of fastidious microorganisms e.g. *H. influenzae*.
- Most of the *K. pneumoniae* consist of SHV resistance genes which can affect the treatment of the patient.
**Recommendations**

1- Further studies are recommended for the detection of the other bacterial species in sputum samples coupled with viral detection using PCR, especially for bacterial PCR-negative samples.

2- Studies addressing the detection of antibiograms resistance genes of the bacteria causing community acquired pneumonia are also needed.

3- Use of advanced molecular techniques e.g. Real Time PCR for detection of respiratory pathogens.

4- Overcome of treatment failure should be solved by health education of patients and standard strategy for antibiotic treatment.

5- Further studies are recommended for the risk factors concerning CAP.
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Appendixes

Appendix I

Sudan University of Science and Technology
Community-acquired bacterial pneumonia among Sudanese: A phenotype and molecular study

Questionnaire
Hospital name: ..........................
-Patient No: ..........................
-Name ..................................
-Age: .................................
-Gender:
-Male .........................
-Female .........................
Researcher signature: ................
Donor signature: ....................
Date: ...../..../..................
Appendix II

Informed consent

إعلام موافقة

هذه دعوة مني: الباحث/ ناصر محمد نصر طالب دكتوراه - مختبرات طبية - بجامعة السودان للعلوم والتكنولوجيا لمشاركتكم في برنامج بحث هدفه الكشف عن الألتهاب البكتيري الروني الغير نمطي في مجتمع السودان بدراسة مصلية وجزئية.

إذا رغبت في إنجاح هذا البرنامج فإلى وفريق البحث سنقوم: أخذ عينة بلغم للكشف عن البكتريا المسببة لهذا المرض.

بملء إستمارة بمعلومات تخصصكم لها علاقة بموضوع البحث.

أي معلومة تخصكم في الإستمارة سوف تكون سرية.

مشاركتكم في البرنامج تسعدنا وتساعد في إنجاح هدف البحث.

لكم كامل الحرية في اختيار عدم المشاركة، المشاركة أو الانسحاب من برنامج البحث في أي وقت تشاءون.

يمكنكم الحصول على إجابة لأي سؤال عن برنامج البحث.

التاريخ ............................................................

توقيع المتبرع .............................................

توقيع الباحث .............................................
Appendix III

Preparation of reagents

A) Preparation of 10 X TBE buffer

Amount of 108 g Tris base were weighed and added to 55gm of boric acid and 40ml of 0.5 EDTA then dissolved into 1 liter deionized water PH 8.0

B) Preparation of 1X TBE buffer

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

C) Preparation of Ethidium bromide

Five milligrams of Ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

D) Preparation of loading dye

Three ml of glycerol were added to 7ml of D.W and 2.5 g of bromophenol blue was dissolved into 100 ml D.W. The mixture was as a loading dye.

E) Preparation of agarose gel

Amount of 1.5% of agarose powder was dissolved by boiling in 100 ml 1X TBE buffer (AppliChem). Then was cooled to 55°C in water bath. Then, 5 µl of (10mg/ml) Ethidium bromides were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to
set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

**F) Visualization of PCR products**

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface, 5 µl of PCR products from each samples was mixed with 0.5 µl of loading dye and then electrophoreses 5 µl of DNA ladder (marker) was mixed with 0.5 µl of loading dye and were added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 125v, 500 mA, UK). The electrophoresis was carried at 75v for 30 minutes after electrophoresis period, the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized by u.vtransilluminater (Uvitec – UK).

**G) Trisbase Boric acid EDTA (TBE) Buffer For 500 ml**

89 mMTrisbase 54 gm
89 mM Boric acid 27.5 gm
2 mM EDTA 3.72 g
## Appendix IV

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