IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE, HAEMOPHILUS INFLUENZAE,* AND *MORAXELLA CATARRHALIS* FROM SPUTUM SAMPLES OF PATIENTS WITH COMMUNITY ACQUIRED PNEUMONIA BY POLYMERASE CHAIN REACTION

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The Graduate School of Natural and Applied Sciences

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

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ABSTRACT

IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE, HAEMOPHILUS INFLUENZAE,* AND *MORAXELLA CATARRHALIS* FROM SPUTUM SAMPLES OF PATIENTS WITH COMMUNITY ACQUIRED PNEUMONIA BY POLYMERASE CHAIN REACTION

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The present work describes the evaluation of the value of polymerase chain reaction in diagnosis of pneumonia caused by the most common three bacterial pathogens; *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* from sputum of patients with community acquired pneumonia admitted to The Department of Pulmonary Diseases of Gulhane Military Medical Academy. In this study, 107 sputa from 142 patients with suspected community acquired pneumonia were used to survey the causative agents.

Identification of the pathogens was performed by sputum Gram stain and conventional microbiological methods. Polymerase chain reaction was performed to investigate the presence of

S.pneumoniae, *H.influenzae*, and *M.catarrhalis* for the same sputum samples as well. PCR products were processed by electrophoresis on 2% agarose gels with visualization of the amplicon with ethidium bromide and UV illumination. The 33 of 107 samples were positive in cultures and 67 in PCR.

S.pneumoniae (48.5%) was the most common etiologic agent as to PCR analysis. The incidences of *H.influenzae* and *M.catarrhalis* were determined as 18.6%, and 4.7% respectively.

The incidence of *S.pneumoniae* in patients with CAP and control group individuals were almost the same. The sputum PCR positives were higher than those reported carriage rates for these three microorganisms. 9 of 107 patients with PCR-positive had evidence of infection with pathogens other than *S.pneumoniae*.

The results indicated that some of the PCR results were false positive due to oropharyngeal contamination. PCR testing of sputum samples for diagnosing pneumococcal pneumonia is unable to distinguish colonization from infection in some circumstances. To distinguish the colonization from infection, sputum Gram stain should be applied to the sputum specimens.

Because of being faster and easier, PCR looks like becoming more reliable technique by the using of valid specimens from patients with community-acquired pneumonia if supported by quantitative techniques.

Keywords: Community-acquired pneumonia, *S.pneumoniae*, *H.influenzae*, *M.catarrhalis*, Identification, PCR, Sputum

ÖZ

TOPLUM KÖKENLİ PNÖMONİ HASTALARININ BALGAM ÖRNEKLERİNDEN *STREPTOCOCCUS PNEUMONIAE, HAEMOPHILUS INFLUENZAE* VE *MORAXELLA CATARRHALIS'İN* POLİMERAZ ZİNCİR REAKSİYONU İLE TANIMLANMASI

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Bu çalışma, Gülhane Askeri Tıp Akademisi, Göğüs Hastalıkları Kliniğine gelen, toplum kökenli pnömoni hastalarının balgam örneklerinden en sık rastlanan üç bakteriyel izolat olan *S.pneumoniae, H.influenzae* ve *M.catarrhalis'* in tanımlanmasında polimeraz zincir reaksiyonunun verimini değerlendirmektedir. Çalışmada, 142 toplum kökenli pnömoni şüpheli hastalardan 107 balgam örneği, etken ajanlaın araştırılması için kullanılmıştır.

Patojen mikroorganizmaların tanımlanmasında balgam Gram boyama ve standart mikrobiyolojik metodlar kullanılmıştır. Polimeraz zincir reaksiyonu, *S.pneumoniae, H.influenzae* ve *M.catarrhalis* varlığının araştırılmasında aynı balgam örneklerine uygulanmıştır. Polimeraz zincir reaksiyonu ürünleri etidium bromid ile UV görüntüleme sistemiyle, %2'lik agaroz jelde

görüntülenmiştir. 107 balgam örneğinin 33 tanesi kültürde; 67 tanesi PCR' da pozitif olarak saptanmıştır.

PCR sonuçlarına göre, *S.pneumoniae* %48.5 ile en sık rastlanan etyolojik ajan olmuştur. *H.influenzae* ve *M.catarrhalis* insidansları sırasıyla; %18.6 ve %4.7 olarak saptanmıştır.

S.pneumoniae insidansı pnömoni hasta grubunda ve kontrol grubunda birbirine çok yakın değerlerde gözlenmiştir. Çalışma grubunda yer alan hastaların PCR pozitiflikleri her üç mikroorganizma için de raporlanmış taşıyıcılık oranlarından daha yüksek bulunmuştur. *S.pneumoniae* için PCR-pozitif 107 hastanın 9' unda *S.pneumoniae* dışındaki etkenlerin oluşturduğu enfeksiyon varlığına dair kanıtların mevcut olduğu görülmüştür.

Sonuçlar, PCR verilerinin bazılarının orofarengial kontaminasyon nedeniyle yalancı pozitif olabileceğini göstermiştir. Bazı durumlarda balgam örneklerinde PCR testi, pnömokoksik pnömoni tanısında kolonizasyonu infeksiyondan ayırmada yetersiz kalabilmektedir. Yalancı pozitif sonuçlardan kaçınmak, kolonizasyonu infeksiyondan ayırt edebilmek için balgam örneklerinin kalitesi Gram boyama tekniği uygulayarak saptanmalıdır.

Sayısal tekniklerle desteklendiği durumda, toplum kökenli pnömoni hastalarından uygun örneğin elde edilmesiyle, daha hızlı ve kolay olan PCR metodunun daha güvenilir hale gelebileceği düşünülmektedir .

Anahtar Kelimeler: Toplum-kökenli pnömoni, *S.pneumoniae, H.influenzae, M.catarrhalis*, tanımlama, PCR, balgam.

To my parents and my husband ……

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INTRODUCTION

1.1 THE LOWER RESPIRATORY TRACT

1.1.1 Anatomy

The respiratory system can be divided into two as upper and lower tracts (Figure 1.1). The lower respiratory tract includes the trachea, bronchi, and bronchiols. The respiratory tract as the gastrointestinal tract is one of the major connections between the interior of the body and the outside environment. The respiratory tract begins with the nasal and oral passages, which serve to humidify inspired air, and extends past the nasopharynx and oropharynx to the trachea and then into the lungs. The trachea divides into bronchi, which subdivide into bronchioles, the smallest branches that terminate in the alveoli. Some 300 million alveoli are estimated to be present in the lungs; these are the primary, microscopic gas exchange structures of the respiratory tract (Forbes et al., 1998).

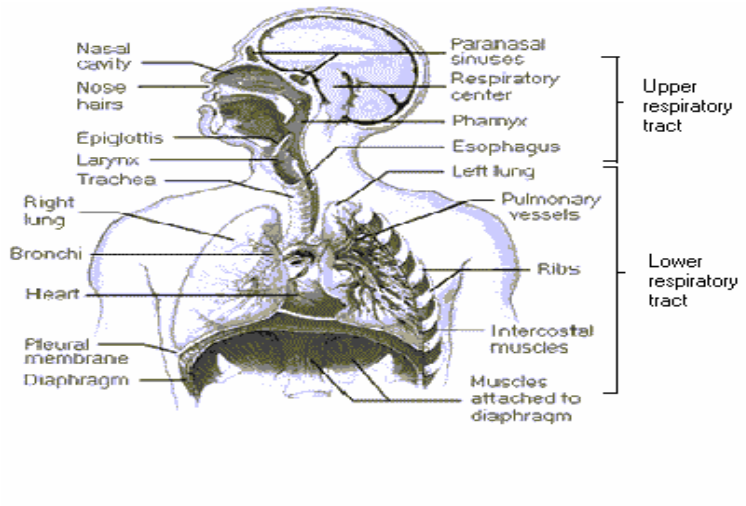

FIGURE 1.1: Anatomy of the respiratory tract, including upper and lower regions.

1.1.2 Pathogenesis

Basic Concepts

Microorganisms primarily cause disease by a limited number of pathogenic mechanisms. Encounters between the human body and microoroganisms occur frequently. However, establishment of infection after such contacts tends to be the exception rather than the rule. Whether an organism is successful in establishing an infection is dependent not only on the organism's ability to cause disease (pathogenicity) but also on the human host's ability to prevent the infection (Forbes et al., 1998).

Normal flora of the nasopharynx and oropharynx help to prevent colonization of the upper respiratory tract. Some of the bacteria that can be isolated as part of the indigenous flora of healthy hosts are listed in Table 1.1. Many species of them may

cause disease under certain circumstances but they are often isolated from the respiratory tracts of healthy persons (Forbes et al., 1998).

Table 1.1 Organisms Present in the Nasopharynx and Oropharynx of Healthy Humans

Possible Pathogens	**Rarely Pathogens**
Acinetobacter spp.	Nonhemolytic streptococci
Viridans streptococci including *S.milleri*	Staphylococci
Beta-hemolytic *streptococci*	Micrococci
Streptococcus pneumoniae	*Corynebacterium* spp.
Staphylococcus aureus	*C*oagulas(-)staphylococci
Neisseria meningititis	Neisseria spp.
Mycoplasma spp.	(Other than
	N.gonorrhoeae
Haemophilus influenzae	and N.meningititis)
Haemophilus parainfluenzae	Lactobacillus spp.
Moraxella (Branhamella) catarrhalis	*Veillonella spp.*
Candida albicans	*Spirochetes*
Herpes simplex virus	Rothia detocariosa
Enterobactericeae	Leptotrichia buccalis
Mycobacterium spp.	Selenomonas
Pseudomonas spp.	Wolinella
Burkholderia (pseudomonas) cepacia	*Stomatococcus mucilaginosus*
Filamentous fungi	Campylobacter spp.
Klebsiella ozanae	
Eikenella corroders	
Bacteroides spp.	
Peptostreptecoccus spp.	
Actinomyces spp.	
Capnocytophaga spp.	
Actinobacillus spp. *A.actinomycetemcomitans*	
Haemophilus aphronilus	
Entamocha gingivais	
Trichomonas tenax	

1.1.3 Respiratory Tract Pathogens:

Certain microorganisms are almost always considered to be etiologic agents of a disease if they are present in any number in the respiratory tract because they possess virulance factors that

are expressed in every host. These organisms are listed in Table 1.2, and Table 1.3 (Forbes et al., 1998).

Table 1.2 Definite respiratory Tract Pathogens

*Corynebacterium diphteriae (*toxin-producing)
Mycobacterium tuberculosis
Mycoplasma pneumoniae
Clamydia trachomatis
Clamydi (TWAR) *pneumoniae*
Bordetella pertusis
Leigonella spp.
Pneumocystis carini
Nocardia spp.
Histoplasma capsulatum
Coccidioides immitis
Cryptococcus neoformans (may also be recovered from patient without disease)
Blastomyces dermatitidis
Viruses (respiratory syncytial virus, adenoviruses, enteroviruses, herpes simplex virus, influenza and parainfluenza virus, rhinoviruses).

Table 1.3 Rare Respiratory Tract Pathogens

Francisella tularensis
Bacillus anthracis
Yersinia pestis
Burkholderia (Pseudomonas) pseudomallei
Coxiella burnetti
Chlamidia psittaci
Brucella spp.
Salmonella spp.
Pasteurella multocida
Klebsiella rhinoscleromatis
Varicella-zoster virus (VZV)
Parasites

1.1.4 Respiratory Infections

Acute respiratory tract infections (ARI) are a major cause of morbidity and mortality worldwide. In a global survey of causes of

mortality, respiratory tract diseases were estimated to be the third most common with 4.3 million deaths in 1990. Although the incidence of ARI does not differ between developed and developing countries, the incidence of acute lower respiratory tract infection is over 12-fold greater in developing countries (Hart et al., 2002).

1.2 PNEUMONIA

Pneumonia (inflammation of the lower respiratory tract involving the lung's airways and supporting structures) is a major cause of illness and death in both the community and hospital settings. Once the microorganism has successfully invaded the lungs, disease can follow that includes the alveolar spaces and their supporting structures, the interstitium, and the terminal bronchioles (Forbes et al., 1998).

1.2.1 Causes of Pneumonia

Pneumonia may result from bacteria, viruses, mycoplasmas, other infectious agents, such as fungi - including pneumocystis, and various chemicals.

Bacterial pneumonia is an important cause of death (Balthasar & Rossi, 2001; Guerrero, 1996). It can attack anyone, from infant to elderly. Alcoholics, the debilitated, post-operative patients, people with respiratory diseases or viral infections and people who have weakened immune systems are at greater risk (Garbino et al., 2002; File, 2003).

Most of all pneumonia incidences is believed to be caused by viruses and bacteria (Guerrero, 1996). After viral pneumonia,

especially influenza, secondary bacterial disease caused by beta-hemolytic streptococci, Pneumococci, *Staphylococcus aureus, Moraxella catarrhalis, Haemophilus influenzae,* and *Chlamidia pneumoniae* is more likely to develope (Murray et al., 2003).

Approximately one third of CAP cases are caused by atypical pathogens *M.pneumoniae, C.pneumoniae, L.pneumophila* (Pinar et al., 2004). Atypical pathogens especially *M.pneumoniae* have an important role in CAP (Liu et al., 2004).

Pneumocystis pneumonia (PCP) caused by the opportunistic fungal agent *Pneumocystis carinii* continues to cause illness and death in HIV-infected patients (Beard et al., 2004).

