ISOLATION AND CHARACTERIZATION OF AN ADHESIN PROTEIN FROM THE SURFACE OF A RESPIRATORY PATHOGEN *MORAXELLA CATARRHALIS*

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BY
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To My Family...

I certify that I read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Dr. Gülay Özcengiz

I certify that I read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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Moraxella catarrhalis is a member of the normal flora of upper respiratory tract. Starting in the early 1980s it gained importance as an important cause of otitis media in children and lower respiratory tract infections in adults with chronic obstructive pulmonary disease. β-lactamase producing strains of *M. catarrhalis* has been increasing at a very fast rate. In some locations, 100% of the strains are β-lactamase producer. The pathogenesis of infection by this bacterium is not clearly understood which hindered the development of a vaccine. In this study, a surface protein of about 55 kDa was isolated from *M. catarrhalis* by celite chromatography. It is a heat stable protein and is not affected by 2-mercaptoethanol or dithiothreitol treatment. The immunogenic property of the protein has been determined by immunizing rabbits with *M. catarrhalis* and detecting the antibody response in serum against 55 kDa protein by Western blotting. In addition, the protein is immunogenic in humans as antibody against 55 kDa protein can be detected in the sputum of patients with *M. catarrhalis* infection. Moreover, we determined upto 40 amino acids at the *N*-terminal and also two fragments of the protein. To determine the function of the protein, attachment inhibition assays were performed and it was found that 55 kDa protein competetively inhibits attachment of *M. catarrhalis* to human pharyngeal epithelial cells (HPEC). Similarly, monoclonal antibody against 55 kDa (mAb) blocks the protein and inhibits the attachment of *M. catarrhalis* to HPEC. These two lines of evidence show that 55 kDa protein is an adhesin of *M. catarrhalis* which mediate attachment to HPEC. In addition, immunoflourescence experiments further verified that 55 kDa protein binds to HPEC. To sequence the gene encoding 55 kDa protein, PCR was done using degenerate primers constructed from the N-terminal amino acid sequence. PCR amplification of the possible gene of 55 kDa protein resulted in a 500 bp fragment, but no homology can be obtained with *N*-terminal amino acid sequence. In addition, a genomic library of *M. catarrhalis* is prepared and screened with mAb and with a radiolabelled oligonucleotide probe. We isolated several positive clones; therefore in future it might be possible to sequence the gene encoding 55 kDa protein from these clones.

Keywords: Moraxella catarrhalis, adherence, attachment inhibition asssay, adhesin.

ÖZET

MORAXELLA CATARRHALIS BAKTERİSİNİN YÜZEYİNDEN BİR ADHESİN PROTEİNİNİN İZOLE EDİLMESİ VE KARAKTERİZASYONU

TOLGA TURAN

Moleküler Biyoloji ve Genetik Yüksek Lisans
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İnsan boğaz florasının bir üyesi olan *Moraxella catarrhalis*, 80'li yılların başından itibaren bir patojen olarak önem kazanmaya başlamıştır. *M. catarrhalis* çocuklarda orta kulak iltahabı ve yetişkinlerde (özellikle kronik bronşit hastalarında) alt solunum yolu enfeksiyonunda en çok karşılaşılan üçüncü bakteridir. Antibiyotik dirençli suşların son zamanlarda çok artması (bir çok yerde 100%) ve bu bakteriye karşı başarılı bir aşı geliştirilememiş olması, *M. catarrhalis* üzerine yapılan çalışmaları arttırmıstır. Bu çalışmada molekül ağırlığı 55 kDa olan bir protein *M. catarrhalis*'in yüzeyinden celite kromatografisi metoduyla izole edilmiştir. Bu proteinin ısı dirençli olduğu ve 2-merkaptoethanol ve DTT tarafından etkilenmediği bulunmuştur. Proteinin immunojenik olduğu tavşan ve insanda yapılan deneyler sonucu ispatlanmıştır. Buna ek olarak proteinin amino kısmından 40 amino asitlik bölümü Edman metoduyla belirlenmiştir. Proteinin bakteride adhsin olarak görev yaptığı bağlanma inhibisyonu deneyleriyle gösterilmiştir. Bu deneylerde 55 kDa proteininin insan boğaz epitel hücreleriyle inkübe edilmesi *M. catarrhalis*'in boğaz hücrelere bağlanmasını azaltmıştır. Benzer mantıkla bu proteine karşı üretilmiş monoklonal antikorun *M. catarrhalis* ile inkübe edilmesi bakterinin hücrelere bağlanmasını azaltmıştır. Bu proteinin boğaz epitel hücrelerine bağlandığı immunofloresans metodu ile de gösterilmiştir. 55 kDa proteini kodlayan genin klonlanması için protein sekansından elde edilen dejenere primerlerle PCR yapılmıştır. Sonuç olarak 500 bazlık bir fragman elde edilmiştir ama bu fragmanın amino asit zinciriyle homolog olmadığı görülmüştür. Buna ek olarak *M. catarrhalis* genomundan hazırlanan gen kütüphanesi monoklonal antikor ve radyoaktif işaretli bir oligonukleotid ile taranmıştır. Çıkan pozitif kolonilerin sekanslanması sonucu elde edilen bilgiler amino asit zinciriyle karşılaştırılmış fakat homoloji bulunamamıştır. Elde edilen pozitif klonlar sonuna kadar henüz sekanslanmamıştır.

Anahtar Kelimeler: *Moraxella catarrhalis*, bağlanma, bağlanma inhibisyon deneyi, adhesin.

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N-terminal amino acid sequencing was done in collaboration with Dr. Tomomitsu Hatakeyama, Department of Chemical Engineering, Nagaski University

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ABBREVIATIONS

RTI	Respiratory tract infections
CTAB	Cetyltrimethylammonium Bromide
TBS	Tris Buffered Saline
PBS	Phosphate Buffered Saline
IgA	Immunoglobulin A
BHI	Brain Heart Infusion
COPD	Chronic Obstructive Pulmonary Disease
OMP	Outer Membrane Protein
HPEC	Human Pharyngeal Epithelial Cell
mAb	Monoclonal Antibody
PCR	Polymerase Chain Reaction
SCID	Severe Combined Immunodeficient
Usp	Ubiquitous Surface Protein
RFLP	Restriction Fragment Length Polymorphism
PFGE	Pulsed Field Gel Electrophoresis
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrohoresis
HRP	Horse Radish Peroxidase
OD	Optical Density
PB	Phosphate Buffer
BSA	Bovine Serum Albumin
APS	Ammonium per Sulphate
TEMED	N, N, N, N-tetramethyl-1,2 diaminoethane
MH	Mueller Hinton

CHAPTER 1:INTRODUCTION

1.1 *MORAXELLA CATARRHALIS*

Moraxella (Branhamella) catarrhalis, formerly called *Neisseria catarrhalis* or *Micrococcus catarrhalis* is a gram-negative aerobic diplococcus frequently found as a normal inhabitant of human respiratory tract. Over the last 20 years, this bacterium has emerged as a pathogen (Johnson 1981) and is now considered as an important cause of respiratory tract infection and otitis media together with *Heamophilus influenzae* and *Streptococcus pneumoniae*.

1.1.1 History of Classification

Although *M. catarrhalis* is now accepted as a pathogen, its classification has generated controversy since Pfeiffer's designation as *Mikrococcus catarrhalis* in 1896 (Frosch 1896). Subsequent studies reported that *M. catarrhalis* was a common inhabitant of the oropharynx of healthy adults. It was classified as *Neisseria catarrhalis* based on phenotypic characteristics and the colony morphology (Enright 1997). Unfortunately, this latter study failed to differentiate *M. catarrhalis* from the commensal bacterium *Neisseria cinera*. Thus *M. catarrhalis* was not considered as a pathogen for a significant portion of this century and most clinical laboratories neglected to test for it in biological fluids collected from patients. In 1970, DNA hybridization studies showed that little homology existed between *M. catarrhalis* and *Neiseriaceae* species, influencing its reclassification as *Branhamella catarrhalis* (Karalus 2000). However, Bovre proposed a division of genus *Moraxella* into two subgenera, *Moraxella* and *Branhamella* (Bovre 1979). To date the controversy over the nomenclature of this bacterium remains unresolved, although most investigators now use *Moraxella catarrhalis*.

1.1.1.1 *Identification and Bacteriological Properties*

Laboratory identification of *M. catarrhalis* is not difficult: It is a gram-negative diplococcus. It is positive for cytochrome oxidase, DNase and tributyrin, 4-methylumbelliferyl butyrate as well as indoxyl acetate esterases. It fails to produce acid from glucose, maltose, sucrose, lactose and fructose; it can grow at 22 °C on nutrient agar; it fails to grow on modified Thayer-Martin medium and it

can reduce nitrate and nitrite (Doern 1990). In addition, modern DNA technology has opened new avenues for the detection of *M. catarrhalis* in clinical materials. Particularly, PCR is the most convenient method of modern molecular biology to identify microorganisms (Verduin, 2002). Recently, multiplex PCR has been designed for detection of major pathogens in middle ear infections (*H. influenzae*, *M. catarrhalis* and *S. pneumoniae*) (Hendolin, 2000).

1.1.2 Importance

M. catarrhalis had been known as a harmless commensal for a significant portion of this century. How or why did it suddenly emerge as a pathogen is not yet known. However, the following two factors might be responsible:

1. The bacterium may have changed such that it is now more virulent than in past decades.
2. Since *M. catarrhalis* has long been regarded as a nonpathogenic commensal, infections by the organism may have been occurring but have been overlooked for years.

M. catarrhalis is an important pathogen to explore because the infections it causes results in a high level of morbidity and mortality and a substantial financial burden. For example, in USA, it is responsible for about 15-20 % of all otitis media cases in children (Enright 1997) and this represents a substantial financial burden ($2 billion/year in US) on the health care system (Murphy 1996).

Although, *M. catarrhalis* is now an established pathogen all over the world, its virulence factors remain to be discovered. Moreover the pathogenesis of this bacterium is also not yet clear. In addition, substantial increase in β-lactamase producing *M. catarrhalis* makes it difficult to treat with commonly used antibiotics (Martinez, 1998). Therefore new preventive and therapeutic strategies are urgently needed to combat infections by this bacterium. All these factors motivated us to study *M. catarrhalis.*

1.1.2.1 *M. catarrhalis Infections*

In children, *M. catarrhalis* is a major cause of otitis media together with *H. influenzae* and *S. pneumoniae* (Catlin 1990, Murphy 1996, Christensen 1999). Studies on infants and young children by various groups in United States,

Norway, Finland, Japan and Spain used tympanocentesis to demonstrate that 15 to 20 % of the otitis media cases were caused by *M catarrhalis* (Del Beccaro 1992). This infection rate is significant as it is estimated that 70 to 80% of all children will have had at least a single episode of middle ear disease by the age of three years. Recent data from various centers throughout the US show that *M. catarrhalis* is responsible for over 3 million episodes of otitis media annually and repeated episodes of otitis media may result in hearing loss and are associated with developmental and learning problems as children reach school age (Murphy 1996).

M. catarrhalis is one of the major cause of respiratory tract infection (RTI) in adults especially in adults having an underlying disease such as chronic obstructive pulmonary disease (COPD). *M. catarrhalis* is one of the most three common causes of exacerbation in COPD patients, together with *H. influenzae* and *S. pneumoniae*. The reason why *M. catarrhalis* infection is low in healthy adults is thought to be due to the strong immune system in these individuals. Therefore *M. catarrhalis* cause more infection in children whose immune system is immature and in old people whose immune system is weak (Verduin 2002).

Occasionally, the bacterium causes systemic infections, e.g., meningitis and sepsis (Catlin 1990). In addition, *M. catarrhalis* may cause sinusitis, tracheitis, bronchitis, pneumonia and less commonly, ocular infections in children (Verduin 2002). Although rare, *M. catarrhalis* can cause sinusitis (Brorson 1976), septic arthritis (Craig 1983), bacteremia (Cimolai 1989), cellulitis (Rotta 1994), osteomyelitis (Prallet 1991), endocarditis (Douer 1977), and pericarditis. (Kostiala 1989). It is also important to note that nosocomial infections could be caused by *M. catarrhalis* (Omori, 1999).

1.1.3 Level of Fight with *M .catarrhalis*

Because very short time has passed since this organism stepped on stage as a pathogen; the level of knowledge on it is scarce. Both the virulence factors and the pathological properties of *M. catarrhalis* remain largely unknown. The virulence factors should be explored further to understand the pathogenesis of this bacterium and to develop protective means against the organism.

One additional reason for the low level of knowledge on *M catarrhalis* is the lack of a good animal model. *M. catarrhalis* is a strict human pathogen and it

doesn't cause infection in mouse or in other small laboratory animals. The best models available are the Murine Pulmonary Clearance Model (Unhanand 1992) and the SCID (Severe Combined Immunodeficient) mouse model (Harkness 1993). In the pulmonary clearance model, mice are challenged by introducing bacteria into their lungs and the rate of clearance is followed as a measure of the immune response. In SCID mouse model, bacteria are introduced by multiple routes including intranasal and intravenous, but the symptoms produced in these animals do not mimic those seen in human infection.

It has been difficult to combat with *M. catarrhalis* as more and more β-lactamase positive strains emerge. The first β-lactamase-positive clinical strain was isolated in 1977 (Hoi-Dang 1978). Subsequently, resistance to β-lactams has increased at very fast rate. Presently, in North America and Europe almost 100% of the strains are β-lactamase producer (Clavo-Sanchez 1997). However it varies from country to country such as in Japan it is 94% (Martinez 1998), in Kuwait it's 57% (Ahmed 1999) and in Uganda it's 100% (Yoshimine 2001). Although the reason is not known, it is thought that increased use of antibiotics has contributed for this dramatic increase of β-lactamase producing strains. However, such increase has not been demonstrated in other bacteria under the same selective pressure (Felmingham 1999).

The β-lactamase enzymes of *M. catarrhalis* have been designated as BRO β-lactamase. BRO is different from other β-lactamases and has only been identified in two other Moraxella species (Wallace 1989). The suspected location of the *bro* genes on the chromosome and the high efficiency of transfer tend to support theory of a conjugal transposon as a method for spread of resistance (Wallace 1989). This different means of spread of resistance might be the reason of the steep increase of antibiotic resistance in *M. catarrhalis.*

The emergence of β-lactamase-positive strains motivated scientists to find a vaccine against *M. catarrhalis.* But vaccine development for *M. catarrhalis* is only in the antigen identification stage.

Surface exposed antigens of *M. catarrhalis* are mainly the outer membrane proteins, other surface proteins and the lipooligosaccharide. Studying surface exposed antigens will not only be a progress in vaccine development but also help us to understand the virulence properties of *M. catarrhalis* since the surface

exposed antigens are the sites where the bacterium interacts with the host. In this respect our work on the characterization of a surface exposed protein of *M. catarrhalis* will be an important contribution to the understanding of pathogenesis and prevention of infection caused by this bacterium.

1.1.4 Surface Exposed Antigens

The outer membrane proteins (OMPs) of *M. catarrhalis* are similar to other gram-negative bacteria in that 10 to 20 OMPs are present with 6 to 8 of these proteins predominating (Murphy 1990). The outer membrane of *M. catarrhalis* contains 8 major OMPs that are labeled OMP A through OMP H. The molecular weight of these proteins ranges from 98 to 21 kDa. An interesting finding was the striking homogeneity of OMPs among 50 strains. This means that an immune response against one strain will recognize a subsequent strain also (Bartos 1988).

Following is the list of the major OMPs of *M. catarrhalis:*

CopB:

CopB was one of the first OMP studied. It exhibits moderate degree of antigenic conservation among strains tested (Karalus 2000). There are 3 lines of evidence to say that an antigen is conserved:

- Antibody reactivity
- Restriction Fragment Length Polymorphism (RFLP)
- Comparison of sequence of the gene

The protein is immunogenic as the antibody against it could be detected in convalescent sera of patients (Sethi 1995). Moreover, passive immunization of mice with the antibody against CopB resulted in enhanced clearance of *M. catarrhalis* from the lungs of these animals (Karalus 2000). Mutation and expression analysis show that CopB is involved in iron acquisition. Iron is an important element for many bacteria to live on their mammalian hosts. Therefore bacteria developed strategies to transfer low concentration of iron from their environment.

OMP CD:

Sequence analysis and RFLP studies on OMP CD show high degree of conservation among different strains of *M. catarrhalis*. It is a heat-modifiable protein that was initially reported as OMP C and OMP D. Then it was realized that these two bands were the two forms of a single antigen. BLAST search shows that the protein has homology with *Pseudomonas aeruginosa* porin protein (Murphy 1993). The antibody against OMP CD elicit complement-mediated bactericidal activity against various strains of *M. catarrhalis* which indicate it may stimulate a protective immune response if used as a vaccine.

UspA1 and UspA2:

They were first named as High Molecular Weight OMP (HMW-OMP) since the protein forms oligomeric complexes. Later on it was realized that the complex was composed of two proteins and named as ubiquitous surface protein A1 and A2 (UspA1 and UspA2). They were characterized as a single protein not only because of a single band in Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) but also because they share a common epitope. In fact they share a common 140-aminoacid region that is 93% identical (Cope 1999). In addition, passive immunization of mice with the antibody against CopB resulted in enhanced clearance from the lungs of these animals (Karalus 2000).

Fimbriae :

Fimbriae have been described on the surface of *M. catarrhalis* by electron microscopy (Ahmed 1994). In *M. catarrhalis,* fimbriae are responsible for adherence and hemagglutination, therefore thought to be an important virulence factor (Ahmed 1992a). It was also found that fimbriae are present on all fresh isolates of *M. catarrhalis,* and it decreases in number upon repeated in vitro passages (Ahmed 1992). Since fimbriae has not been isolated, little information available regarding the characterization of these structures.

Lipooligosaccharide(LOS)

Another prominent bacterial surface component of *M. catarrhalis* is the LOS. *M. catarrhalis* LOS shares homology with LOS of other gram-negative bacteria. In addition, these shared LOS epitopes have been implicated as a virulence factor

for *M. catarrhalis* i.e. resistance to bactericidal activity mediated by normal human serum (Zaleski 2000). There are three major LOS serotypes in *M. catarrhalis:* A, B and C. these structures encompass 95% all strains studied to date (Rahman 1996). Recently, it has been suggested that LOS may act as an adhesin of *M. catarrhalis* (Hu, 2001).

1.1.5 Typing and Epidemiology

The worldwide distribution of *M. catarrhalis* as well as nosocomial outbreaks by this bacterium, provide an opportunity for considering epidemiological studies. Several epidemiological typing methods have been used to characterize strains of *M. catarrhalis*, such as phenotypic characterization, electrophoretic mobility of esterases, OMPs, whole-cell proteins, plasmid profile, isoelectric focusing of β-lactamase enzymes, bacteriocin typing, haemagglutination, fimbriation and serotyping; however, these methods could not satisfactorily discriminate interstrain variability. The RFLP has been also used for *M. catarrhalis*; however the result was not satisfactory due to many fragments, which are difficult to compare. Pulsed field gel electrophoresis (PFGE) was successfully used to find out inter-strain variation of *M. catarrhalis* isolated from sputum of patients with RTI from various parts of the world (Martinez 1999). The PFGE analysis also showed that there were wide variations among the strains of *M. catarrhalis* isolated from the same and different geographical location. However, strains isolated from same location showed some common bands shared by the strains. In conclusion, the study showed that the strains of *M. catarrhalis* may originate from ancestor strains and they have been proliferating with slow genomic rearrangement.