Rickettsia, Q fever, psittacosis, tularaemia, and endemic fungi (histoplasmosis, coccidioidomycosis, blastomycosis) diseases, and *Mycobacterium tuberculosis* are other causes of CAP (File et al., 2003).

1.2.2 Epidemiology / Ethiologic Agents

There are two major categories of pneumonia: those that are considired community-acquired (patients are believed to have acquired their infection outside the hospital setting) and those that are considered hospital-acquired (patients are believed to have acquired their infection within the hospital setting, usually at least 3 days following admission) (Forbes et al., 1998).

Hospital acquired pneumonia can be caused by numerous pathogenic bacteria most commonly including *Staphylococcus*

aureus, *Pseudomonas aeruginosa* and *Enterobacteriaceae* (Gordon et al., 2003).

1.3 COMMUNITY-ACQUIRED PNEUMONIA

The Infectious Diseases Society of America (IDSA) defines community-acquired pneumonia (CAP) as an acute infection of the pulmonary parenchyma that is associated with at least some symptoms of acute infection, accompanied by acute infiltrate on the chest radiograph or auscultatory findings consistent with pneumonia (such as altered breath sounds) in a patient who is not hospitalized or residing in a long-term-care facility for 14 or more days before symptoms onset (Barlett et al., 2000).

CAP is a disease with substantial morbidity, mortality, and economic impact, especially during the winter and early spring (Capelasteguia et al., 2004). In the United States, pneumonia is the sixth leading cause of death. It is the number one cause of death from infectious diseases, and accounts for at least 600,000 hospital admissions each year (Sanraj et al., 2004; Miyashita et al., 2004; Mark et al., 2001).

CAP remains to be a common cause of a serious disease. In spite of the recent advances in the field, including the identification of new pathogens, new methods of microbial detection, new antimicrobial agents, and effective vaccines, lower respiratory infections are still the most frequent causes among infectious mortality worldwide. In 1998, the World Health Organization reported 3.7 million deaths due to lower respiratory infections (WHO, 1998). In the United States, it has been estimated that more than 5 million cases of CAP occur annually, and nearly 1.1

million of these require hospitalization (Niederman et al., 1998). In the outpatient setting, the mortality rate of pneumonia remains low (5%); however, when patients with CAP require hospitalization, the mortality increases to an average of 12% (Gales et al., 2002).

1.3.1 Pathogenesis

Organisms can cause infection of the lung by four possible routes: by upper airway colonization or infection that subsequently extends into the lung; by aspiration of organism, thereby avoiding the upper airway defenses; by inhalation of airborne droplets containing the organism; or by seeding of the lung via the blood from a distant site infection. Viruses cause primary infections of the respiratory tract, as well as inhibit host defenses that, in turn, can lead to a secondary bacterial infection (Forbes et al., 1998).

Aspiration of oropharyngeal contents is important in the pathogenesis of many types of pneumonia. Aspiration happens most often during a loss of consciousness such as might occur with anesthesia or seizure, or after alcohol or drug abuse, but other individuals, particularly geriatric patients, may also develop aspiration pneumonia. Neurologic disease or esophageal pathology and periodontal disease or gingivitis are other important risk factors. Aided by gravity and often by loss of some host nonspecific protective mechanisms, organisms reach lung tissue, where they multiply and attract host inflammatory cells. Other mechanisms include inhalation of aerosolized material and hematogenous seeding. The buildup of cell debris and fluid contributes to the loss of lung function and thus to the pathology (Forbes et al., 1998).

1.3.2 ETIOLOGY OF CAP

More than 75% of patients presenting with CAP are treated as outpatients. When cultures are obtained, a pathogen is identified in approximately 50% of patients with CAP, and *S.pneumoniae* is the most common identified pathogen (Thomas et al., 2004; Miyashita et al., 2004). *S.pneumoniae* may be the pathogen in some "culture-negative" CAP cases, because *S.pneumoniae* was the most frequent pathogen identified through transthoracic needle aspiration, even when traditional sputum cultures were negative. More importantly, *S.pneumoniae* infection in CAP is frequently linked to serious illness requiring hospital admission and/or admission to the intensive care units. Additional bacterial etiologies of CAP have been identified less frequently and include *Haemophilus influenzae*, *Moraxella catarrhalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus* spp. Moreover, atypical pathogens (*Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella* spp) have been noted in previous investigations and may constitute up to 25% of CAP isolates, although they are not identified by Standard microbiological evaluations. In addition, patients with CAP may be infected with both typical and atypical pathogens. Recent studies show that coinfection rates with both typical and atypical pathogens can range from 7% to 37.1% (Mark, 2001).

The most frequently isolated respiratory pathogens are *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* (Pfaller et al., 2001).

1.4 STREPTOCOCCUS

1.4.1 Taxonomy

In traditioal and clinically useful taxonomic schemes, the streptococci belong to the family *Streptococcaceae.* The nomenclature for streptococci, especially the nomenclature in medical use, has been based largely on serogroup identification of cell wall components. For several decades, interest has focused on two major species that cause severe infections: *S.pyogenes* (group A streptococci) and *S.pneumoniae* (pneumococci). In 1984, two other members were assigned a new genus - the group D enterococcal species (which account for 98% of human enterococcal infections) became *Enterococcus faecalis* (the majority of human clinical isolates) and *E faecium* (associated with a remarkable capacity for antibiotic resistance) (Koneman et al., 1992).

In recent years, increasing attention has been given to other streptococcal species, partly because innovations in serogrouping methods have led to advances in understanding the pathogenetic and epidemiologic significance of these species. A variety of cell-associated and extracellular products is produced by streptococci, but their cause-effect relationship with pathogenesis has not been defined. Some of the other medically important streptococci are *S.agalactiae* (group B), *E.faecalis* (group D) and the viridans streptococci. Particularly for the viridans streptococci, taxonomy and nomenclature are not yet fully reliable or consistent. Important members of the viridans streptococci, normal commensals, include *S.mutans* and *S.sanguis*, *S.mitis S.milleri* (Madigan, 1999).

1.4.2 General Characteristics

The genus *Streptococcus,* a heterogeneous group of Gram-positive bacteria, has broad significance in medicine and industry. Various streptococci are important ecologically as part of the normal microbial flora of animals and humans; some can also cause diseases that range from subacute to acute or even chronic ones (Madigan, 1999).

They are Gram-positive cocci, nonmotile, and nonsporulating; they usually require complex culture media. S *pneumoniae* appears as a 0.5-1.25 µm diplococcus, typically described as lancet-shaped but sometimes difficult to distinguish morphologically from other streptococci. Streptococcal cultures older than the exponential phase, may lose their Gram-positive staining characteristics (Madigan, 1999).

Unlike *Staphylococcus*, all streptococci lack the enzyme catalase. Most are facultative anaerobes but some are obligate anaerobes (Madigan, 1999; Koneman, 1992). Streptococci often have a mucoid or smooth colonial morphology, and *S pneumoniae* colonies exhibit a central depression caused by rapid partial autolysis. As *S pneumoniae* colonies age, viability is lost during fermentative growth in the absence of catalase and peroxidase because of the accumulation of peroxide (Madigan, 1999).

1.4.3 Streptococcus pneumoniae

Streptococcus pneumoniae are Gram-positive, lancet-shaped cocci (elongated cocci with a slightly pointed outer curvature). Usually they are seen as pairs of cocci (diplococci), but they may

also occur singly and in short chains. When cultured on blood agar, they are alpha hemolytic. Individual cells are between 0.5 and 1.25 micrometers in diameter. They do not form spores, and they are nonmotile. Like other streptococci, they lack catalase and ferment glucose to lactic acid. Unlike other streptococci, they do not display an M protein, they hydrolyze inulin, and their cell wall composition is characteristic both in terms of their peptidoglycan and their teichoic acid (Todar, 2002).

The pneumococcus is the major cause of community-acquired bacterial pneumonia (Murdoch et al., 2003; Balthasar, 2001; Koneman, 1992; Brown, 1998). The organism is a member of the normal upper respiratory tract flora in many individuals (Madigan, 1999; Koneman, 1992).

1.4.4 Pathogenesis

Streptococci vary widely in pathogenic potential. Despite the remarkable array of cell-associated and extracellular products previously described, no clear scheme of pathogenesis has been worked out. *S.pneumoniae* and, to a lesser extent, *S.pyogenes* are part of the normal human nasopharyngeal flora. Their numbers are usually limited by competition from the nasopharyngeal members of the microbial community and by nonspecific host defense mechanisms, but failure of these mechanisms can result in disease. More often disease results from the acquisition of a new strain following alteration of the normal flora (Madigan, 1999).

Invasion of other portions of the upper or lower respiratory tract results in infections of the middle ear (otitis media), sinuses (sinusitis), or lungs (pneumonia). In addition, meningitis can occur by direct extension of infection from the middle ear or sinuses to

the meninges or by way of bloodstream invasion from the pulmonary focus. Bacteremia can also result in infection of bones (osteomyelitis) or joints (arthritis) (Madigan, 1999).

Antiphagocytic capsules are an important virulence factor of the pathogen (Madigan, 1999). Encapsulated organisms are more virulant than noncapsulated organisms. There are 84 capsular types of *S.pneumoniae*; 23 of these types account for over 88% of pneumococcal bacteremia and meningitis (Koneman, 1992). The antiphagocytic *S pneumoniae* capsule is the most clearly understood virulence factor of these organisms; type 3 *S.pneumoniae*, which produces copious quantities of capsular material, are the most virulent. Unencapsulated *S.pneumoniae* are avirulent (Patterson, 2004).

The capsule of *S.pneumoniae* renders it resistant to phagocytosis. The ability to evade this important host defense mechanism allows *S.pneumoniae* to survive, multiply, and spread to various organs. The cell wall of S.*pneumoniae* contains teichoic acid. The inflammatory response induced by Gram-positive cell walls differs from that induced by the endotoxin of Gram-negative organisms, but does include recruitment of polymorphonuclear neutrophils, changes in permeability and perfusion, cytokine release, and stimulation of platelet-activating factor. The role of other *S.pneumoniae* moieties in virulence is less clear: protein A, pneumolysin, and peptide permeases. *S.pneumoniae* is the leading cause of bacterial pneumonia beyond the neonatal period. Pleural effusion is the most common and empyema (pus in the pleural space) one of the most serious complications of *S.pneumoniae*. This organism is also the most common cause of sinusitis, acute bacterial otitis media, and conjunctivitis beyond early childhood.

Dissemination from a respiratory focus results in serious disease: outpatient bacteremia in children, meningitis, occasionally acute septic arthritis and bone infections in patients with sickle cell disease and, more rarely, peritonitis (especially in patients with nephrotic syndrome) (Madigan, 1999)

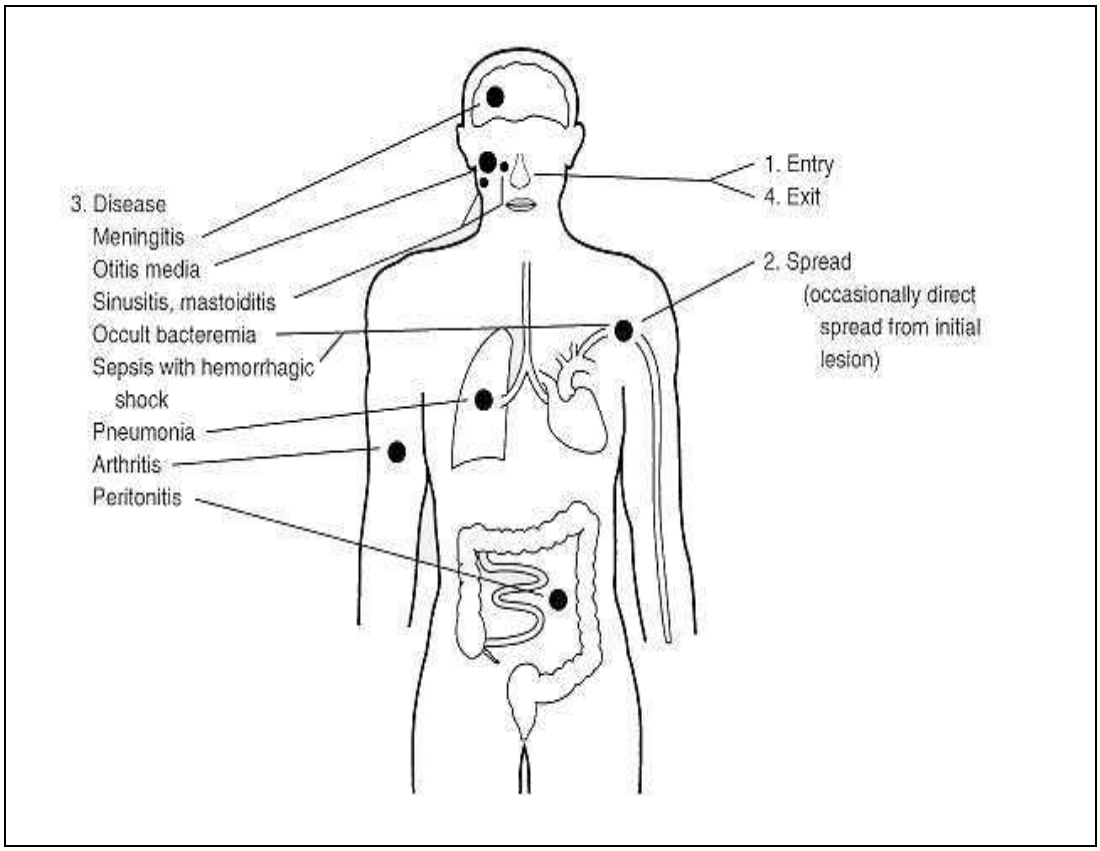

FIGURE 1.2 Pathogenesis of *S.pneumoniae* infections

1.4.5 Epidemiology

S.pneumoniae is a transient member of the normal flora, colonizing the nasopharynx of 40% of healthy adults and children with no adverse effects. Children carry this pathogen in the nasopharynx asymptomatically for about 4-6 weeks, often several serotypes at a time. New serotypes are acquired approximately every 2 months. Serotypes 6, 14, 18, 19, and 23 are the most prevalent accounting for 60-80% of infections depending on the

area of the world. Pneumococcal infection accounts for more deaths than any other vaccine-preventable bacterial disease. Those most commonly at risk for pneumococcal infection are children between 6 months and 4 years of age and adults over 60 years of age. Virtually every child will experience pneumococcal otitis media before the age of 5 years. It is estimated that 25% of all community-acquired pneumonia is due to pneumococcus (1,000 per 100,000 inhabitants). Recently, epidemics of disease have reappeared in settings such as chronic care facilities, military camps and day care centers, a situation not recognized since the pre-antibiotic era (Todar, 2002).

Also of concern, is the increased emergence of antibiotic resistance, especially in the past decade. Multiple antibiotic resistant strains of *S. pneumoniae* that emerged in the early 1970s in Papua New Guinea and South Africa were thought to be a fluke, but multiple antibiotic resistances now covers the globe and has rapidly increased since 1995. Resistance has followed increases in penicillin resistance to cephalosporins and multidrug resistance. The incidence of resistance to penicillin increased from <0.02 in 1987 to 3% in 1994 to 30% in some communities in the United States and 80% in regions of some other countries in 1998. Resistance to other antibiotics has emerged simultaneously: (Todar, 2002).

1.4.6 Clinical Manifestations

In humans, diseases associated with the streptococci occur chiefly in the respiratory tract, bloodstream, or as skin infections; it is the leading cause of community-acquired pneumonia and a frequent cause of meningitis, sinusitis, and otitis media in both

children and adults (Messmer et al, 2004), also cause of bacteremia, peritonitis and arthritis (Madigan, 1999)

Pneumonia

S. pneumoniae is a leading cause of pneumonia in all ages (particularly the young and old), often after "damage" to the upper respiratory tract (e.g. following viral infection). The organism may be harbored in the upper respiratory tract of 5% to 10% of adult. The organism gains access to the alveolar spaces of the lungs and eventually causes labor pneumonia commonly of the right middle lobe, with consolidation and bacteremia (Brown, 1998; Koneman, 1992).

Meningitis

Bacterial meningitis is one of the most dangerous infections in children. The organism often spreads causing bacteremia and meningitis. This organism is the most common cause of bacterial meningitis in adults (Musher et al., 2000).

Otitis media

Otitis media is both the most common bacterial infection in childhood and the most commonly diagnosed bacterial illness in the pediatric age group (Tele et al., 1989). Between 6 month and 4 years of age *S. pneumoniae* account for about 40-50% of infections (Musher et al., 2000). In children up to 10 years of age, *S. pneumoniae* accounts for about 40% of infections (Barry et al., 1994). In adults, pneumococci are the most common pathogen in otitis media (Musher et al., 2000)

Bacteremia

Peumococcal bacteremia is most common in children aged 3-36 months. Pneumococcal bacteremia can occur in 25-30% of

patients with pneumonia, and more than 80% of patients with meningitis (Murray et al., 1994)

1.4.7 Laboratory Diagnosis of *S. pneumoniae*

Gram-stained smears of clinical specimens that yeild streptococci on culture will generally show gram-positive or Gram-variable cocci arranged in pairs and chains. Chains of cells in both specimens and broth cultures tend to appear as chains of pairs of cells rather than as chains of induvidual cells. Indivudial cell shapes range from those that resemble diplococci to those that are coccobacillary or coryneform in appearance. These morphologies are often observed on smears from broth cultures and from solid media as well. *S.pneumoniae* will most often appear as pairs of lanceolate-shaped cells. On smears of specimens yielding mucoid, heavily encapsulated strains, the capcule may appear as a pink halo or as a nonstaining area surrounding the cells in relief against a pink background surrounding the organism (Koneman, 1992).

Definite identification of *S.pneumoniae* involves the serologic detection of pneumococcal capsular polysaccharides using specific antisera. This is complicated by the fact that there are more than 84 different capsular serotypes. Omniserum is capable of detecting all pneumococcal serotypes (Koneman, 1992).

Specimens that may be expected to yield *s*treptococci on culture should be plated onto a suitable blood containing peptone rich medium to support this fastidious organism (Koneman, 1992).

Streptococci recovered from human clinical specimens are identified on the basis of their hemolytic qualities, serologic tests

for the detection of cell-wall or capsular antigens, and physiologic and biochemical tests. Some of the tests performed in the laboratory for identification of these organisms provides presumptive results, while others provide definitive results. Before proceeding with identification tests, however, one must be sure that the gram-positive cocci under considiration are calase negative, placing them in the *Streococcoccus* bacterial groups (Koneman, 1992).

To identify *S.pneumoniae* from the sputum specimens, sheep blood agar with added gentamicine (5μg/ml) should be used as a selective media (Murray et al., 1994). Plates shoul be incubated at 35 ^{0}C in ambient atmosphere or in 5% to 7% CO_2. *S.pneumoniae* displays a spectrum of colony types the appearance of which is dependent on the degree of encapsulation. In all cases, these colonies are generally surrounded by large zone of intense green α-hemolysis. Colonies of heavily encapsulated strains may be several milimeters in diameter, are highly mucoid, appear gray, and may ressemble drops of oil (Koneman, 1992).

Susceptibility to optochin is used to differantiate *S pneumoniae* from the other viridans streptococci. The optochin susceptibility test is performed on blood agar media. Zones of inhibition must be measured prior to interpretation. A zone of 14 mm or greater around the 6mm disk indicates susceptibility to optochin and identifies the organism as a pneumococcus. Optochin resistant *S pneumoniae* isolates are rarely encountered (Ruoff et al., 2003; Koneman, 1992).

1.5 HAEMOPHILUS

1.5.1 Taxonomy

The genus *Haemophilus* is classified in the family *Pasteurellaceae,* which also includes *Pasteurella* and *Actinobacillus.* There are ten human related species and several animal related species in the genus (Pfaller et al., 2001; Koneman, 1992).

Human related species in the genus *Haemophilus* are, *H.influenzae, H.parainfluenzae, H.haemolyticus, H.parahaemolyticus, H.aphrophilus, H.paraphrophilus, H.paraphrophaemolyticus, H.aegyptius, H.segnis, H.ducrei* (Pfaller et al., 2001; Koneman, 1992; Campos et al., 1999).

Pfeiffer isolated *H.influenzae* from sputum and lung tissues of persons who died during the 1892 influenza pandemic. In 1931, 6 serotypes of *H.influenzae* was determined (a, b, c, d, e and f) by Pittman (Campos et al., 1999).

1.5.2 General Characteristics

Members of the genus *Haemophilus* are small, nonmotile, gram-negative bacilli that require growth factors present in blood (Koneman, 1992).

H.influenzae requires hemin (factor X) and NAD+ (factor V) for growth. It grows on chocolate agar but not on blood agar (Koneman, 1992). It may appear on a blood agar plate as tiny satellite colonies around the colonies of other bacteria that have lysed red blood cells, like *Staphylococcus aureus* (Koneman et al., 1997). Other *Haemophilus* species require only NAD+ and

therefore grow on blood agar. NAD+ is released into the medium by red blood cells and is available to the bacteria in blood agar, hemin is bound to red blood cells and is not released into the medium unless the cells are broken up, as in chocolate agar (Murray et al., 1994; Koneman et al., 1997).

H.parainfluenzae requires only factor V and therefore is able to grow on blood agar. The long-prevailing notion that *H.ducreyi* grows only in clotted rabbit blood has been dispelled by recent studies that show slow growth of this organism in Mueller-Hinton agar containing 5% sheep blood. All *Haemophilus* species grow more readily in an atmosphere enriched with CO_2; *H.ducreyi* and some nontypable *H.influenzae* strains will not form visible colonies on culture plates unless grown in CO_2-enriched atmosphere (Murray et al., 1994; Todar, 2002).

1.5.3 *Haemophilus influenzae*

H.influenzae strains are classified as either serotypable (if they display a capsular polysaccharide antigen) or nontypable (if they lack a capsule). The word "type" as applied to *H.influenzae* refers to this serotyping scheme. There are six generally recognized types: a, b, c, d, e, and f (Campos et al., 1999; Koneman et al., 1997; Murray et al., 1994). Type b *H.influenzae* is plainly the most virulent of the *Haemophilus* species; 95 percent of bloodstream and meningeal Haemophilus infections in children are due to these organisms. In contrast, in adults, nontypable strains of *H.influenzae* are the most common cause of Haemophilus infection, presumably because most adults have acquired antibody to PRP (Koneman et al., 1997).

H.influenzae infections are generally due to direct spread from the blood stream of colonization area (Hand, 2000).

1.5.4 Pathogenesis

The pathogenesis of *H.influenzae* infections is not completely understood, but the presence of a polyribosyl ribitol phosphate (PRP) capsule is an important virulence factor: it renders type b *H.influenzae* resistant to phagocytosis by polymorphonuclear leukocytes in the absence of specific anticapsular antibody (Koneman et al., 1997; Todar, 2002). Encapsulated organisms can penetrate the epithelium of the nasopharynx and invade blood capillaries directly. Nontypable strains are less invasive, but they, as well as typable strains, induce an inflammatory response that causes disease; production of exotoxins is not thought to play a role in pathogenicity (Murray et al., 1994).

Nontypable *H.influenzae* strains colonize the nasopharynx of most normal individuals, but type b *H.influenzae* strains are found in only 1 to 2 percent of normal children. Outbreaks of type b infection occur, especially in nurseries and child care centers (Harabuchi et al., 1994; Murphy & Apicella, 1987).

Type b *H.influenzae* colonizes the nasopharynx, and may penetrate the epithelium and capillary endothelium to cause bacteremia. Meningitis may result from direct spread via lymphatic drainage or from hematogenous spread. Nontypable H influenzae colonizes the nasopharynx and, to a lesser extent, the trachea and bronchi and may infect mucosa damaged by viral disease or cigarette smoking. Lipooligosaccharide is largely responsible for

inflammation; exotoxins do not play a role (Hand, 2000; Brown, 1998; Pfaller et al., 2001).

1.5.5 Epidemiology

Haemophilus organisms spread directly among individuals without a known contribution from environmental sources or animal reservoirs. Nontypable *H.influenzae* strains are found in the nasopharynx of many healthy subjects, depending upon the frequency and intensity with which they are sought. By contrast, type b H influenzae is found only in 1 to 2 percent of healthy children, and its spread to previously uncolonized children in the early years is associated with a substantially increased risk of infection. Families and day care centers are important sources for dissemination of these organisms (Sell & Wright, 1990).

1.5.6 Clinical Manifestations

The genus Haemophilus includes a number of species that cause a wide variety of infections (Figure 1.2) but share a common morphology and a requirement for blood-derived factors during growth that has given the genus its name. Type b *H.influenzae* is by far the most virulent organism in this group, commonly causing bloodstream invasion and meningitis in children younger than 2 years. Nontypable strains are frequent causes of respiratory tract disease in infants, children, and adults. Type b *Haemophilus influenzae* can cause meningitis, epiglottitis, bacteremia, and cellulitis. Nontypable *H.influenzae* can cause otitias media, sinusitis, tracheobronchitis, and pneumonia (Koneman et al., 1997; Murray et al., 1994).

Other Haemophilus species cause disease less frequently. *Haemophilus parainfluenzae* sometimes causes pneumonia or bacterial endocarditis. *Haemophilus ducreyi* causes chancroid. *Haemophilus aphrophilus* is a member of the normal flora of the mouth and occasionally causes bacterial endocarditis. *Haemophilus aegyptius*, which causes conjunctivitis and Brazilian purpuric fever, and *Haemophilus haemolyticus* used to be separated on the basis of their ability to agglutinate or lyse red blood cells, but both are now included among the nontypable *H.influenzae* strains. *H.ducreyi* cause genital chancre (Murray et al., 1994).