1.1.5.1 Colonization

M. catarrhalis is capable of colonizing humans without causing disease, which was one of the reasons it was characterized as a commensal bacterium. The rate of colonization is affected by age, health, socioeconomic condition, season and geographic location. *M. catarrhalis* is a frequent inhabitant of oro- and nasopharynx of children 0-10 years of age with a prevalence rate of 25-35%. Healthy adults are less frequently colonized (Ejlertsen 1991). In a study done on 1520 patients (Ejlertsen 1994), *M .catarrhalis* colonization was examined with

respect to prevalence, time of colonization and association with respiratory tract infections (RTI). In the age group of 1-47 months, children with healthy respiratory tracts were colonized with a prevalence of 36%. However there was a significant increase in colonization rate in children with upper and lower RTI. (68 %). Children with RTI may be prone to be colonized as long as their respiratory epithelium is damaged. Subsequently, they eliminate *M .catarrhalis* as they recover from their RTI, their colonization rate returning to the non-RTI children.

Colonization with *M .catarrhalis* is not static. Molstad *et al.* (Molstad 1988) found a high rate of acquisition and elimination of *M .catarrhalis* in children. Nearly 100% of children in their study group were colonized at various times with a mean prevalence rate of 62%.

In the study of Chen *et al* (1999), they found that antibody levels against an OMP of *M.catarrhalis* (UspA1) are higher in 2 months old children when compared to 18 months old children due to the passage of maternal antibodies to the fetus during pregnancy. The low antibody levels and the low bactericidal activities seen in sera of children in 6-18 months of age are consistent with the epidemiological finding that children at this age have the highest rate of colonization and highest incidence of *M. catarrhalis* infection.

In a different study on nasophryngeal cultures from children of 0-2 years of age demonstrated that 66% of the infants become colonized within first year of life and increasing to 77.5% by two years of age (Faden 1994). This study also revealed that in the first 2 years of life a child acquires and clears 3 to 4 different strains of *M. catarrhalis.* Therefore, the rate of colonization in infants and young children is much more greater than that of adults.

1.2 ADHERENCE:

1.2.1 Definition and Importance

Bacterial adherence (also called attachment) is the term used to describe when bacteria attach to different types of surfaces present in the environment. Bacterial attachment is the initial step in the pathogenesis of infections. In *M. catarrhalis*, attachment to human pharyngeal epithelial cells (HPEC) has been

shown to be the initial event of infection (Mbaki 1987). Therefore studies on the attachment mechanism of *M. catarrhalis* is helpful for the understanding of pathogenesis as well as to find new strategies for the treatment and prevention of infection.

1.2.2 Mechanism of Adherence

Several nonspecific factors are involved in the process of adhesion such as surface electric charge, hydrophobicity and van der Waals forces etc. It is important to note that these are initial factors; they are required, but not enough for firm binding. The specific factor for attachment is the interaction of the *adhesin* on the bacterium with the *receptor* on the host cell. Adhesin is a molecule on the surface of bacteria that mediates adherence by interacting with a receptor on the substratum. Chemically, adhesins may be proteins, polysaccharides, teichoic acids or lipids in nature. Many bacterial species have been found to express more than one type of adhesins and, in some cases, more than 10 have been described (London 1996). A *receptor* is any surface molecule of a substratum that is complementary to a bacterial adhesin. Chemically mammalian cell receptors are composed of proteins, glycoproteins and glycolipids.

Other factors to affect adherence are the seasonal changes (high in winter and low in summer), defects in immune system, repeated sub-culturing of bacteria *in vitro*, prior viral infection and underlying COPD (Rikitomi 1997, Rikitomi 1986).

Before the interaction of adhesin and the receptor, bacteria should overcome the repulsive electrostatic forces since both the bacterium and the epithelial cell are negatively charged. At this time point the filamentous structures such as fimbriae, flagella and fibrillae, act as bridging structures helping bacteria to overcome repulsion forces. The bacterium can now move closer to the surface of the epithelial cell, but the amount of water adsorbed around the surface of the epithelial cell or bacterium is the last barrier to overcome. Hydrophobic groups located on fimbriae or proteins of the cell surface, may play a major role, allowing the displacement of water molecules (Braga 2000). On the contrary, a recent study showed that bacteria with its negative charge bind with the positively charged micro domains (microplicae) on the HPEC. This study also

showed that HPEC is not entirely negatively charged rather composed of positively and negatively charged domains (Ahmed 2000).

1.2.3 Mucus and Mucociliary Action

During a normal day a person inhales about 10,000 microorganisms per 24 hours (Mims 1995). To prevent infection by this huge amount of microorganisms, mucosal surfaces have several mechanisms:

- Secretion of mucus
- Mucociliary clearance
- Cellular defenses
- Immunological defenses.

The mucus in the respiratory tract is a mixture of the secretions of different types of cells present in or under the mucosa: goblet cells, epithelial serous cells, secretory cells in submucosa and ciliated cells. It is a gel like structure consists of mucus glycoproteins called mucins. It acts as a barrier for microorganisms to overcome before reaching to the epithelial cell layer. Other functions of bronchial mucosa are to entrap microorganisms and repel them out of the respiratory tract by the mucociliary action. In addition, mucosa acts as an extracelullar surface for immunoglobulin action.

Some bacteria can produce factors that disturb the mucociliary system by slowing or disorganizing the beating of cilia (Wilson 1996). Some of these factors exert their effects by increasing the mucus production, which in turn, decrease the mucociliary action. Till now no such effect has been reported in *M. catarrhalis*. However, report has shown that *M. catarrhalis* can bind with human nasopharyngeal mucin (Reddy 1996).

1.2.4 Adhesins and Receptors for *M. catarrhalis*

Adhesion is a complex process which might include more than one adhesin-receptor interaction. For *M. catarhalis,* fimbriae are found to be adhesins. Fimbriae are composed of repeating subunits, the part that interacts with the host cell is on the tip of the pilus (London 1996). Other adhesins for *M. catarrhalis* are hemagglutinins, UspA1(Aebi 1988) and CD protein(Murphy 1993). In addition, in this study we proved that our protein of interest is an adhesin of *M.*

catarrhalis. The receptors for *M. catarrhalis* identified so far are ganglioside GM2 (Ahmed 1996) and asialoganglioside GM1 (Ahmed 2002).

1.2.5 How to Exploit Adherence

The study on bacterial adherence to host is not only a basic science to understand the pathogenesis and virulence factors, but also it has several practical applications. These applications center on the prevention of adhesion before or after the bacterium attaches to host. One of them is to provide the adhesin or its analogue into the respiratory tract. This might be done by spraying it to the pharynx of the patient. The adhesin or its analogue will bind to complementary receptor on host cells. This will block the receptor and will prevent the attachment of bacteria to the epithelial cells. The same logic can also work for the receptor. There are receptor analogs, which can mimic the receptor and bind to the adhesin on the surface of bacterium. The binding of the receptor analog to the adhesin will block the adhesin-receptor interaction.

Another strategy is to use the adhesin as a vaccine. Since it is a surface protein, the immune response primed by adhesin, would be protective. The immune system will not only act by conventional means to kill the bacterium, but also prevent the adherence by the binding of antibodies to adhesin.

The scope of adhesion research can be enlarged by using anti-attachment drugs. These can be divided into two:

1. Low-dose antibiotics.
2. Non-antibiotic drugs.

Low-dose antibiotics don't kill the bacteria but prevent the attachment by changing the surface structure of the bacterium. But the weakness of this strategy is the rapid emergence of the antibiotic resistant bacteria.

The non-antibiotic drugs have the ability to prevent attachment of *M. catarrhalis* and nontypeable *H. influenzae.* One of them is a widely used mucolytic drug: S-carboxymethyl cysteine(S-CMC). The mechanism how S-CMC prevent attachment is not yet clear, however, it has been shown that it can remove the carbohydrate from the surface of HPEC (Zheng 1999). The receptors for bacteria are mainly glycolipid in nature therefore it is thought that S-CMC may remove the receptor from cell surface. Another study showed that S-CMC

can decrease the positive charge of the microplicae hence decrease the attraction needed for bacteria to attach with HPEC (Ndour 2001).

1.3 Aim of the Project

The aim of this project can be categorized as the following:

1. Isolation of an adhesin from the surface of *M. catarrhalis.*
2. To understand the biochemical properties of the protein such as heat sensitivity, molecular weight, sensitivity to reducing agents and glycosylation.
3. To determine the immunogenic potential of the protein:
    a. In animal by inoculating bacteria
    b. In human during *M. catarrhalis* infection
4. Production of monoclonal antibody
5. Attachment inhibition assay to prove the protein as an adhesin.
6. *N*-terminal amino acid sequence.
7. Identification of the gene of the protein

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of analytical grade and supplied by Sigma-Aldrich Chemie GmbH (Germany) except for:

- 4-chloro-1-naphthol was purchased from Wako Pure Chemical Industries Ltd. (Japan).
- Ampicillin and kanamycin were purchased from Roche Diagnostics GmbH (Germany).
- IPTG, X-Gal were purchased from MBI Fermentas Inc. (USA).

2.1.2. Bacterial Strains

In this study, for all purposes *M. catarrhalis* was used and only for cloning purposes *E. coli* was used.

2.1.2.1 *E. coli* Strains

Strain	Genotype	Usage	Source
M15	F^-,Nal^S,Str^S,rif^s,lac^-,ara^-,gal^- mtl^-,rec^+,uvr^+	Production of expression libraries	Bio-Rad (Hercules USA)
DH5α	*F-supE44 hsdR17 recA1gyrA96 endA1 thi-1 relA1 deoR lsmbda-*	Production of library and propagation of plasmids	Bio-Rad (Hercules ,USA)

2.1.2.2 *M. catarrhalis* strains

Strains of *M. catarrhalis*, B-87-34, B-87-69, B-87-94, B-88-152 and strain F isolated from sputum of patients with RTI were used in this study. Strain B-88-

152 was mainly used unless otherwise stated. Except strain F, all the strains were fimbriated (Ahmed 1990).