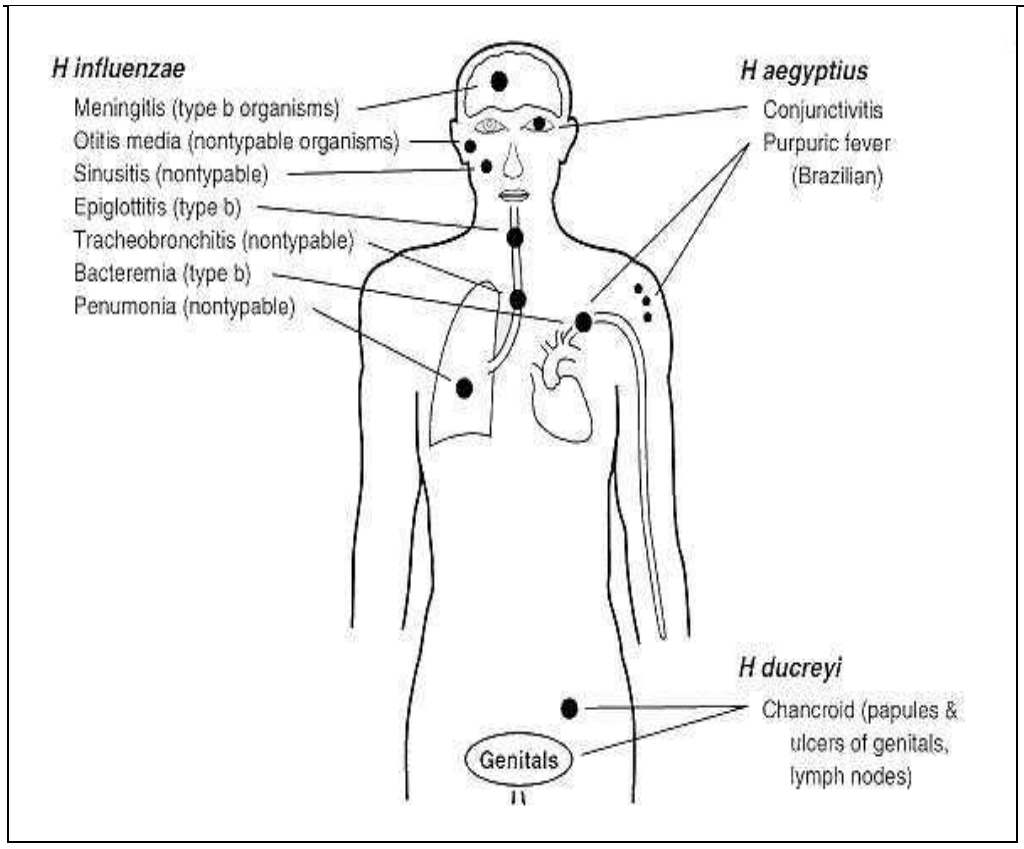

FIGURE 1.3 Clinical presentation of Haemophilus infections.

Meningitis

The pathogenesis of meningitis due to type b *H.influenzae* has been well studied. These organisms colonize the nasopharynx and spread from one human to another by direct contact or via secretions and/or aerosol. They penetrate epithelial layers and capillary endothelium by unknown mechanisms, reaching the meninges either directly via lymphatic drainage from the nasopharynx or indirectly by causing bacteremia with subsequent seeding of the highly vascular choroid plexus (Murray et al., 1994).

Most cases of *H.influenzae* meningitis in adults are due to nontypable strains. The pathogenesis of these infections differs from that of type b *H.influenzae*. Nontypable strains are unencapsulated and therefore less virulent, and they are unable to penetrate directly into capillaries. Rather, they gain entry to the central nervous system by direct extension, often associated with infection of the sinuses or middle ear and/or with trauma involving the sinuses or skull. Thus, about 50 percent of adults with *H.influenzae* meningitis have a history of prior head trauma with or without a documented cerebrospinal fluid leak, and another 25 percent have chronic otitis media (Murphy & Apicella, 1987).

Cellulitis and Epiglottitis

Cellulitis and epiglottitis are discussed together because their pathogenesis is probably quite similar. Both are due to the type b *H.influenzae*, are likely to cause associated bacteremia, and occur more frequently in children than adults. Epiglottitis can be regarded as a cellulitis of the relatively loose submucosal connective tissues of the epiglottis. In this syndrome, a sore throat rapidly progresses to difficulty in breathing, stridor, obstruction of the airways, and

respiratory arrest. Local extension from the colonized nasopharynx through soft tissues is probably responsible for epiglottitis. Cellulitis often involves the face or neck. It sometimes seems to start at the buccal mucosa and extend outward, supporting the idea that it also results from local extension. The often-repeated teaching that facial cellulitis due to Haemophilus causes a distinctive bluish tinge enabling it to be distinguished from cellulitis caused by other bacteria, defies reason and is best ignored (Koneman et al., 1997; Murray et al., 1994).

Bacteremia

Bactremia is a frequent and early manifestation of acute *H.influenzae* type b infection. Hematogenous spread of the microorganism, however, may results in several other clinical manifestations of infection. Seeding of the soft tissues results in cellulitis. Septic arthritis and osteomyelitis may also complicate *H.influenzae* bacteremia (Koneman et al., 1992).

Respiratory Diseases
(sinusitis, otitis media, acute tracheobronchitis, and pneumonia)

Nontypable *H.influenzae* is a major pathogen that colonizes the human respiratory tract. Adherence of bacteria to mammalian tissues, which is mediated by fimbriae, is thought to be an important precursor to colonization, and infection of the upper airways is associated with the presence of fimbriae. Respiratory infections caused by these organisms include sinusitis, otitis media, acute tracheobronchitis, and pneumonia (Murphy & Apicella, 1987).

Purulent material aspired from acutely infected paranasal sinuses in children or adults or from behind an infected tympanic

membrane in babies and young children commonly contains nontypable *H.influenzae*. Studies of outer membrane protein profiles have shown that middle ear and nasopharyngeal isolates are identical, supporting the notion that colonization of the eustachian tube, followed by obstruction and infection, is probably responsible. Repeated bouts of otitis media are thought to be due to different strains; each infection may be associated with emergence of antibody to distinctive surface proteins. The decreasing frequency of otitis media with age is due in part to anatomic changes and in part to immunity to *H.influenzae* (Murray et al., 1994).

Nontypable *H.influenzae* is found in the nasopharynx and in sputum cultures of nearly one-half of adults with chronic bronchitis. Not surprisingly, this organism is also recovered from the large airways via bronchoscopy, since upper-airway bacteria are carried along by the bronchoscope (Murphy & Apicella, 1987; Musher et al., 1983).

Nontypable *H.influenzae* is certainly a prominent cause of acute tracheobronchitis or pneumonia in patients who have underlying chronic bronchitis, emphysema, or obstructive pulmonary disease. Other debilitating diseases such as malnutrition, lung cancer, and alcoholism are also often present (Murphy & Apicella, 1987).

Nontypable *H.influenzae* is second only to *S.pneumoniae* as the cause of bacterial pneumonia in middle-aged men. Although type b *H.influenzae* is more virulent, pneumonia due to this organism is much less common, probably because of its vastly lower incidence of colonization (Murray et al., 1994).

1.5.7 Laboratory Diagnosis

A rapit presumptive diagnosis of *H.influenzae* infection can be made by direct examination of appropriate clinical material using Gram stain. If sufficient amounts (≥1ml) of cerebrospinal fluid (CSF) is received, the specimen should be centrifuged to obtain a pellet of material for examination and culture (Campos et al., 1999; Forbes et al., 1998; Koneman, 1992). The CSF in untreated patients contains an average of 2 X 10^7 bacteria/ml, so that microscopic examination, especially in the absence of prior antibiotic therapy, should reveal the infecting organisms. On Gram stain preparations *Haemophilus* cells appers as small, pale-staining, gram-negative coccobacilli (Koneman et al., 1992).

Although *H.influenzae* may be likely pathogen on the basis of Gram-stained appearance and the clinical presentation, the organisms cannot be identified on the basis of the Gram-stained smear alone (Koneman et al., 1992).

For rapid diagnosis of *H.influenzae* type b infections, immunologic techniques are available for the detection of the type b PRP capsular antigen in CSF, serum, and urine (Murray et al., 1994; Koneman et al., 1992). These methods include counterimmunoelectrophoresis (CIE), latex particle agglutination (LA), staphylococcal protein A coagglutination (COA), and enzyme immunoassay (EIA) (Koneman et al., 1992).

Detection of capsular material in the cerebrospinal fluid by counter immunoelectrophoresis is helpful in cases in which the Gram stain is not conclusive; this technique is especially important in patients who have received enough antibiotic to suppress the

growth of organisms in cultures of cerebrospinal fluid, but not enough to be curative (Forbes et al., 1998).

A monoclonal antibody directed against a specific outer membrane of *H.influenzae;* P6, was conjugated to an immunoperoxidase enzyme and evaluated for its ability to detect and specifically identify *H.influenzae* directly in sputum smears.probe for direct detection of *H.influenzae* in clinical samples*.* For spurum specimens, the sensitivity of the probe was directly related to the length of time. The probe also detected *H.influenzae* in a small number of positive blood and CSF specimens that were tested (Koneman et al., 1992).

Nucleic acid detection tests are more sensitive than immunological methods in microbial diagnosis. PCR has been known as a sensitive and specific method in microbial diagnosis. The importance of PCR amplification in detection of *H.influenzae* was first detected by Ketel (Ketel et al., 1990).

Recent studies using the polymerase chain reaction (PCR) have identifies bacterial genomic sequences in nonculturable states (Gok et al., 2001; Aul et al., 1998)

Conventional sheep blood agar is not suitable for recovery of *Haemophilus* species that require V factor for growth due to the presence of V factor-inactivating enzymes in native sheep blood. Rabbit or horse blood does not contain these enzymes, and agar media containing either of these blood products will support the growth of most *Haemophilus* species. Media contain rabbit or horse blood is not routinely used in most clinical laboratories, so other techniques or media must be used (Koneman et al., 1992).

Primary isolation of *Haemophilus* species from clinical specimens is accomplished by using chocolate agar, *Haemophilus* isolation agar, or the *Staphylococus streak technique.* Chocolate agar is prepared by adding sheep blood to an enriched agar base medium that is at a high enough temperature (about 80 ^{0}C) to lyse the red cells and release X and V factors (Koneman et al., 1992; Campos et al., 1999).

Many commercial media producing companies currently market antibiotic-containing media for the selective isolation of *Haemophilus* species. These media contain beef heart infusion, peptones, yeast extract, and defibrinated horse blood (5%), which contain both X and V factors. In addition, bacitracin (300 mg/liter) is added to inhibit the other normal respiratory tract flora (Koneman et al., 1992).

Many bacteria and yeast synthesize and secrete V factor during growth on bacteriological media. In mix culture *Haemophilus* species that require V factor may grow as pinpoint colonies around the colonies of these other microorganisms. This phenomenon is called *satellitism.* This property provides a technique for detection of these organisms in mixed culture as well as a presumptive test for genus-level identification (Campos et al., 1999; Forbes et al., 1998; Murray et al., 1994; Koneman et al., 1997).

Chancres due to *H.ducreyi* are tender, somewhat irregular, and slightly indurated; they may be confused with primary syphilitic chancres, traumatic lesions of the penis (especially with bacterial superinfection), fixed drug eruptions, or ulcerated

herpetic lesions. The diagnosis is established by culturing the causative organism on Mueller-Hinton agar supplemented with 5 percent sheep blood and incubating it for 96 hours in a CO_2-enriched atmosphere (Forbes et al., 1998).

Optimal recovery of *Haemophilus* species from clinical specimens depend on proper collection and transport and the use of appropriate culture media and incubation environments. Isolation of *Haemophilus* requires incubation in a moist environment with increased CO_2 (3%-5%). The plates are incubated in 3% to 5% CO_2 at 35 ^{0}C for 18 to 24 hours (Koneman et al., 1992).

Commonly, *Haemophilus* species are identified on the basis of their hemolytic reaction on horse blood agar and their growth requirements for X and V factors. In identification of the type of *H.influenzae,* serotyping can be used (Koneman et al., 1992, Murray et al., 1994).

1.6 *MORAXELLA CATARRHALIS*

1.6.1 Taxonomy

Originally, *M.catarrhalis* was thought to be a commensal organism and scientists classified this bacterium as *Neisseria catarrhalis* based on phenotypic characteristics and colony morphology (Enright & McKenzie, 1997). *M. catarrhalis* was catalogued as a respiratory pathogen in the early 1900s by Berk using evidence gathered by Ghon and Pfeiffer (Paykel, 2002). However, subsequent studies reported that *M.catarrhalis* was a common inhabitant of the oropharynx of healthy adults, influencing

the reclassification of the organism as a harmless commensal. Unfortunately this latter study failed to differentiate *M.catarrhalis* from the commensal bacterium *Neisseria cinerea*. Thus, *M.catarrhalis* was not considered a pathogen for a significant portion of this century and most clinical laboratories neglected to test for it in biological fluids collected from patients with respiratory infections. In 1970, DNA hybridization studies showed that little homology existed between *M. catarrhalis* and the *Neisseriaceae* species, influencing its reclassification as *Branhamella catarrhalis* (Karasula & Campagnaria, 2000). However, Bovre proposed a division of the genus *Moraxella* into two subgenera, *Moraxella* and *Branhamella* (Bovre, 1979).

1.6.2 General characteristics

Moraxella species are parasites of the mucous membranes of humans and other warm-blooded animals. Many species are nonpathogenic. *M.catarrhalis* is a human mucosal pathogen that causes middle ear infections in infants and children and lower respiratory tract infections in adults with chronic pulmonary disease (Karasula & Campagnaria, 2000). *M.catarrhalis* is an aerobic, gram-negative diplococcus that grows well on blood or chocolate agar. The organism is oxydase positive, nitrate negative, and demonstrates negative carbohydrate fermentation tests for glucose, sucrose, maltose, fructose, and lactase (Paykel, 2002; Murray, 2003).