2.1.3 Enzymes

All restriction enymes (*Hind*III*, Pst*I*, and EcoRV*), DNA modifying enzymes (T4 ligase, calf intestine alkaline phosphatase) and *Taq* polymerase were purchased from MBI Fermentas Inc. (USA). RNase A, Proteinase K and Lysozyme were purchased from Roche Diagnostics GmbH (Germany). Lysine endopeptidase was purchased from Wako Pure Chemical Industries Ltd. (Japan).

2.1.4 Oligonucleotides

All primers used in this study were custom synthesized and purchased from Iontek (Bursa, Turkey). The sequences of the primers were designed by converting the *N*-terminal amino acid sequence of 55 kDa protein to DNA sequence. These primers were used to amplify possible gene of 55 kDa protein by PCR for sequencing. The sequences of the primers were as follows:

Forward: 5'-ATTACTCATCAAAATATGCTTCC-3'

3rd base is degenerate: T, C or A
6th base is degenerate: T, C, A or G
9th base is degenerate: T or C
12th base is degenerate: A or G
15th base is degenerate T or C
19th base is degenerate: T or C
21st base is degenerate: T, C, A or G

Reverse: 5'- TTAGCAGGAAACATATTTTGATG- 3'

3rd base is degenerate: T, C, A or G
6th base is degenerate: T, C, A or G
9th base is degenerate: T, C, A or G
11th base is degenerate: A or G
15th base is degenerate: A or G
18th base is degenerate: T or C
21st base is degenerate: A or G

Apart from these degenerate primers, a 23-mer oligonucleotide has been designed according to the preferred codon usage in gram-negative bacteria. This oligonucleotide was used in colony hybridization experiment as the radiolabelled probe.

Probe: 5'- AAAATCCAYACCCCGAGCATCAC-3'

Y= C or T

We also used several primers for sequencing purposes which are not shown here for limitation of space.

2.1.5 Cloning Vectors

pQE-31 (Qiagen Inc. USA) and pBluescript SK (Stratagene Inc. USA) were the vectors used in this study.

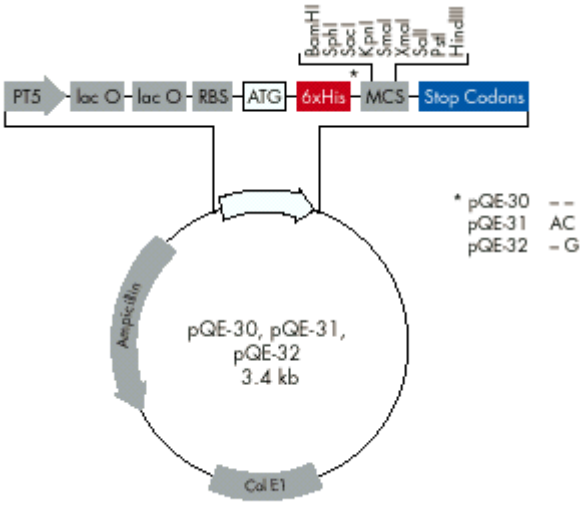

Figure 2.1: The map of pQE expression vector. It contains *lac* operon and is suitable for expression studies.

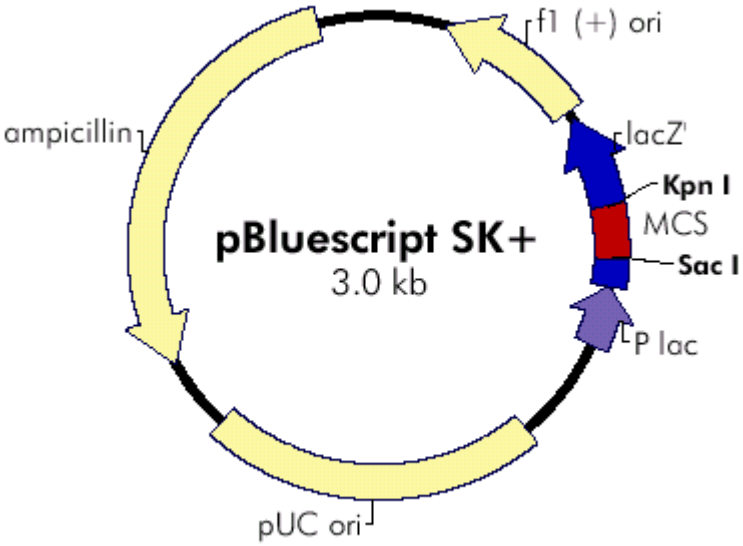

Figure 2.2: The map of pBluescript cloning vector. It contains *lacZ* gene and suitable for α-complementation

2.1.6 Antibodies

The primary antibodies used in this study were the monoclonal antibody against 55 kDa protein and polyclonal antibody against whole cell *M. catarrhalis.*

2.1.6.1 Generation of monoclonal antibody against 55 kDa protein

mAb 4H3, a murine antibody of IgM class (Hybridoma Subisotyping Kit, Calbiochem-Novabiochem Corporation, USA), was prepared by fusing spleen cells from BALB/c mouse immunized with 55 kDa protein with SP-2 mouse myeloma fusion partner (Oishi 1996) and screening the resulting hybridomas by enzyme-linked immunosorbent assay (ELISA) employing microtiter wells coated with 55 kDa protein. mAb to 55 kDa protein was measured in polystyrene microtiter plate caoted with 1 μg of purified protein per ml suspended in coating buffer and left to stand overnight. mAb 4H3 was cloned and adapted as an ascites tumor in pristine-primed BALB/c mice. Ascitic fluid was collected, centrifuged and the supernatant was stored at -80 °C until use.

2.1.6.2 Generation of polyclonal antibody against M. catarrhalis

Whole cell antibody against strain B-88-152 was generated by injecting a rabbit with live organism as described previously (Ahmed 1992). A dose of 1 ml bacterial suspension in 1 ml Freund's adjuvant (Difco Laboratories, USA) was injected each time in equally dividing doses into two subcutaneous and two intramuscular sites. A total of four doses was administered at 2-week intervals. Two weeks after the last injection, blood was collected and the serum was stored at -80 °C.

2.1.6.3 Secondary Antibodies

Secondary antibody for colony lift, dot blot and Western blotting was purchased from Sigma (Sigma-Aldrich Chemie GmbH Germany). It was anti-mouse IgM antibody conjugated to horse radish peroxidase (HRP) enzyme to permit the visualization of the signals. The secondary antibody for immunofluorescence was purchased from Zymed (Zymed Laboratories, Inc., USA). It was goat anti-mouse IgM antibody conjugated to fluorscein isothiocyanate (FITC). Secondary antibodies for sputum Western blot were anti-human IgA, IgG and IgM conjugated with HRP. The secondary antibody for

rabbit antiserum experiment was anti-Rabbit IgG conjugated with HRP and it was purchased from Zymed.

2.1.7 Commercially Available Kits

Qiaex II Gel Extraction Kit (by Qiagen Inc., USA) was used to purify DNA from agarose gels.

*MN Nucleospin Plasmid (*Macherey-Nagel, GmbH& Co, Germany) was used to purify plasmids from *E. coli.*

DIG Glycan Detection Kit (Boehringer Mannheim Corp. USA) was used to determine the gycosylation status of proteins.

HexaLabel Plus DNA Labeling Kit (MBI Fermentas Inc. NY, USA) was used to radioactively label the oligonucleotide probe for colony hybridization experiment.

Silver Stain Kit (Wako Pure Chemical Industries Ltd. Japan) was used to stain the SDS-PAGE gels.

Mouse IgM LL Nanorid Kit *(Binding Site Limited, England*) *was used to determine the concentration of the primary antibody.*

Hybridoma Serotyping Kit (Calbiochem –Novabiochem Corporation, USA) was used to determine the class of the antibody produced.

DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences UK Limited, England) was used for sequencing reactions.

Bio Rad Protein Assay Kit (Bio Rad Laboratories, USA) was used to determine concentration of proteins.

2.1.8 Apparatus and Equipment

Vertical mini gel apparatus for polyacrylamide gel electrophoresis was a product of Atto Corp. (Japan). The power supply was Power Pac 200 from Bio Rad Laboratories (USA). Horizontal mini gel apparatus used for agarose gel electrophoresis was Mupid-2 purchased from Advance Co., Ltd., Japan. Thermal cycler for PCR was a product of Perkin Elmer (USA). Slab gel dryer is from Savant Instruments Inc. (USA). Wet transfer unit for Western Blotting and GelDoc 2000Image analyzer for agarose gel electrophoresis were purchased from Bio Rad Laboratories (USA). Cytospin for attachment assays was purchased

from ThermoShandon Inc. (USA). UV cross linker, the UV Stratalinker 1800, was from Stratagene Inc (USA). Fluorescence microscope was from Zeiss (Carl Zeiss Jena GmbH, Germany)

2.1.9 Materials for Autoradiography

Radiolabelled dCTP (containing ^{32}P) was purchased from NEN (NEN Life Science Products, Inc. USA) The light-proof film cassette (Hypercassette) and film developing unit were products of Amersham Biosciences UK Limited (England) Medical x-ray films were purchased from Fuji (Tokyo, Japan).

2.1.10 DNA and Protein Size Markers

As DNA size marker 1 kb DNA ladder from MBI FERMENTAS Inc. (USA) was used.

SDS-PAGE protein size markers, the Rainbow Marker and Low Range Protein Size Marker were purcahsed from Bio Rad Laboratories (CA, USA).

2.1.10 Membranes

Nitrocellulose membranes for colony lift and colony hybridization were purchased from Shleicher & Schuell GmbH (Germany)

PVDF membranes for Western blots were purchased from Bio Rad Laboratories (USA)

2.1.11 Computer Programs

DNASIS Mac V3.6 and Oligo 4.0s were used to design oligonucleotide primers.