1.6.3 Pathogenesis

In general, the pathogenicity and virulence of a microorganism are determined by its ability to avoid host defense

mechanisms. Five cardinal requirements for a bacterium to be virulent: (i) binding, colonization, and infection of mucous surfaces; (ii) entry into host tissues; (iii) multiplication in the in vivo environment; (iv) interference with host defense mechanisms; and (v) production of damage to the host. Relatively little is known about the precise virulence traits of *M. Catarrhalis* (Verduine et al., 2002).

The surface antigen of *M.catarrhalis,* including Lipooligosaccharide (LOS) is an important virulence factor (Verduine et al., 2002). LOS, a predominant surface-exposed component of the outer membrane, has been implicated as a virulence factor in the pathofgenesis of *M.catarrhalis* (Luke et al., 2003)

1.6.4 Epidemiology

M. catarrhalis is capable of colonizing humans without causing disease, which was one of the reasons it was characterized as a commensal bacterium. The primary site of colonization is in the respiratory tract (Paykel, 2002; Karasula & Campagnaria, 2000).

The rates of colonization appear to vary with many contributing factors including age, health, socioeconomic condition and geographical location. In addition, seasonal variation may be involved in colonization and subsequent infection as certain reports suggest infection rates increase during the fall and winter seasons (Paykel, 2002; Karasula & Campagnaria, 2000).

The rate of colonization of infants and young children is much greater than that of adults. Studies indicate that between 28 to

100% of infants are colonized with *M. catarrhalis* within the first year of life (Karasula & Campagnaria, 2000; Gleckman, 1991). Although it is not known how geographic location may contribute to colonization and infection, overall living conditions may have a role. In general, people of a lower socioeconomic level experience greater overcrowding and poorer hygiene which may contribute to the more efficient spread of *M. catarrhalis* from one individual to another (Karasula & Campagnaria, 2000).

1.6.5 Clinical Manifestations

Infections in infants and young children

M. catarrhalis is a significant cause of middle ear disease in both infants and young children . *M. catarrhalis* is one of the three major causes of otitis media along with *Streptococcus pneumoniae* and *Haemophilus influenzae* (Karasula & Campagnaria, 2000).

In addition to the more common upper respiratory tract infections, a review by Meyer et al. suggests that *M. catarrhalis* may become an increasing cause of unsuspected bacteremia in children with no identifiable underlying conditions. Although these cases of bacteremia are rare, there is concern as more clinical isolates are found to be beta-lactamase producers. The emergence of antibiotic resistance is even more disturbing as the first case of fatal neonatal meningitis caused by *M.catarrhalis* has been reported (Karasula & Campagnaria, 2000).

Infections in adults

M.catarrhalis also causes disease in adults, particularly those with predisposing conditions, such as chronic obstructive pulmonary disease (COPD). However, the frequent isolation of

M.catarrhalis from healthy individuals continued to delay the acceptance of this organism as a pathogen in this population. Multiple lines of evidence were necessary to prove that this bacterium caused disease in adults. *M. catarrhalis* was identified as the predominant organism in Gram stains of sputum in a subset of patients with exacerbations of COPD. Treatment with appropriate antibiotics in patients with suspected *M.catarrhalis* infection resulted in clinical improvement. In addition, pure *M.catarrhalis* was isolated by transtracheal needle aspiration in chronic bronchitics and patients with clinical evidence of lower respiratory tract infections. Occasionally, the organism has been isolated from blood and pleural effusion of patients with COPD and pneumonia. *M. catarrhalis* has emerged as an important cause of acute exacerbations in adults with COPD (Karasula & Campagnaria, 2000).

Nosocomial infections

There have been nosocomial outbreaks of *M.Catarrhalis* infections reported from several centers indicating that clusters of infections occur in hospitals. Most of these outbreaks involved respiratory tract infections and some occurred exclusively in pulmonary units. In addition, studies have implicated *M. catarrhalis* as a newly identified source of community-acquired pneumonia (Karasula & Campagnaria, 2000).

Other infections

There have been documented cases of other serious infections caused by *M.catarrhalis*. Based on culture isolation and serological studies, *M.catarrhalis* has been implicated as a cause of sinusitis in both children and adults. In addition, *M.catarrhalis* occasionally causes severe infections, but these are rare

occurrences. There have been case reports of septic arthritis, bacteremia, cellulitis, osteomyelitis, endocarditis, and pericarditis (Karasula & Campagnaria, 2000).

1.6.6 Laboratory Diagnosis

Diagnosis of *M.catarrhalis* infection can be made by direct examination of appropriate clinical material using Gram stain. On Gram stain preparations *M.catarrhalis* organisms apper as in 0.5-1.5 mm diameter, Gram-negative diplococci. Strict adherence to the staining protocol is required (Murray et al., 2003).

Confirmation of the diagnosis of *M.catarrhalis* infection is based on the isolation of the organism in culture. Cultures can be taken from middle ear effusion, the nasopharynx, sputum, sinus aspirates, transtracheal or transbronchial aspirates, blood, peritoneal fluid, wounds, or urine (Murray et al., 2003).

Colonies are approximately 0.2 cm in diameter, opaque, and nonhemolytic after incubation on chocolate or blood agar for 48 hours. Characteristically, colonies can be pushed along the surface of the agar like a hockey puck (Murray et al., 2003; Koneman et al., 1997).

With standard methods of identification, *M.catarrhalis* can be differentiated from *Neisseria* species by not using sucrose, glucose, maltose, and lactose. Because *Neisseria cinerea* has the same reaction pattern, the superoxol test must be added. For definitive identification, deoxyribonuclease (DNase) and nitrate reduction are

performed; *M catarrhalis* produces DNase and reduces nitrate and nitrite levels (Murray et al., 2003; Koneman et al., 1997).

Several rapid confirmatory tests are available to identify *M.catarrhalis,* and they are all based on the ability of *M.catarrhalis* to hydrolyze tributyrin. This provides immediate identification and separation from human *Neisseria* species, which do not hydrolyze tributyrin (Paykel, 2002).

Serologic tests for infections with *M.catarrhalis* are not widely used; cross-reactivity with *Neisseria* species in the detection of complement fixation antibodies by immunoelectrophoresis has been demonstrated. Serum antibodies to whole-cell proteins, to lipo-oligosaccharides, and to outer membrane antigens have proved useful in the diagnosis of *M.catarrhalis* infections. Other lab studies may be needed, depending on the site of infection and underlying conditions (Paykel, 2002).

1.7 DIAGNOSIS OF CAP

Diagnostic testing is important in 3 aspects of the clinical management of CAP: confirming pneumonia, assessing its severity by chest radiography, and finally, identifying the responsible pathogens (Smith, 2001; Saranglao & Smith, 2002).

Altough distinctive patterns of pulmonary infection can be occasionally recognized, the isolation of the causative microorganism is almost always required to make a definitive diagnosis.

1.7.1 Serologic Testing

Serologic tests are available for the atypical organisms with CAP; *Legionella pneumophila, M.pneumoniae, C.pneumoniae,* and viruses. However, since a fourforld increase in titer between the acute and convalescent sera is required to make a diagnosis, these studies have limited usefulness in the routine management of CAP. One exception is an acute agglutinin that, if elevated, is suggestive of *M.pneumoniae* (Smith, 2001).

1.7.2 Antigen Testing

Antigen testing has the added benefit over Gram stain and culture in that it is not affected by prior antibiotic therapy (Smith, 2001; Skerrett, 1999). For pneumococcal pneumonia, the specifity of sputum antigen detection is greater than 70%, but sputum antigen testing may yield a high rate of false positive results (Skerrett, 1999).

1.7.3 Sputum examination and culture

The Gram stain and culture of a pretreatment sputum sample are the most useful tests, but have significant limitations. Gram stain and culture are not able to detect atypical pathogens such as *M.pneumoniae, C.pnemoniae, Legionella spp,* and respiratory viruses (Smith, 2001). The Gram-stained smears had to fulfill the following two criteria to be considered valid: there had to be more than 25 polymorphonuclear leukocytes (PMNL) and less than 10 squamous epithelial cells on low-power magnification (Ronald et al., 2000)

1.7.4 Invasive procedures

Ussually these studies are reserved for identification of pathogens in the sickest patients who are not responding to therapy. Studies may include transtracheal aspiration, thoracentesis of pleural effusions, fiberoptic bronchoscopy, and transthoracic needle aspiration (Smith, 2001).

1.7.5 Polymerase Chain Reaction (PCR)

Nucleic acid amplification techniques, such as PCR, are under actice investigation for the diagnosis of respiratory tract infections. (Ruiz-Gonzalez et al., 1999; skerrett, 1999; Menendez et al., 1999). High sensitivity and specificity make this approach appealing for the detection of organisms. The test can detect fewer than 10 organisms and is greater than 95% specific (Skerrett, 1999).

The role of these new tools is under investigation and they are not yet in routine use; however, they could offer the potential for rapid diagnosis and have been shown to be useful in clinical situation (Menendez et al., 1999).

PCR is a simple, in vitro, chemical reaction that permits the synthesis of essentially limitless quantities of a targetes nucleic acid sequence. This is accomplished through the action of a DNA polymerase that, undervthe proper conditions, can copy a DNA strand. RT-PCR, Nested PCR, Multiplex PCR are some types of this technique (Murray et al., 2003).

PCR offers many potential advantages; results are positive early in the course of infection, it is unaffected by prior administration of antibiotics, and it is not dependent on a host responce. In addition, the samples can be stored and tested or retested months or years later (Falguera et al., 2002).

1.8 Aim of the Study

Pneumonia (inflammation of the lower respiratory tract involving the airways and supporting structures and lungs) is a major cause of illness and death in both the community and hospital settings, especially during the winter and early spring periods. Community acquired pneumonia is the leading cause of infection-related mortality and morbility, ranks sixth among all causes of death.

Diagnostic testing is important in 3 aspects of the clinical management of CAP: confirming pneumonia, assessing its severity by chest radiography, and finally, identifying the responsible pathogens. Altough distinctive patterns of pulmonary infection can be occasionally recognized, the isolation of the causative microorganism is almost always required to make a definitive diagnosis.

Current criteria of diagnosis are based on sputum Gram stain and sputum culture. Some investigators have, however, questioned the reliability of the sputum Gram stain. Clinicians are interested in rapid, simple, inexpensive, and readily available tests that will assist them in prescribing proper medications for life-endangering infections.

For the best choise of antimicrobial therapy, the responsible pathogen should be determined with a fast, easy and reliable procedure such as polymerase chain reaction.

The aim of this study to evaluate the value of polymerase chain reaction in diagnosis of the most common three bacterial pathogens; S.pneumoniae, H.influenzae, and M.catarrhalis, from sputum of patients with community acquired pneumonia.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

Table 2.1 Materials and suppliers

Chemicals	**Suppliers**
Agarose	Sigma
Boric acid (55 gr/lt)	Sigma
Chlorophorm	Merck
DNA Size Marker	Fermentas
DNTP	Amresco
Dryslide PYR	Becton, Dickinson & Com.
EDTA	Sigma
Ethidium Bromide	Boehringer Mainheim
H.influenza antiserum	Difco
Lauryl Sulfate	Sigma
Optochin discs	Becton, Dickinson & Com.
Orange G	Sigma
Phenol	Amresco
Primers	Qbiogene
2-propanole	Riedel-deHaén
Proteinase K	Amresco
Taq DNA polymerase	Bioron
Tris (108 gr/lt)	Sigma

2.2. Equipments

Table 2.2 Equipments and Suppliers

Equipments	**Suppliers**
Analytical Balance	Precisa 125 A SCS
API System	BioMerieux
Centrifuge	Eppendorf
Deepfrezer	Snijders scientific
Electrophoresis tank and accessories	BioRad
Gel illuminator	GelDoc 2000-BioRad
Incubator	Nüve Jouan EC150
Power supply	BioRad
Thermal cycler	MWG-Biotech
Various Micro Pippets	Biohit Proline, Genex Beta
Vortex	IKA-Works

2.3 Media

Table 2.3 Media and suppliers

Media	**Suppliers**
Blood Agar	Salubris
Chocolate Agar	Salubris
Eosin Methylene Blue Agar	Salubris

2.4 Control Strains

- *Haemophilus influenzae* **ATCC 49247**
- *Streptococcus pneumoniae* **ATCC 49619**
- *Moraxella catarrhalis* **ATCC 25238**

2.5 Methods

2.5.1 Patient characteristics

Sputum cultures of adult patients with suspected bacterial pneumonia admitted to The Department of Pulmonary Diseases of Gulhane Military Medical Academy between January 2003 and June 2004 were collected. One hundred and seven adult patiens with community-acquired bacterial pneumonia who necessitated treatment during 1.5-year period were candidates for this study.

Patients eligible for inclusion in the study met the following criteria: older than 18 years and hospitalized with a primary diagnosis of CAP; a medical and clinical history of pneumonia; a new pulmonary infiltrate revealed by chest X-ray compatible with pneumonia; and two or more signs and symptoms associated with a lower respiratory tract infection (i.e.body temperature >38 ^{o}C or <36.1^{o}C, new or increased cough, production of purulant sputum, findings on examination such as rales or evidence of pulmonary consolidation, blood leukocyte count >10 000/mm3 or <4000mm3).

2.5.2 Control group characteristics

30 adult healthy individuals were selected as control group. Members of control group are older then 18 years old, and do not have any symptoms of community-acquired pneumonia.