GENETYX-MAC 10.1 was used to translate the DNA sequence into amino acid sequence in three reading frames. Pairwise BLAST and protein BLAST was used for homology search.

2.2 SOLUTIONS AND MEDIA

2.2.1 Agarose Gel Electrophoresis Solutions

50X Tris-Acetic acid-EDTA (TAE)	*Per Liter:* 242 g Tris Base 57.1 ml Glacial Acetic acid 100 ml of 0.5 M EDTA
Ethidium bromide	10 mg/ml in water (stock solution) 30 ng/ml(working solution)

2.2.2 Microbiological Media and Antibiotics

Luria-Bertani Medium(LB)	*Per Liter:* 10 g Bacto tryptone, 5 g Yeast extract 5 g NaCl 15 g Agar(for solid media)
Blood Agar	%7 Rabbit blood in BHI agar
Brain Heart Infusion (BHI) Agar	*Per Liter:* 52 g of BHI agar powder
BHI Broth	*Per Liter:* 37 g of BHI broth powder
Mueller Hinton (MH) Broth	*Per Liter:*21 g of MHB powder
Ampicillin	100 mg/ml(stock solution) 100 µg/ml (working solution) in dH_2O
Kanamycin	25 mg/ml (stock solution) 25 µg/ml (working solution) in dH_2

2.2.3 Polyacrylamide Gel Electrophoresis Solutions

Separating Gel (10%)	7.8 ml dH_2O 5 ml 1.5 M Tris(pH 8.8) 6.7 ml acylamide-bisacrylamide(30:0.8) 200 µl 10% SDS 200 µl 10% APS 6.7 µl TEMED
Stacking Gel (5 %)	3.3 ml dH_2O 1.5 ml 0.5 M Tris(pH 6.8) 1 ml acylamide-bisacrylamide(30:0.8) 60 µl 10% SDS 120 µl 10% APS 3 µl TEMED
Sample Buffer	10 ml 10 % SDS 4 ml 0.25 M Tris-HCl (pH 7.6) 6 ml dH_2O 2 ml Glycerol 30 mg EDTA.2Na *and per ml of the above solution:* 20 µl Bromophenol Blue (Saturated Soln)/ml 75 µl 2-mercaptoethanol OR 0.13 g of DTT/ml
Electrophoresis Buffer(10X)	*Per 500 ml:*15.14 g Tris 72 g Glycine 5 g SDS
Staining Solution	2 g Coomassie Blue 100 ml Trichloro acetic acid (100%) 400 ml Ethanol 500 ml dH_2O
Destaining Solution	1950 ml dH_2O 750 ml Ethanol 240 ml Acetic Acid

2.2.4 Colony Lift Solutions

10X TBS	*Per Liter*: 80 g NaCl 2 g KCl 30 g Tris(pH 8.0)

Blocking Solution	5% non-fat dry milk or 5% BSA

2.2.5 Western Blot Solutions

10X Tris-HCl Buffer	*Per Liter:* 12.4 g Tris-HCl (pH 7.3) 85 g NaCl
Electrode Solution	*Per Liter:* 6 g Tris 28.8 g Glycine 600 ml Methanol
Blocking Solution	5% non-fat dry milk

2.2.6 Colony Hybridization Solutions

Denaturation solution	0.5 NaOH 1.5M NaCl 0.1% SDS
Neutralization Solution	1.0 M Tris-HCl, pH 7.5 1.5 M NaCl
20X SSC	3M NaCl 0.3M Sodium Citrate, pH7.0
Blocking Solution	0.5M Sodium Phosphate Buffer, pH7.0 7% SDS 1mM EDTA
2X Wash Solution	2X SSC 0.1% SDS
0.1X Wash Solution	0.1X SSC 0.1% SDS

2.3 METHODS:

2.3.1 Growth and Maintenance of Bacteria

2.3.1.1 *M. catarrhalis*

M. catarrhalis strains were stocked in MH broth containing 5% rabbit blood and stored at -20C until use. For stocking, the bacteria were cultured on 7% rabbit blood agar plates and the colonies were taken with a sterile cotton swab and transferred to the cryotube containing 1 ml of media. During experiments, the bacteria were cultured on BHI agar or BHI broth. The plates were incubated in a box containing 5% CO_2 at 37 °C. The CO_2 was generated by the CO_2 Gen Compact (Oxoid Limited, UK). All media were purchased from Merck Kga, (Germany).

2.3.1.2 *E. coli*

E. coli strains were stored in 50% sterile glycerol at -70 °C until use. Overnight grown cultures to saturation were mixed with sterile glycerol in a ratio of 1:1, mixed to homogeneity and stored at -70 °C until required. Bacteria were recovered by scraping a small amount of cells from the stock with an inoculation loop and streaking onto a LB-agar plate. Experiments with *E. coli* were done by culturing the bacteria on LB agar or LB broth. Liquid cultures were incubated in a rotary shaking incubator at 37 °C (~200 rpm).

2.3.2 DNA EXPERIMENTS

2.3.2.1 Preparation of Competent Cells and Transformation of *E. coli*

2.3.2.1.1 Simple and Efficient Method (SEM) for Preparation of Competent Cells

A single colony of appropriate *E. coli* strain was inoculated into 15 ml of LB broth (containing the appropriate antibiotics) and grown overnight at 37 °C. The starter culture was diluted to OD_{600} of 0.2-0.3 in 250 ml of SOB medium (2% Bacto-tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, sterilized by autoclaving) and grown to an OD_{600} of 0.6 at 18 °C

with shaking at 200-250 rpm. The culture was chilled on ice for 10 minutes, centrifuged at 2500 Xg for 10 minutes at 4 °C. The pellet was then resuspended in 80 ml of ice-cold TB (10mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl, pH 6.7, sterilized by filtration through 0.45µm filter, stored at 4 °C), and incubated on ice for 10 minutes. The mixture was pelleted as above, resuspended gently in 20 ml of TB. DMSO was added to a final concentration of 7%, mixed gently and incubated on ice for 10 minutes. Aliquots of this mixture were then immediately chilled in liquid nitrogen, and stored at -70 °C up to 3 months without loss of transformation efficiency (Inoue et al. 1990).

2.3.2.1.2. Transformation of *E. coli*

For transformation using supercompetent cells, an aliquot of frozen competent cells was thawed on ice. 200 µl of the cells was mixed with plasmid DNA (less then 100ng) in an eppendorf tube and incubated on ice for 30 minutes. The cells were then exposed to a 90 seconds heat-shock at 42 °C and then chilled on ice for 2 minutes. 800 µl of SOC (SOB with 20mM glucose) was added and the cells were grown at 37 °C for 45 minutes with vigorous shaking at 250 rpm. The culture was then centrifuged and the pellet was resuspended in 100µl of SOC, and spreaded on LB agar plates containing the appropriate antibiotic.

2.3.2.2 Plasmid DNA Isolation

The plasmid DNA was isolated with MN Nucleospin Plasmid kit according to the manufacturers instructions.

2.3.2.3 DNA Ligation Reactions

DNA fragments were ligated into plasmid vectors in 15 µl reaction volumes containing 0.3-1.0 µg of linearized plasmid vector and 3-5 times molar excess of insert DNA in the presence of 1-4 Weiss units of T4 ligase and 1 X concentration of the standard ligation buffer supplied with the enzyme. The reaction mixture was incubated at 16 °C for overnight.

2.3.2.4 Restriction Enzyme Digestion

Restriction enzyme digestions were performed in 10-20 µl reaction volume for 1.5 to 4 hours and typically 1-10 µg of DNA and 5-15 units of restriction

enzymes were used. Reactions were carried out with the appropriate reaction buffer and conditions according to the manufacturers recommendations.

2.3.2.5 Alkaline Phosphatase Reaction

Calf intestine alkaline phosphatase enzyme was used in order to prevent the religation of the linearized plasmids in the ligation reaction. In 50 µl of total volume, 1 µl of enzyme and 5 µl reaction buffer was used to remove phosphates from DNA.

2.3.2.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to amplify and sequence the possible gene encoding 55 kDa protein. PCR reactions were performed in Thermowell^{TM} tubes (Corning Costar Corp.) using GeneAmp PCR system 9600 (Perkin Elmer).

PCR reactions were carried out in a reaction volume of 50 µl containing 100 ng of *M. catarrhalis* genomic DNA, 5 µl 10X PCR buffer, 4 mM $MgCl_2$, 0.32 mM of each dNTP 20 pmol of each primer and 2.5 unit *Taq* DNA polymerase.

The reaction mixture was preheated to 94 °C for 5 minutes and subjected to 30 cycles of denaturation (30 seconds at 94 °C), annealing (45 seconds at 55 °C) and elongation (2 minutes at 72 °C). At the end of the 30 cycles a final extension at 72 °C for 10 minutes was also applied. PCR products were assessed by agarose gel electrophoresis and EtBr staining.

2.3.2.7 Genomic DNA Isolation from *M. catarrhalis*

Large scale genomic DNA isolation was performed by the standard methods of Current Protocols (John & Wiley press 1998)for gram-negative bacteria.

100 ml BHI broth was inoculated with 1 or 2 colonies of *M. catarrhalis.* After overnight incubation the bacteria were harvested by centrifugation at 10000 rpm. The pellet was resuspended in 8.75 ml of 10X TE (100mM Tris-HCl, pH 7.5, 10 mM EDTA) by repeated pipetting and then transferred to a disposable 50 ml centritube. The cell suspension was freezed at -20 °C for 60 minutes. 1.0 ml of freshly prepared lysozyme(10 mg/ml in 0.25 Tris-HCl, pH 8.0) solution was added to the frozen cells and the cells were thawed by mixing in a room-temperature water bath. Just after thawing, the cells were put on ice for 45

minutes. 0.25 ml of 20 % SDS, 50µl of 20 mg/ml proteinase K and 10 µl of 10 mg/ml DNase-free RNase was added to the cell suspension. After mixing thoroughly the solution was incubated at 55 °C for 1 hour with occasional gentle mixing. Then 1.8 ml of 5 M NaCl was added and the tube was mixed thoroughly by inverting for 5 minutes. 1.5 ml of CTAB/NaCl solution was added and the tube was mixed thoroughly and then it was incubated at 65 C for 20 minutes with occasional shaking.

Approximately equal volume of chloroform: isoamyl alcohol (49:1) was added, the tube was mixed thoroughly by inverting the tube for 5 minutes, and it was spin down 10 min at 3000 rpm. The chloroform phase was removed and the supernatant and the interphase were homogenized with a plastic pipette. The suspension was centrifuged 10 min at 3000 rpm again and the aqueous, viscous supernatant was transferred to a fresh centritube, leaving the interface behind.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), was added and the solution was extracted thoroughly by inverting the tube and was centrifuged 10 minutes at 3000 rpm. The aqueous, viscous supernatant was transferred to a fresh centritube, leaving the interface behind.

0.6 volume of isopropanol was added to precipitate the nucleic acids. The DNA pellet was transferred to a fresh tube containing 70 % ethanol by hooking it onto the end of a glass Pasteur pipette. The DNA was washed with 70 % ethanol to remove the residual CTAB and recentrifuged to pellet it. The supernatant was removed carefully and the pellet was dissolved in TE.

2.3.2.8 Phenol-Chloroform Extraction

Phenol-chloroform extraction was done to purify DNA after each enzymatic manipulation. First, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA solution containing the impurities. The optimum starting volume was about 100 µl. If the solution was less than this volume it was diluted with dH_2O accordingly. After mixing 45 seconds by vortexing, the tube was centrifuged for 5 minutes at 13000 rpm. The upper (water phase) was transferred to a new eppendorf tube . Then 1/10 volume of 3M NaOAc was added. After mixing by inverting the tube 2.5 volume of cold absolute ethanol was added. After mixing by inverting the tube was kept in -80C for 20 minutes. Then the tube was centrifuged for 10 minutes at 13000 rpm. After discarding the

supernatant, the pellet was washed with 200 µl of 70% ethanol. After drying all the ethanol the pellet was dissolved in TE or water.

2.3.2.9 Electroelution

This method was done to purify DNA from agarose gels that were too big for Qiagen Gel Extraction kit. First, the DNA was electrophoresed by using freshly prepared TAE buffer. Using the long wavelength UV the band was located and cut with a scalpel. The gel slice was put in the dialysis membrane containing some TAE. Then the membrane was put in the electrophoresis tank and run for 1 hour at 150 V. The electric field was reverted for 10 seconds. Then the buffer containing the DNA was taken out with a Pasteur pipette and the membrane was washed with 300 µl TE buffer.

2.3.2.10 Butanol Concentration

High volume DNA solution obtained after electroelution was concentrated using this method. Equal volume of butanol was added to DNA solution. After vortexing for 10 seconds the tube was centrifuged for 15 seconds. The upper butanol phase was discarded. This process was repeated until desired volume was reached.

2.3.2.11 Agarose Gel Electrophoresis

DNA fragments of less than 1 kb were generally separated on 1.0 % agarose gel , those greater than 1 kb were separated on 0.8 % gels. Required amount of agarose gels were completely dissolved in 1X TAE buffer by heating in microwave, then ethidium bromide was added to a final concentration of 30 ng/ml. The DNA samples were mixed with one volume loading buffer and loaded onto gels. The gel was run at room temperature in 1X TAE at 100 V.

2.3.2.12 Purification of Linear Plasmid DNA from Agarose Gels

This method was used to isolate the linear plasmid from the restriction enzyme digested plasmid DNA that contained both linear and circular bands. For this experiment Qiagen Gel Extraction Kit was used. The instructions of the manufacturer were pursued.

2.3.2.13 DNA Sequence Analysis

DNA sequence analysis of the positive clones was performed by the dideoxy method. Cycle sequencing reactions were set up in 0.2 ml Greiner tubes. The 20 µl reaction volume contained 30-180 ng template miniprep plasmid product, 3.2 pmol of primer and 8 µl terminator ready reaction mix (DYEnamic ET Terminator Cycle Sequencing Kit, Amersham Biosciences UK Limited, England). The cycle sequenase reactions were performed in Perkin Elmer GeneAmp PCR 9600 system thermal cycler with the following parameters: 25 cycles of denaturation (95 °C for 20 seconds), annealing (50 °C for 15 seconds) and extension (60 °C for 1 minutes).

Electrophoresis was performed on the ABI PRISM^{TM} 377 Genetic Analyzer (Perkin Elmer, USA) by loading the samples into the lanes of a vertical polyacrylamide slab gel. The separated DNA fragments were exposed to a laser which in turn would excite the flourescent dyes attached to the fragments. These data were then collected and analyzed by the help of ABI Sequencing Analysis Software (version 3.0.1).

2.3.3 PROTEIN EXPERIMENTS

2.3.3.1 Isolation of 55 kDa Protein

Celite chromatography was done to isolate the 55 kDa protein with the procedure described previously (Lu 1998). 1 g of activated celite was suspended in 100 mM Tris-buffered saline, pH 7.6 (TBS) and added to the column. Surface proteins from bacteria were harvested by water extraction as described previously (Pei 1988). The water extract (WE) in 1 mM phenylmethylsulphonyl flouride at 4 °C was applied on the matrix at a flow rate of 0.5 ml/minute. The column was washed with 100 ml 100 mM TBS to remove unbound proteins. Bound protein was eluted with 10 ml stepwise gradient of 5~10 mM EDTA and finally with 1M Tris, pH 10.5.

Eluted fractions were checked by spectrophotometer (OD_{280}) to determine the presence of protein. Finally, the protein elution was confirmed by running the elutions on SDS-polyacrylamide gels. Protein concentration was determined by Bio Rad Protein Assay Kit according to the manufacturer's instructions.

2.3.3.2 *N*-Terminal Amino Acid Sequencing

The single eluted protein was sequenced by directly applying protein through automated *N*-terminal Edman degradation with the High Performance Liquid Chromatography system. For determination of *N*-terminal amino acid sequence of 55 kDa protein fragments, the protein was dialyzed against dH_2O for overnight, then lyophilized. The protein was dissolved in 300 ml of 0.1 M NH_4HCO_3, then 20 ml, 1 mg/ml of lysine endopeptidase was added and incubated at 37 °C in an incubator for 4 hours. 15 ml of the product was applied onto a C4 column and eluted with graded concentration (0-70%) of acetonitrile at a flow rate of 1 ml/min. Elutions were collected at peak. The two highest peaks were subjected to *N*-terminal sequencing.

2.3.3.3 Glycan Detection

DIG Glycan Detection Kit was used to determine whether our protein was glycosylated or not. Adjacent hydroxyl groups in sugars of glycoconjugates are oxidized to aldehyde groups by mild periodate treatment. The spacer linker steroid hapten digoxigenin (DIG) is then covalently attached to these aldehydes via a hydrazide group. DIG labelled glycoconjugates are subsequently detected in an enzyme immunoassay using a digoxigenin specific antibody conjugated with alkaline phosphatase.

The 55 kDa protein, transferrin and creatinase (5μg/lane) was subjected to SDS-PAGE. After transferring to PVDF membrane, the protocol was followed according to the instruction of the manufacturer.

2.3.3.4 SDS Polyacrylamide Gel Electrophoresis and Western Blot

Electrophoretic separation of proteins under denaturing conditions was performed by using the vertical mini gel apparatus. For all experiments 5 % stacking gel and 10% separating gel was used and run at 40 mA. After performing SDS-PAGE, the proteins were transferred from the gel to nitrocellulose or PVDF membrane at 100 V for 1 hour. Membranes were blocked by 5 % non-fat dry milk or BSA. For detection of the transferred proteins, 4-chloro-1-naphthol method was used (explained in section 2.3.3.5).

2.3.3.5 Colony Lift

This method was used to detect the bacteria expressing the protein of interest on their surfaces. Firstly the bacteria were streaked or spreaded on LB agar plates containing the appropriate antibiotic(s). The membrane was placed onto the plate for 1minute to lift the colonies and was incubated in petri dish containing 10 ml 5% non-fat dry milk (in TBS) for overnight at 4 °C. Then the membrane was washed 3 times with TBS each time for 3 minutes in a rotary shaker. Then the membrane was treated with primary antibody for 90 minutes at a dilution of 1:5000 (0.1 μg/ml) and was washed 3 times with TBS. After the washing step, the membrane was treated for 90 minutes with secondary antibody at a dilution of 1:5000 in TBS. Then the membrane was washed again 3 times with TBS. The detection part of the experiment was done in two different ways:

1. By using ECL reagents: The ECL reagents were mixed and spreaded over the membrane for 1 minute. The film was exposed to the membrane for 1 minute. Then the film was developed in a (name company of the machine)
2. By using 4-chloro-1-naphthol: Solution A (15 mg 4-chloro-1-naphtol in 5 ml cold methanol) and Solution B(15 μl 30% H_2O_2 in 20 ml Tris-HCl) were prepared. A and B were mixed and the solution was incubated with the membranes until the color reaction was seen.

2.3.3.6 Dot Blot

This method was used to confirm the positive signals obtained in the colony lift protocol. 2-3 colonies were picked up from the LB agar and suspended in TBS. 2-3 drops of this suspension were given on to the nitrocellulose membrane. After drying the membrane the procedure was followed as described in colony lift.

2.3.3.7 Attachment Inhibition Assay

Attachment assay is used to measure the attachment ability of bacterial cells to the human pharyngeal epithelial cells. In attachment inhibition assay, whether a molecule can prevent attachment of bacteria to human pharyngeal epithelial

cells is determined. In this study, 2 types of attachment inhibition assays were done:

1. Attachment inhibition assay with protein
2. Attachment inhibition assay with antibody.

In the first type of attachment experiment, the 55 kDa protein was incubated with the pharyngeal epithelial cells. If the protein was an adhesin as we hypothesized, the exogenously added 55 kDa protein would bind and block the receptor on the epithelial cell and attachment of bacteria would decrease with respect to control. In the second type of attachment experiment, the mAb against 55 kDa protein was incubated with *M. catarrhalis*. If the protein was an adhesin as we hypothesized, the antibody would bind and block the 55 kDa protein on *M. catarrhalis* and attachment of bacteria would decrease with respect to the control.