2.5.3 Specimen collection

Spontaneously expectorated sputa were collected within 2 h of hospital admission. Deep cough sputum was obtained and transported immediatelly to the bacteriology laboratoty in a commertioly available sterile wide mouth jar with a tightly fitted scew-cap lid.

2.5.4 Microbiological investigations

Gram Stain

From all patients, for routine culture and Gram stain, a sputum specimen was obtained prior to study entry. For the purpose of this study, the Gram-stained smears had to fulfill the following two criteria to be considered valid: there had to be

- more than 10 polymorphonuclear leukocytes (PMNL)
- less than 10 squamous epithelial cells on low-power (x100) magnification.

We rejected all the specimens that have more than 10 squamous epithelial cells. Specimens including more than 25 PMNL were selected as group A, and between 10-25 PMNL were selected as group B.

Culture

All sputa which have the criteriae described above were cultured. Sputa were incubated for 48 hours on chocolate, Eozine Methylen Blue, and %5 sheep blood agar plates. These plates were incubated in 5% CO_2.

Identification

Organisms were identified by standard microbiologic techniques. The sputum plates were read on the day after the Gram-stained preparations were interpreted. The following organisms were considered potential pathogens: *S.pneumoniae, H.influenzae, N.meningitidis, S.aureus, S.pyogenes, S.agalactiae, M.catarrhalis,* and facultatively anaerobic gram-negative bacilli. Standart respiratory pathogens, when isolated from purulent respiratory secretions, were considered to be the microorganism responsible for the infection.

The gray mucoid (Gram-negative bacilli) colonies grew on the chocolate agar but not on the sheep blood agar were considered as *H.influenzae*. This organism appears as small, poorly staining coccobacilli on gram stains. Gives agglutination with *H.influenza* antiserum Poly I-V.

Very mucoid, gray (resemble drops of oil) colonies surrounded by a large zone of intense green α-hemolysis on the sheep blood agar were considered as *S.pneumoniae.* Gram-positive, catalase negative colonies were tested for PYR to differentiate α-hemolytic Streptococci from Enterococci. PYR negative colonies were tested for optochin susceptibility to

differentiate *S.pneumoniae* from other viridans streptococci. A zone of 14 mm or greater around the 6 mm disk indicates susceptibility to optochin and was identified as a pneumococcus.

M.catarrhalis colonies grew well on both blood and chocolate agar. Smooth, white, opaque, oxidase positive, Gram-negative diplococci colonies were identified as *M.catarrhalis*.

To confirm the identification of the microorganisms, API system was used according to the directions the manifacturer (API ID32STR, API NH, bioMerieux, France)

2.5.5 PCR

After Gram-stain and cultuvation, the sputum samples assigned for PCR test were stored at -80°C until testing was performed. *Haemophilus influenzae* ATCC 49247, *Streptococcus pneumoniae* ATCC 49619, and *Moraxella catarrhalis* ATCC 25238 were used as positive control.

2.5.5.1 Primers

- The common lower primer (Pcl) is a 21-mer

5'-CTA CGC ATT TCA CCG CTA CAC-3'

It is located at positions 679 to 699 in the *H.influenzae* sequence; positions 630 to 650 in the *M.catarrhalis* sequence; and positions 567 to 587 in the S*.pneumoniae* sequence.

- The species-specific upper primers are as the following:

H.influenzae primer (Phinf), a 24-mer

5'-CGT ATT ATC GGA AGA TGA AAG TGC -3'

M.catarrhalis primer (Pmcat), a 20-mer

5'-CCC ATA AGC CCT GAC GTT AC-3'

S.*pneumoniae* primer (Pspneu), a 22-mer

5'-AAG GTG CAC TTG CAT CAC TAC C-3'

2.5.5.2 DNA Extraction

Sputum samples from patients with pneumonia and from control group were tested for the presence of *S. pneumoniae, H.influenzae,* and *M.catarrhalis* DNA by performing PCR.

DNA was extracted from sputum samples (200 μl) according to the following protocol.

1. 17,5 μl Proteinase K (20mg/ml)
   50 μl SDS (10%)
   450 μl K buffer
   200 μl specimens were added into sterile eppendorf tubes, and were incubated over nigth at 55 ^{0}C.
2. 700 μl Phenol: Choloform: Izoamylalcohol (25:24:1) was added and centrifugated 10 min. at 12000 rpm at 4 ^{0}C.
3. Upper phase was transfered into a new sterile eppendorf tube, and step two was repeated.
4. Upper phase was transfered into a new tube.
5. 500 μl isopropile alcohol was added and vortexed. Mixture was centrifugated 10 min. at 12000 rpm at 4 ^{0}C.

6. Supernanant was removed. 500 μl ethyl alcohol was added and vortexed. Mixture was centrifugated 5 min. at 12000 rpm at 4 ^{0}C.
7. Supernatant was removed at tubes were placed into 37 ^{0}C to be dried.
8. After drying, 100 μl sterile distilled water was added.
9. Extracts were stored at –20 ^{0}C until use.

2.5.5.3 PCR Mixture

An aliquot of 10 μL of template DNA was added to 50 μL of PCR mixture consisting of:

1x PCR buffer,
0·2 mM (each) dATP, dCTP, dGTP and dTTP,
2·5 mM $MgCl_2$,
50 pmol of primers , and
1·2 U *Taq* DNA polymerase.
For each sample, three PCRs were performed for each strain.

2.5.5.4 Amplification

Extraction, mixture preparation and amplification steps were performed at different rooms to avoid contamination.

The samples were subjected to 35 PCR cycles, each consisting of 1 min at 94 °C, 2 min at 59 °C for each primer, and 2 min at 72 °C. Final elongation step was carried out at 72 °C for 5 min.

2.5.5.5 Analysis of the PCR Products

The PCR mixtures were analyzed with electrophoresis on 2% agarose gels with visualization of the amplicon with ethidium bromide over UV illumination.

1. 30 ml TBE buffer was mixed with 0.6 mg agarose, and boiled to prepare 2% agarose gel.
2. 5 μl ethidium bromide was added into agarose, and replased into the electrophoresis tank.
3. 20μl PCR product was mixed with 2-3 μl loading buffer, and subjected to agarose gel electrophoresis.
4. DNA size marker was loaded into one of the wells.
5. Electroforesis was performed at 100 volt for 20 minutes into the 0.5X TBE buffer solution.
6. The amplicons were visualized over a UV light box.
7. Both DNA size marker and positive controls and negative control were compared to comfirm the results.

CHAPTER III

RESULTS

In this study, 107 sputa of patients with pneumonia and 30 salivary samples of control group individuals were examinated to investigate bacterial pathogen using PCR comparing with standart bacteriological techniques. The results are shown on table 3.1 and table 3.2.

The patients ranged in age from 20 to 80 years, with a mean of 42.8 years. Sputa which have more than 25 PMNL and less than 10 squamous epithelial cells on low-power (x100) magnification were selected as group A. We achived valid sputa with 55 of 142 patients (38.7%) (Table 3.1). We rejected all the specimens that have more than 10 squamous epithelial cells. Specimens with less than 10 squamous epithelial cells and with between 10-25 PMNL were grouped as B (Table 3.2). The 52 of 142 sputa were group B. Total 107 sputa were selected to be cultured and tested for PCR tests.

Table 3.1 Results of both sputum cultures and PCR of group A

NO	Age	*H.influenzae* PCR	*S.pneumoniae* PCR	*M.catarrhalis* PCR	Culture
1	20	-	-	-	*P. aeruginosa*
2	21	+	-	-	-
3	56	+	-	-	-
4	20	-	-	-	-
5	20	-	-	-	-
6	20	-	+	-	*S. pneumoniae*
7	55	-	-	-	-
8	63	+	+	-	*S. pneumoniae*
9	23	-	+	-	-
10	20	-	-	-	*Klebsiella pneumoniae*
11	64	-	+	-	-
12	23	+	-	-	*H. influenzae*
13	65	-	-	-	*Klebsiella pneumoniae*
14	62	-	-	+	-
15	20	-	-	-	*H. influenza*
16	55	-	-	-	*H. parainfluenzae*
17	20	+	+	-	*S.pneumoniae+ H. iİnfluenzae*
18	65	-	-	-	-
19	71	-	-	-	-
20	21	-	-	-	*P. aeruginosa*
21	21	-	-	-	*H. parainfluenzae*
22	24	-	+	-	*S. pneumoniae*
23	20	-	-	-	-
24	21	-	-	-	-
25	45	-	+	-	-
26	21	-	+	-	-
27	74	-	-	-	*Candida spp*
28	20	-	-	-	-
29	21	-	-	-	-
30	22	-	+	-	-
31	30	-	-	-	-
32	21	+	-	-	-
33	72	+	-	-	-
34	69	-	-	-	*Proteus spp.*
35	79	-	+	-	-
36	39	-	+	-	*S. pneumoniae*
37	65	-	+	-	-
38	69	-	-	-	-
39	73	-	-	-	-
40	61	-	-	-	*P. aeruginosa*
41	70	+	-	-	-

Table 3.1 Cont'd.

NO	Age	*H.influenzae* PCR	*S.pneumoniae* PCR	*M.catarrhalis* PCR	Culture
42	20	-	+	-	-
43	70	-	-	-	*Staphylococcus aureus*
44	64	-	+	-	-
45	70	-	+	-	-
46	24	-	-	+	-
47	21	-	-	-	-
48	41	-	+	-	-
49	64	-	-	-	*Escherichia coli*
50	31	-	+	-	-
51	63	-	-	-	-
52	66	-	-	-	-
53	66	-	+	-	-
54	26	+	-	-	*H. influenzae*
55	69	+	-	-	*H. influenzae*

Table 3.2 Results of both sputum cultures and PCR of group B

NO	Age	*H.influenzae* PCR	*S.pneumoniae* PCR	*M.catarrhalis* PCR	Culture
56	21	+	+	-	-
57	55	-	-	-	-
58	20	-	+	-	-
59	21	-	+	-	-
60	21	-	+	-	-
61	43	-	+	-	*group C β-hemolytic streptococci*
62	21	-	+	-	-
63	49	-	+	-	-
64	50	-	+	-	-
65	71	-	-	-	*Enterobacter cloaceae*
66	21	-	+	-	-
67	70	-	+	-	*Escherichia coli*
68	22	-	+	+	*Moraxella catarrhalis*
69	24	-	+	-	-
70	24	+	-	-	*H. influenzae*
71	21	-	+	-	-
72	71	+	-	-	-
73	58	-	+	-	*E.coli + Acinetobacter spp.*
74	79	-	+	+	*Candida spp*
75	56	-	+	-	*Klebsiella oxytoca*
76	21	-	-	-	-
77	56	-	-	-	-
78	21	-	-	-	-
79	55	-	+	-	-

Table 3.2 Cont'd.

NO	Age	*H.influenzae* PCR	*S.pneumoniae* PCR	*M.catarrhalis* PCR	Culture
80	25	+	-	-	-
81	21	-	+	-	-
82	25	+	+	-	-
83	21	+	-	-	*Candida spp*
84	63	+	+	-	-
85	22	+	+	-	-
86	21	-	-	-	-
87	63	-	-	-	-
88	65	-	+	-	-
89	52	-	+	-	*Candida spp*
90	61	-	+	-	-
91	20	+	+	-	*Staphylococcus aureus*
92	65	-	+	-	-
93	20	-	+	-	-
94	79	-	-	-	-
95	67	-	-	-	-
96	64	-	-	-	-
97	70	+	-	+	-
98	23	-	+	-	-
99	40	-	+	-	-
100	40	-	+	-	-
101	80	-	+	-	-
102	44	-	-	-	*P. aeruginosa*
103	51	-	-	-	-
104	21	-	-	-	-
105	22	-	+	-	*Klebsiella pneumoniae*
106	20	-	+	-	-
107	53	-	+	-	-

3.1 Results of Sputum Culture

The etiology of the pneumonia, as determined by sputum cultures, was shown in table 3.1. The 33 of 107 sputum cultures were positive, and 74 were negative (Figure 3.1), included; 4 infections caused by *Streptococcus pneumoniae*, 5 caused by *Haemophilus influenzae*, 1 caused by *Moraxella catarrhalis*, 2 caused by *Staphylococcus aureus*, 2 caused by *Escherichia coli*, 1 caused by *Enterobacter cloaceae,* 4 *Psudomonas aeruginosa,* 1

Klebsiella oxytoca, 3 *Klebsiella pneumoniae,* 1 *Proteus spp*., 1 caused by group C β-hemolytic streptococci, 2 caused by *Haemophilus parainfluenzae*, 4 caused by *Candida* spp., 1in which both *Escherichia coli* and *Acinetobacter spp.*, and 1in which both *Haemophilus influenza* and *Streptococcus pneumoniae* were isolated.