2.3.3.7.1 Attachment Inhibition Assay with 55 kDa Protein

Attachment inhibition assay was performed as described previously (Ahmed 1996). Pharyngeal epithelial cells were taken from the throat of a healthy adult individual by scraping the oropharynx with a sterile cotton swab. The cells were transferred to 1/15 mM phosphate buffer pH 7.2 (PB) and filtered to remove particles. Then in order to get rid of the normal flora that might interfere with the attachment, the cells were washed 3 times with PB each time for 10 minutes by centrifugation at 80 Xg at room temperature. The number of cells were determined by Neubauer counting chamber and adjusted to a concentration of 2.5X 10^4 cell/ml.

Then cells were treated with the 55 kDa protein in a shaking water bath at 37 °C for 30 minutes. Cells without 55 kDa protein treatment was taken as a control. Overnight grown *M. catarrhalis* was suspended in phosphate buffered saline pH 7.2 (PBS) and adjusted to a concentration of 1X 10^8 cfu/ml by spectrophotometer. Then the cells and the bacteria were mixed in a 1:1 ratio and centrifuged at 750 Xg for 10 minutes at room temperature and incubated for 30 minutes at 37 °C. After the incubation, 5 times washing was done with PBS to remove the unattached bacteria. The cells in the suspension, then, collected on a slide with cytospin. The slides were Gram stained and the attached bacteria were counted by observing 50 consecutive cells under an oil immersion objective. We

also similarly performed attachment inhibition assay with exogenously added BSA to rule out nonspecific effect on attachment inhibition by 55 kDa protein.

2.3.3.7.2 Attachment Inhibition Assay with Antibody Against 55 kDa protein

Pharyngeal epithelial cells were collected and adjusted to a concentration of 2.5 X 10^4 cells/ml as described above. Overnight grown *M. catarrhalis* was suspended in PBS and adjusted to a concentration of 1X 10^8 cfu/ml by spectrophotometer. Then bacteria were treated with antibody in a shaking water bath at 37 °C for 30 minutes. Bacteria treated in the same way without any antibodies were taken as control. Then the attachment assay was done as described above. We also similarly performed attachment inhibition assay with exogenously added unrelated mouse IgM to rule out nonspecific effect on the attachment inhibition by mAb against 55 kDa protein.

2.3.3.8 Quantitative Culture

This method was used to calculate the *M. catarrhalis* number via spectrophotometric measurement. *M. catarrhalis* colonies grown on BHI agar were suspended in PBS in different dilutions. After measuring the turbidity of these suspensions by a spectrophotometer (OD_{660}), the bacteria were enumerated by quantitative culture. This was done by constructing a calibration curve, which made possible the determination of number of viable bacteria from A_{660} readings.

2.3.3.9 Radial Immunodiffusion (RID)

Radial immunodiffusion is a technique that is routinely used for measuring the concentrations of various soluble antigens. The method involves antigen dissolving radially from a cylindrical well through an agarose gel containing an appropriate mono-specific antibody. Antigen-antibody complexes are formed, which under the right conditions, will form a precipitin ring. The ring size will increase until equilibrium is reached between the formation and break down of these complexes. At this stage, a linear relationship exists between the square of ring diameter and the antigen concentration. By measuring the ring diameters produced by a number of samples of known concentration, a calibration curve

can be constructed. The concentration of an antigen in an unknown sample may then be determined by measuring the ring diameter produced by that sample and reading of the calibration curve.

In this study, the radial immundiffusion was performed for determination of mAb concentration in ascitic fluid by Mouse IgM LL Nanorid Kit.

2.3.3.10 Colony Hybridization

This method was used to screen the genomic DNA library of *M. catarrhalis* with radioactively labeled oligonucleotide probe. First, the plates of *E. coli* containing the genomic library were chilled at 4 °C for 1 hour. After that, nitrocellulose membranes were exposed to the colonies for 1 minute and labeled properly, A blotting paper (Whatmann) was put into denaturation solution until it absorbs enough solution, then the membrane was put on the blotting paper (colony side up) and incubated for 15 minutes. The neutralization solution was applied similarly for 5 minutes. Lastly, 2X SSC was applied for 15 minutes to the membrane by the same method. Then the membrane was UV cross linked by *UV Stratalinker 1800* at auto cross-link option. After that, the membrane was incubated in 3X SSC/0.1% SDS for 1 hour with shaking. After wiping the surface of the membrane by a wet towel, it was incubated for 1 hour at 37°C in TBS containing 20µg/ml proteinase K. To inactivate proteinase K, 40µg/ml PMSF in TBS was incubated with the membrane for 5 minutes at room temperature. Then the membrane was washed twice with 2X SSC for 5 minutes. After washing, the membrane was incubated with the blocking solution for 2 hours at 42 °C. Then the labeled probe was diluted to a concentration of 20 ng/ml in the blocking solution and hybridized for overnight at 68 °C. Next day, the membrane was washed twice with 2X wash solution for 5 minutes each. The washing step was repeated with 0.1 X wash solution for 15 minutes each. Then the membrane was exposed to an X-ray film overnight at -80°C.

To confirm the positive signals, the positive colonies were picked up and grown overnight at 37 °C. Then the colonies were suspended in normal saline and 2-3 drops were applied to membrane. Rest of the procedure was the same as explained above.

2.3.3.11 Immunofluorescence

Immunofluorescence was done to prove that 55 kDa protein bind to the complementary receptor on HPEC. HPEC were collected on a glass slide by the method described in section 2.3.3.7.1. 55 kDa protein (5µg) was incubated with the cells on the slide for 30 minutes. After washing the cells, the slide was blocked with 3% BSA to prevent the background signals. To remove the excess BSA the slide was washed with 0.01 M phosphate buffer, pH7,2. Then mAb against 55 kDa protein was incubated for 45 minutes on the slide at a concentration of 5 µg/ml (1:100 dilution). After washing three times with PB, secondary antibody conjugated to FITC was incubated for 45 minutes on the slide at a dilution of 1:500. After washing the excess antibody the slide was analyzed under fluorescence microscope.

2.3.3.12 Isolation of Sol Phase of Sputum

The sol phase of sputum was collected by centrifuging at 15000 rpm for 90 minutes at 4 °C. Supernatant was collected and kept at -80 °C until use. Among 8 sputum samples obtained during RTI, 6 were pure *M. catarrhalis* infections and other two were *M. catarrhalis* mixed infection with *H. influenzae* and *S. pneumoniae.*

2.3.3.13 Detection of Antibody in Patients' Sputum

The 55 kDa protein (5 µg/lane) was subjected to SDS-PAGE. After transferring protein, PVDF membrane was treated with sol phase of the patients' serum (1:100) for overnight at 4°C. HRP conjugated goat anti-human IgG, IgM and IgA at a dilution of 1:100 were used as a secondary antibody and treated for 90 minutes at room temperature.

2.3.3.14 Detection of Antibody in Rabbit Serum

The 55 kDa protein (1 µg/lane) was subjected to SDS-PAGE. After transferring the protein, PVDF membrane was treated with preimmune rabbit serum and immunized rabbit serum for strain B-88-152 and strain F at a dilution of 1:1000 for 90 minutes at room temperature. Peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:1000 was used as secondary antibody anf treated for 90 minutes at room temperature.

CHAPTER 3: RESULTS

3.1 INTRODUCTION

The results of our study can be divided into 3 parts. First, the protein of interest was isolated from the surface of *M. catarrhalis*, and the biochemical properties of the protein were determined (heat sensitivity, immunogenicity, glycosylation etc). Second, the function of the protein was confirmed by using attachment inhibition assays. In the third part we determined the *N*-terminal amino acid sequence of the protein and we attempted to amplify the partial gene of 55 kDa protein with PCR, sequence and clone the whole gene.

3.2 ISOLATION AND CHARACTERIZATION OF 55 kDa PROTEIN

3.2.1 Isolation of 55 kDa Protein

Figure 3.1 shows the elution profile of 55 kDa protein isolated by celite chromatography. As shown, protein peaks were found in elution fraction 9, 10, 11 and 12. Figure 3.2 consists of SDS-PAGE profile of the corresponding proteins. We see a single band of protein with a molecular size of about 55 kDa at elution fraction 9, 10, 11 and 12.

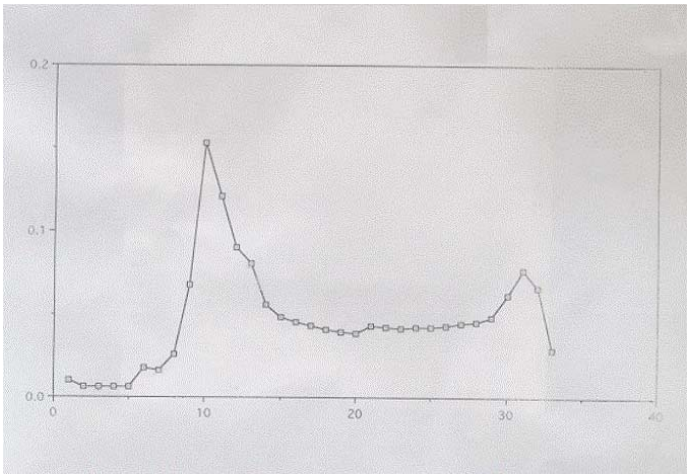

Figure 3.1: Elution profile of celite chromatography. X-axis shows the elution fractions and the Y-axis shows the absorbance at OD_{280}

Figure 3.2: Silver-stained gel after SDS-PAGE analysis of elution fractions showing pure protein was isolated with a molecular weight of 55 kDa. The numbers 6~32 show the elution fractions subjected to electrophoresis. LM marker indicates the protein molecular weight marker and the numbers besides each band indicates their corresponding molecular weight in kDa.