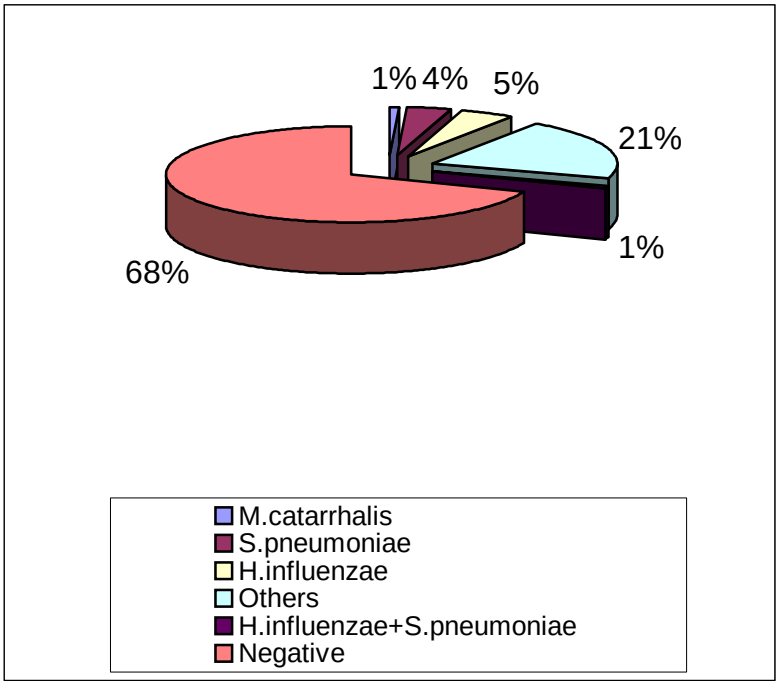

Figure 3.1: Culture results of sputum samples.

In terms of *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis;* 11 (10.3%) positive samples were detected in culture. Table 3.3 concludes culture results in separated group. 9 were positive (4 for *Streptococcus pneumoniae,* 4 for *H.influenzae*, and 1 for both *S.pneumoniae* and *H.influenzae)* were detected in group A, 2 wre positive (1 for *H.influenzae,* and 1 for *M.catarrhalis*) were detected in group B. Culture results of both group A and Group B were shown in table 3.3.

Table 3.3 Culture Results of Group A and Group B for *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis*.

Organisms	Group A (%) (n=55)	Group B (%) (n=52)
Streptococcus pneumoniae	5 (9 %)	0 (0%)
Haemophilus influenzae	5 (9%)	1 (1.9%)
Moraxella catarrhalis	0 (0%)	1 (1.9%)

3.2 Results of PCR Analysis

The PCR mixtures were analyzed followed by electrophoresis on 2% agarose gels with visualization of the amplicon with ethidium bromide and UV illumination (Figure 3.2).

The sixty-seven (63%) of 107 samples were positive in PCR analysis. The results of both group A and group B were babulated in table 3.4.

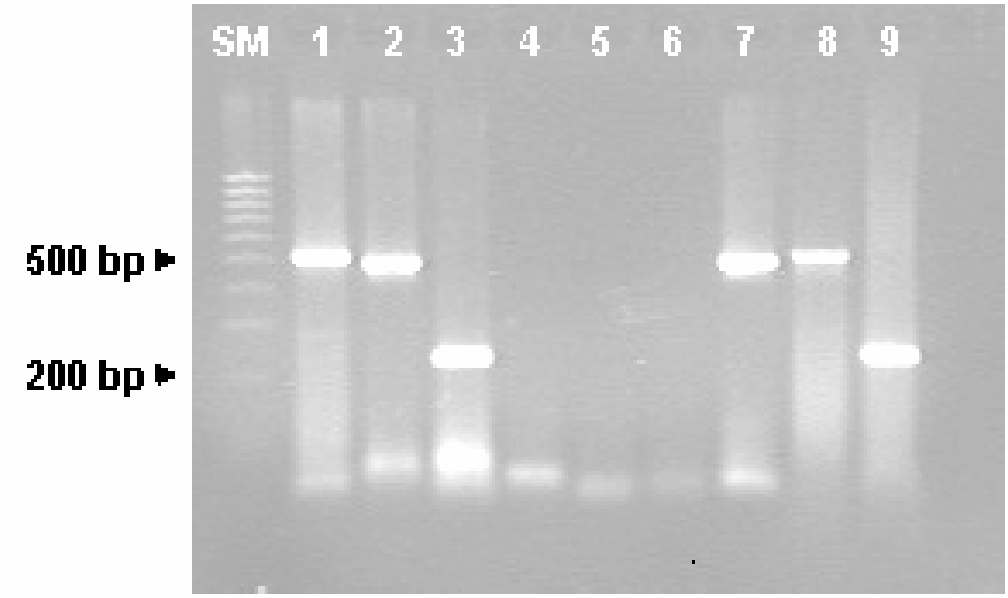

Figure 3.2: Examples of agarose gel for sputum samples. The numbers are given above indicate the samples. Lane SM, molecular size marker (MBI Fermentas, 100bp DNA Ladder yielding the following fragments, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000bp), and the sizes of 200 and 500 bp are indicated on the left. Lane 1 is *H.influenzae* positive control (525 bp); Lane 2 is *S.pneumoniae* positive control (484 bp), Lane 3 is *M.catarrhalis* positive control (237 bp); Lane 4, 5, and 6 are for negative controls of *H.influenzae, S.pneumoniae,* and *M.catarrhalis* respectively. Lane 7, 8, and 9 are for positive PCR samples for *S.pneumoniae, H.influenzae,* and *M.catarrhalis* respectively.

In group A there were 2 mixed positive, whereas there were 8 in group B (Figure 3.3). *S.pneumoniae* alone was positive for 16 cases in group A, but positive for 27 cases in group B. *H.influenzae* was positive for 8 in Group A, and 4 in group B. *M.catarrhalis* was positive in 2 cases in group A but there was no positive cases for only *M.catarrhalis* in group B. However, there were *M.catarrhalis* positivities in mixed infections (n=3) in goup B patients.

Table 3.4 Positive results of PCR

Organisms	**Group A (%) (n=55)**	**Group B (%) (n=52)**	**Total (%) (n=107)**
S.pneumoniae	16 (29%)	27 (52%)	43 (40%)
H.influenza	8 (14.5%)	4 (7.6%)	12 (11%)
M.catarrhalis	2 (3.6%)	-	2(1.8%)
H.influenza + S.pneumoniae	2 (3.6%)	5 (9.6%)	7(6.4%)
H.influenza +M.catarrhalis	-	1 (2%)	1(0.9%)
M.catarrhalis +S.pneumoniae	-	2 (4%)	2 (1.8%)
Total	28 (51%)	39 (75%)	67 (62.6%)

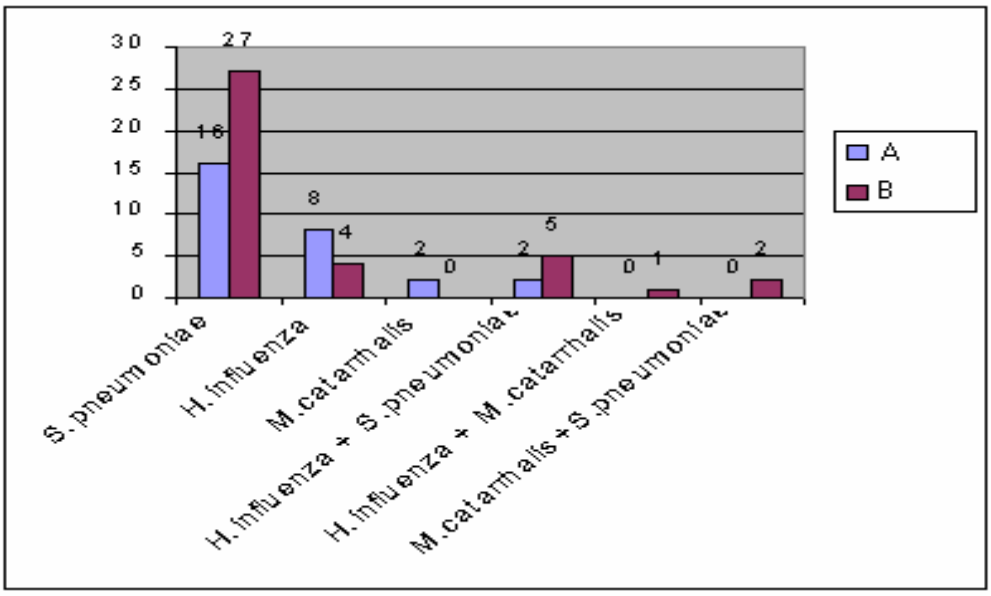

Figure 3.3: Results of PCR analysis in two groups. A indicates the patients belong to group A, and B indicates group B. Mixed infections were predominant in Group B. The number of *S.pneumoniae* also increased in Group B.

3.3 Comparison of culture and PCR

As shown in table 3.5; PCR results are quite different from culture results. The 11 (10.3%) (4 *Streptococcus pneumoniae*, 5 *Haemophilus influenzae*, 1 *Moraxella catarrhalis*, and 1 both *H.influenzae* and *S.pneumoniae*) of 107 samples were positive in cultures; whereas, 67 (62.6%) were positive in PCR analysis. The positive rates of *S.pneumoniae, H.influenzae,* and *M.catarrhalis* rised to 48.5%, 18.6%, and 4.7% by PCR technique, whereas they were ; 4.7%, 5.6%, and 0.9% in conventional culture studies respectively.

All samples which were positive for *S.pneumoniae, H.influenzae,* and *M.catarrhalis* in culture, were also positive in PCR. Only two patients have mixed infection in culture, whereas 10 mixed infection were screened in PCR analysis.

Table 3.5: Comparison of Culture and PCR.

Organisms	Group A (%)		Group B (%)	
	PCR	Culture	PCR	Culture
S.pneumoniae	18 (32.7%)	5 (9 %)	34 (65%)	0 (0%)
H.influenzae	10 (18%)	5 (9%)	10 (19%)	1 (1.9%)
M.catarrhalis	2 (3.6%)	0 (0%)	3 (5.7%)	1 (1.9%)

3.3.1 Comparison of culture and PCR analysis for group A

The 32.7% of 55 CAP infections were caused by *S.pneumoniae*; 18% by *H.influenzae; and 3.6% by M.catarrhalis*. These rates were determined as; 9%, 9% and 0% in cultures respectively (Figure 3.5).

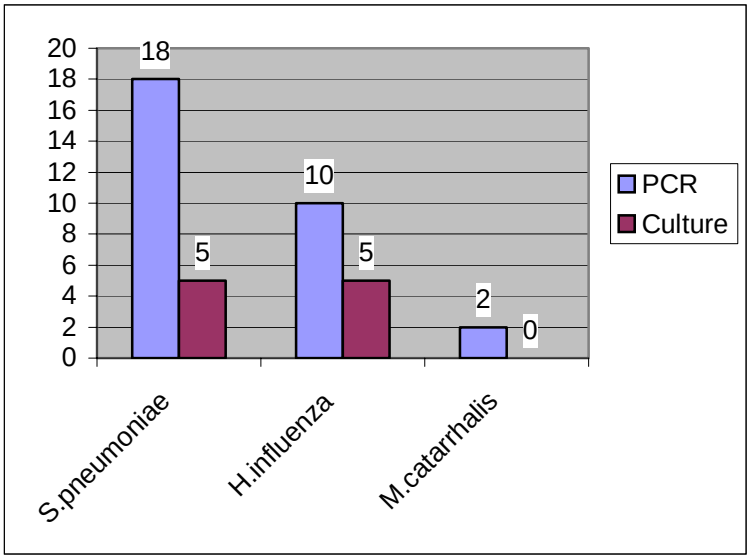

Figure 3.4 Comparison of PCR and Culture of group A

3.3.2 Comparison of culture and PCR analysis for group B

65% of 52 CAP infections were caused by *S.pneumoniae*; 19% by *H.influenzae; and 5.7% by M.catarrhalis*. These rates were determined as; 0%, 1.9% and1.9 0% respectively (Figure 3.5).

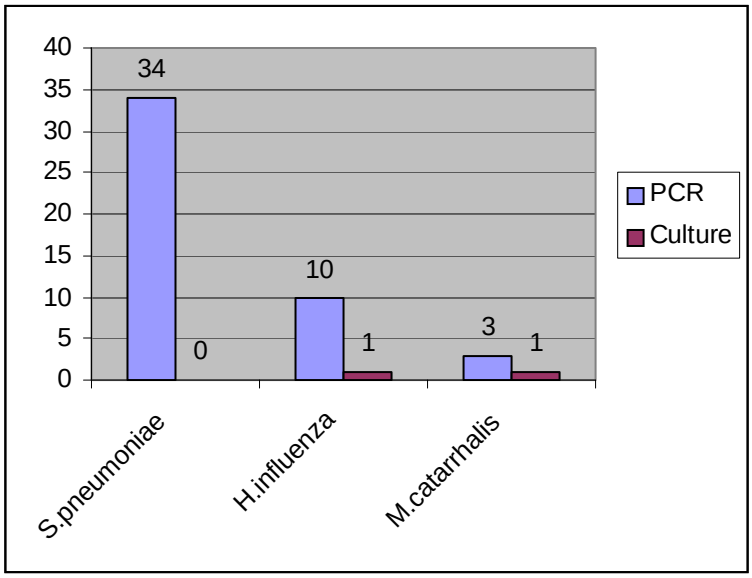

Figure 3.5 Comparison of PCR and Culture of group B

3.4 Results of control group

30 healthy adults were selected as control group. Salivary samples were obtained from these adults. Candidates ranged in age from 20 to 82 years, with a mean of 40 years. All specimens were processed by the same methods applied to sputa. All samples were negative in culture examinations; whereas the 17 (13 (43,3%) were positive for *S.pneumoniae*, 2 (6.6%) for *H.influenzae*, and 2 (6.6%) for *M.catarrhalis*) of 30 samples were positives in PCR analysis.

CHAPTER IV

DISCUSSION

Community-acquired pneumonia is a common clinical disorder with an estimated incidence of 12 cases per 1000 of the population per year. 258 cases per 100 000 population, and 962 cases per 100 000 aged over 65 years need hospital treatment for CAP (Brown et al., 1998). Since CAP also is a potentially fatal disease, even in previously healthy persons, early appropriate antibiotic treatment is vital (Miyashita et al., 2004).

Clinicians are interested in rapid, simple, inexpensive, and readily available tests that will assist them in prescribing proper medications for life-endangering infections and, in the present era of prospective payment, will guide them in the selection of cost-effective treatments (Gleckman et al., 1988). Rapid diagnosis of the etiologic agents of CAP is difficult (Garbino et al., 2002).

For management of CAP, the sputum Gram stain has traditionally served this function. Gram stain result is part of inclusion criteria in lower respiratory tract infections. When comparing it with conventional culture techniques to determine the cause of the pneumonia, some investigators have, however, questioned the reliability of the sputum Gram stain (Miyashita et al., 2004; Gleckman et al., 1988).

To enhance the diagnostic value of a sputum sample and to preclude assessment of respiratory secretions contaminated by oropharyngeal flora, microbiologists, infectious disease consultants, and pulmonary disease specialists recommend that only valid respiratory secretions be processed (Murdoch et al., 2003).

Even under the best conditions, the results of bacteriological examination of expectorated sputum specimens are difficult to interpret. When the potential pathogens are recovered, the physician must decide whether the isolate represent a true pathogen or one that simply colonization the oropharynx (Wong et al.,1982).

The amount of oropharyngeal contamination can be judged by examining the cellular components in a stained smear of the specimen. Since squamous epithelial cells are found only in the upper respiratory tract, their presence suggests oropharyngeal contamination. On the other hand, inflammatory cells (primarily polymorphonuclear leukocytes) suggest material derived from the site of an active infection (Wong et al., 1982).

Wong et al. (1982), compared the six different criteria for judging the acceptibility of sputum specimens. In 1975, Murray and Washington described a simpler scheme for judging the quality of sputum specimens. All specimens with more than 10 squamous epithelial cells per average low-power field (LPF) were rejected (Murray and Washington, 1975). In 1977, all specimens with 25 leukocytes per LPF was accepted regardless of the number of epithelial cells present by Van Scoy. At the end, the question of which method is most reliable remains to be determined, however; they emphasized that Murray and Washington (1975) (All

specimens with more than 10 squamous epithelial cells per average LPF were rejected) tended to give somewhat more reproducible results (Wong et al., 1982).

Gleckman and co-workers assessed the sputum Gram stain in community-acquired bacteremic pneumonia, and they used the following two criteria to be considered valid: there had to be more than 25 polymorphonuclear leukocytes and less than 10 squamous epithelial cells on LPF (100x). Their experience underscores the difficulty of collecting valid respiratory secretions from adult patients with pneumonia. They achieved with 59 (41%) of 144 patients. They concluded that Sputum Gram stain may be a reliable indicator to guide initial antibiotic therapy (1988).

This study was performed on patients with bacterial community-acquired pneumonia, who are admitted to Gulhane Military Medical Academy hospital. We had difficulty in collecting valid sputum specimens from patients, when we performed the way used by Gleckman we achieved with 55 (38.7%) of 142 specimens; however, If we consider the way of Murray and Washington we achieved with 107 (75.3%) of 142 patients. We collected our specimen in two groups to be able to evaluate the effect of oropharyngeal contamination. We eliminated all the specimens with more than 10 squamous epithelial cells. We rejected 35 specimens by this way.

Many rapid diagnostic tests for CAP such as; nucleic acid amplification tests (ie, PCR) are still in early stages of development, or are not commonly available, or are not sufficiently accurate (File, 2003).

We used PCR for sputum samples to determine whether this technique could offer the potential for rapid diagnosis of bacterial CAP, since; PCR is a current diagnostic method that offers many potential advantages; results are positive early in the course of infection, it is unaffected by the prior administration of antibiotics, and it is not dependent on a host response. In addition the sample can be stored and tested or retested months or years later (Falguera et al., 2002).

This study aimed to evaluate PCR for the diagnosis of causative agents of pneumonia in adults by testing valid sputum. We compared PCR results with both sputum Gram stain and sputum culture for the three most common bacterial pathogens; *S.pneumoniae, H.influenzae*, and *M.catarrhalis*.

We found a high PCR positivity rate (63%) (48.5% *S.pneumoniae;* 18.6% *H.influenzae;* and 4.7% *M.catarrhalis)* in this study. By culture we determined these numbers as; 4.7%, 5.6%, and 0.9% respectively. PCR is more sensitive than culture in detecting the causative agents. According to the PCR results the most common isolated pathogen of our study was *S.pneumoniae*.

S. pneumoniae is one of the most frequently isolated pathogen in all respiratory tract infections (Jones et al.,2000). *S. pneumoniae* also is the most frequent etiology of CAP, and may even account for pneumonia in patients who have no etiology identified after a full diagnostic work-up (Ioachimescu et al., 2004). *S.pneumoniae* may be responsible for 20-70% of cases of pneumonia admitted to hospital (Gil et al., 2002). In the hospital setting, it is frequently implicated with *H. influenzae* as the cause of polymicrobial pneumonias (Schleupner and Cobb, 1992). These

two pathogens are frequently the cause of early-onset infections, as occurs in ventilator-associated pneumonia, probably as a consequence of their colonization of the oropharyngeal cavity (Francioli et al. 1997). *H. influenzae* is regularly cited as one of the three most important respiratory tract infection pathogens for community-acquired pneumonia (Baquero et al. 1998).

During the last decade pathogen such as *Moraxella catarrhalis, Legionella pneumuphila and Chlamydia pneumoniae* have been identified frequently (Garbino et al.,2002). Third or fourth leading pathogens are atypical pathogens (Miyashita et al., 2004). *S.pneumoniae, H.influenzae*, and *M.catarrhalis* are the most common community-acquired bacterial respiratory pathogens (Gordon et al., 2003; Pfaller et al., 2001) and they are also the most common three pathogens in CAP (Gotfried et al., 2002). In this study ve did not evaluate samples in terms of atypical pathogens. Our results match with these studies when we compare them with PCR; however, we identified only 4 infections (3.7%) caused by *S.pneumoniae*, 5 (4.6%) caused by *H.influenza*, 1 (0.9%) caused by *M.catarrhalis*, and 1 (0.9%) in which both *H.influenza* and *S. pneumoniae* by culture method.

The reported incidence of *S. pneumoniae* ranged from 4.1 to 39% in various studies (Brown, 1998). We calculated this number as 4.7 % (n=5) in culture and 49% (n=54) in PCR. The reported incidence of *H.influenza* ranged from 2.3 to 10.3 % in various studies (Brown, 1998). In this study we found 5.6% (n=6) in culture and 18.6% (n=20) in PCR. Incidence of *M.catarrhalis* in various samples ranged from 0% to 3% (File , 2001), and we determined this incidence as 0.9% (n=1) by culture, and 4.7% (n=5) by PCR.

Garbino and co-workers calculated the percentage of the respiratory tract pathogens by sputum culture and nonculture methods as 12.6% for *S. pneumoniae,* 6% for *H.influenzae*, and 1.6% for *M.catarrhalis* (Garbino et al., 2002). When our PCR results were compared with the reported studies, the incidence of *S.pneumoniae, H.influenzae,* and *M.catarrhalis* in this study is higher. False-positive results may be due to colonization with these pathogens; but they have been recorded in adult populations in whom the carriage rates of these strains are generally low .

Possible explanations for this variation may include different geographical areas, demographic variation between study populations, and different laboratory techniques used in the microbial diagnosis of pneumonia.

In terms of the quality of the sputum samples we should evaluate the results in separated groups. The incidences of *S.pneumoniae, H.influenzae*, and *M.catarrhalis in* group A are; 32.6%, 18%, 3.6%; and in group B are; 65.6%, 19%, and 6% respectively.

For several reasons, we came to believe that PCR testing of sputum samples is not highly reliable tool for diagnosing pneumococcal pneumonia and that this approach is unable to distinguish colonization from infection in some circumstances. First, the sputum PCR positivity rates of *S.pneumoniae* were almost the same for patients with pneumonia (48.5%) and control individuals (43.3%), suggesting that the most of the positive results are more likely to represent colonization rather than infection. These findings imply that many of the positive *S.pneumoniae* PCR results for

sputum samples are detecting colonizing organisms rather than pathogens. However, if we select the valid sputum samples to be tested, we may obtain more reliable results by PCR technique.

The results from group A seemed more reliable than group B, since sputa from group B are more likely to indicate colonization rather than pathogen. Another reason to question the specificity of the PCR results from sputum is that 28% (n=9) of the group B patients with PCR-positive sputum samples had evidence of infection with pathogens other than *S. pneumoniae* (Table 3.2). It implies that some of these PCR results are falsely positive.

The sputum PCR positivity rates for both patients and controls are higher than reported carriage rates for these three pathogens. In most cases these organisms are carried without causing clinical symptoms. The reported rates of bacterial acquisition and carriage vary extensively between different studies and geographical sites (García and Martínez, 2002). The overall isolation frequencies for adults found by Gunnarsson *et al*. in Sweden are: 2% for *M. catarrhalis*, 0.8% for *S. pneumoniae* and 3% for *H. İnfluenzae* (Gunnarsson et al.,1998). Although variable, the colonization rates in healthy adults are lower than in children. However, some studies have shown higher rates of colonization. *S. pneumoniae* colonizes the nasopharynx of up to 40% of healthy adults and carriage of up to four different serotypes for several months has been documented (García and Martínez ,2002). Given that PCR is more sensitive than culture, it is not surprising that PCR will detect additional cases. In terms of *H.influenzae* and *M.catarrhalis,* PCR techniques seemed more useful in testing valid sputum samples.

The reported sensitivities of PCR for detecting only *S. pneumoniae* in blood samples have ranged from 35 to 100% in adults (Murdoch et al., 2003). The detection limit of *S.pneumoniae, H.influenzae*, and *M.catarrhalis* by PCR is 10 bacterial cells (Hendolin et al., 1997). It implies that some of these PCR results are falsely positive. In any sample including even 10 bacterial cells would have given falsely positive result.

It is possible that quantitative PCR may be more useful, and that infection can be distinguished from colonization by a higher bacterial burden, but this has yet to be systematically examined using nucleic acid amplification methods. Real-time PCR offers a sensitive, efficient, and routinely reproducible approach to quantification. Since during infections changes in the amounts of *S. pneumoniae* may also occur in the upper respiratory tract, quantification of these bacteria in nasopharnygeal secretions (NPSs) may offer a suitable diagnostic approach (Greiner et al., 2001). Same researchers have also performed the same prosecure for *M.catarrhalis*, and they developed a new diagnostic assay on the basis of real-time PCR that allowed the fast (analysis of numerous patient samples, from biological material to analyzed results, within 24 h), sensitive, specific, reproducible, and simple high-throughput detection and quantification of the pneumolysin gene from both typical and atypical *S. pneumoniae and* the *copB* outer membrane protein gene from *M.catarrhalis* (Greiner et al., 2001; Greiner et al., 2003).

These findings indicate the need for more specific PCR targets for *S.pneumoniae,* especially given that the most commonly used targets in diagnostic PCR assays are the pneumolysin and autolysin genes.

Further research should focus on the comparative specificities of different PCR assays, the role of quantitative PCR. These findings indicate the need for more specific PCR targets for these three pathogens

CHAPTER V

CONCLUSION

107 sputum samples of patients with community-acquired pneumonia were used to investigate the absence of *S.pneumoniae, H.influenzae*, and *M.catarrhalis* by PCR.

- 33 of 107 samples were positive in conventional culture in terms of various species.

- In terms of *S.pneumoniae, H.influenzae*, and *M.catarrhalis* 11 of 107 samples were positive in culture, 67 in PCR technique.

- The most common identified agent is *S.pneumoniae* with the incidence of 48.5% followed by *H.influenzae* (18.6%) and *M.catarrhalis* (4.7%). Also, there is a significant number of patients who have mixed infentions.

- Althoug sputum Gram stain, culture and PCR techniques have an important role in the identification of the etiology of CAP, false positive results are still unavoidable due to colonization of these three pathogens in oropharynx.

- PCR testing of sputum samples is not highly reliable tool for diagnosing pneumococcal pneumonia and that this approach is unable to distinguish colonization from infection in some

circumstances. PCR positivity rates of *S.pneumoniae* were almost the same for patients with pneumonia (48.5%) and control individuals (43.3%), suggesting that the most of the positive results are more likely to represent colonization rather than infection.

- All the specimens should be stained and examined for the presence of PMNL, epithelial cells and microorganisms to assess specimen quality. Acceptable specimens will reduce the number of false positive results.

- The finfing indicate the need for more specific PCR targets for related pathoges to eliminate the question for realibitity of sputum samples.

- Real-time PCR seems like a possible solution to differentiate the colonization from infection to avoid false positivity.

- Because of being faster and easier, PCR looks like becoming more reliable technique by the using of valid specimens from patients with community-acquired pneumonia if supported by quantitative techniques.

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