3.2.2 Biochemical Properties

The DIG Glycan Detection kit shows that 55 kDa protein is not glycosylated (Figure 3.3). To prove the validity of the test, we used glycosylated and nonglycosylated proteins, transferrin and creatinase as a positive and negative control, respectively. As seen in the Figure 3.3, transferrin gave a positive signal while no signal was found for the 55 kDa protein and creatinase.

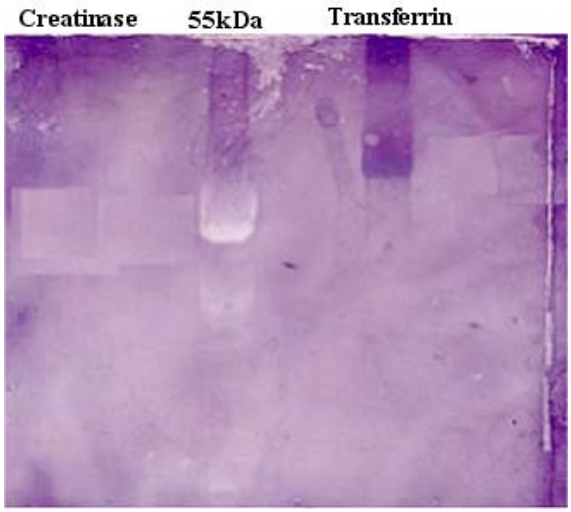

Figure 3.3: Glycosylation determined by DIG glycan detection kit. Glycosylation test was done after Western blotting of 55 kDa protein, transferrin (a glycosylated protein) and creatinase (a nonglycosylated protein). Only transferrin showed positive reaction, indicating creatinase and 55 kDa protein are nonglycosylated.

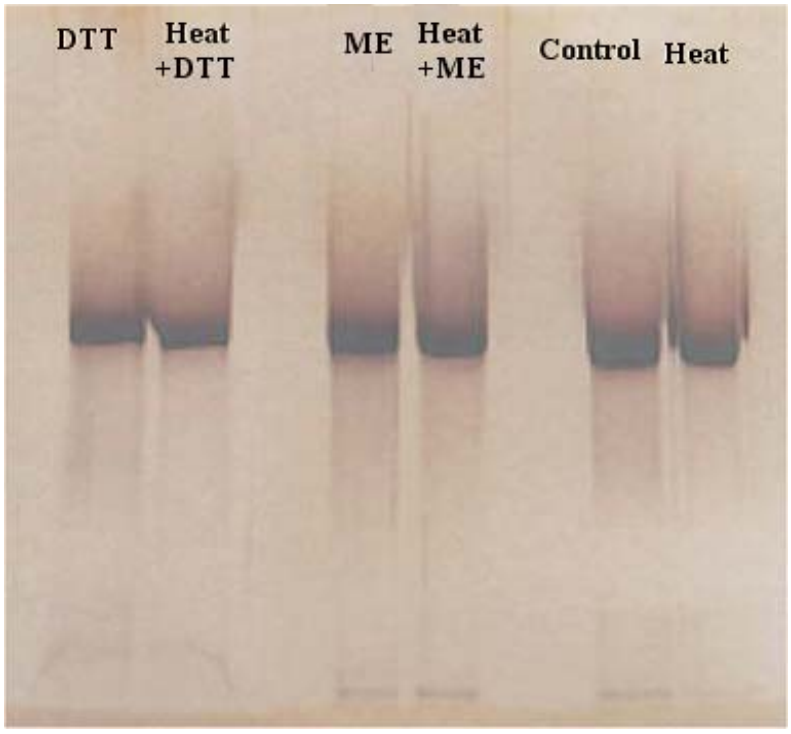

Figure 3.4: Silver stained gel after SDS-PAGE of dithiothreitol(DTT), 2-mercaptoethanol (ME) and heat treatment of 55 kDa protein. Control indicate unheated sample. No change of molecular weight of 55 kDa protein observed.

To determine the heat sensitivity, 55 kDa protein was heated at 100 °C for 5 minutes. In addition, to examine the presence of disulphide bonds, we treated the protein with dithiothreitol (DTT) and 2-mercaptoethanol separately. Moreover, in the presence of these reducing agents, samples were compared by heating at 100 °C for 5 minutes with unheated samples. Silver staining of SDS-PAGE gel seen in Figure 3.4 shows that our protein is resistant to heat, DTT and β-mercaptoethanol.

3.2.3 Immunogenic Properties

Western blot experiment shows that when rabbit was immunized with whole cell *M. catarrhalis*, it produced antibody against 55 kDa protein (Figure3.5). This is not only in the strain B-88-152 but also in strain F, which is a nonfimbriated strain. It indicates that 55 kDa is possibly conserved in other strains and not a fimbrial protein. We only used IgG as a secondary antibody as this is the dominant antibody in serum.

Figure 3.5: Western blot of 55 kDa protein after membrane treated with antiserum of rabbit immunized with whole cell *M. catarrhalis.* No band is visible when membrane was treated with pre-immune rabbit serum(left). While positive band is visible after treatment with antiserum of rabbit immunized with *M. catarrhalis* strain B-88-152 (center) and strain F (right).

To search whether the protein was immunogenic in humans, we did Western blot analysis to detect the antibodies against 55 kDa protein in patients' sputum. After Western blot (figure 3.6), when the membrane was treated with sol phase of sputum, all the samples showed positive reactivity bands with IgG, IgM and IgA, indicating that the patients produced antibody against 55 kDa protein during the acute phase of *M. catarrhalis* infection in their sputum.

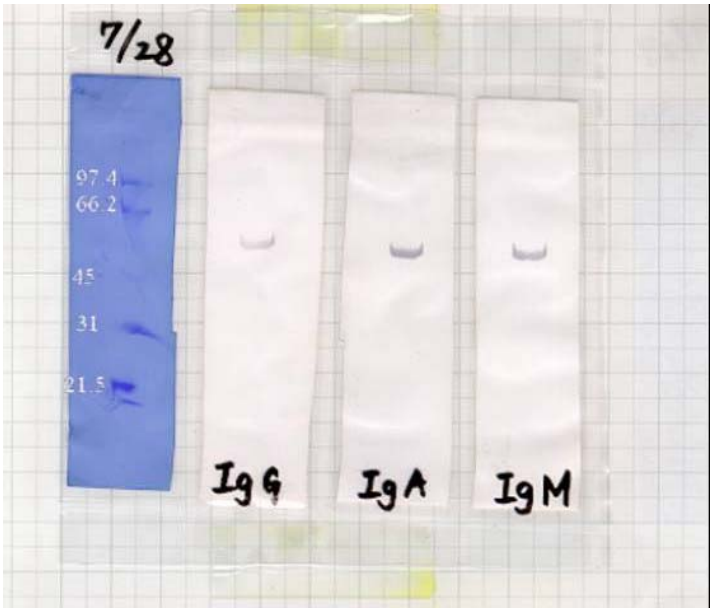

Figure 3.6: Western blot analysis of the 55 kDa protein, detected three different types of antibodies (IgG, IgM and IgA) in sputa of patients infected with *M. catarrhalis*. Left side shows the protein molecular weight marker on PVDF membrane stained with Coomassie blue.

3.2.4 *N*-Terminal Amino acid Sequence

The *N*-terminal amino acid sequencing determined 40 amino acids of 55 kDa protein which were found to be composed of the following amino acids:

- KPSKIHTPSITHQNMLPSNTLAQLDALPQLTQGDGDTGKS…….

It may be noted that 55 kDa protein did not start with methionine. Two other fragments of 55 kDa protein showed the following amino acid sequence

- …..IHTPSITHQNMLPANTLAQ……
- …..KYDHXVKT……

X indicating the amino acid that could not be determined.

3.1 EFFECT OF 55 kDa PROTEIN ON THE ATTACHMENT OF *M. CATARRHALIS*

To check whether 55 kDa protein is an adhesin of *M. catarrhalis*, we employed two strategies. In the first one, we incubated HPEC with exogenous 55 kDa protein to find out whether the protein would block the receptor and inhibit the attachment. We also compared the attachment inhibition ability of an unrelated protein, BSA, using similar conditions. In the second strategy, we incubated the mAb against 55 kDa protein with *M. catarrhalis* to find out whether the antibody would bind to 55 kDa protein and would inhibit the attachment. We also compared the attachment inhibition ability of an unrelated commercially obtained mouse IgM.

3.1.1 Attachment Inhibition Assay with 55 kDa Protein:

When cells were treated with 0.01, 0.1 and 1 μg /ml of 55 kDa protein, there was a significant decrease in attachment as shown in Table 3.1. On the other hand, at a concentration of 0.001and 0.0001 μg/ml, there was no difference of attachment as compared to the control. More significantly, we found a dose dependent effect between attachment inhibition and concentration of 55 kDa protein as shown in percentage data in Figure 3.7. Compared to the control (45.6 ± 6.6 bacteria /cell) there was no significant decrease of *M. catarrhalis* attachment, when cells were treated with 1 μg/ml of BSA (46.5 ± 15.6 bacteria/cell)

Table3.1: Results of attachment inhibition assay with 55 kDa protein. Each experiment was done in duplicate and the values are expressed as mean ± SD. The values indicate number of bacteria attached per cell.

Dilution	Control	Experiment	No. of Exp
0.0001μg/ ml	31.7±6.4	30.1±14.3	3
0.001μg/ml	31.7±6.4	29.2±12.5	3
0.01μg/ml	22.9±10.5	13.4±12.0 *	8
0.1μg/ml	22.2±11.2	12.6±7.1 *	7
1μg/ml	22.9±10.5	10.0±7.5 **	8

*** p<0.05**

****p<0.005**

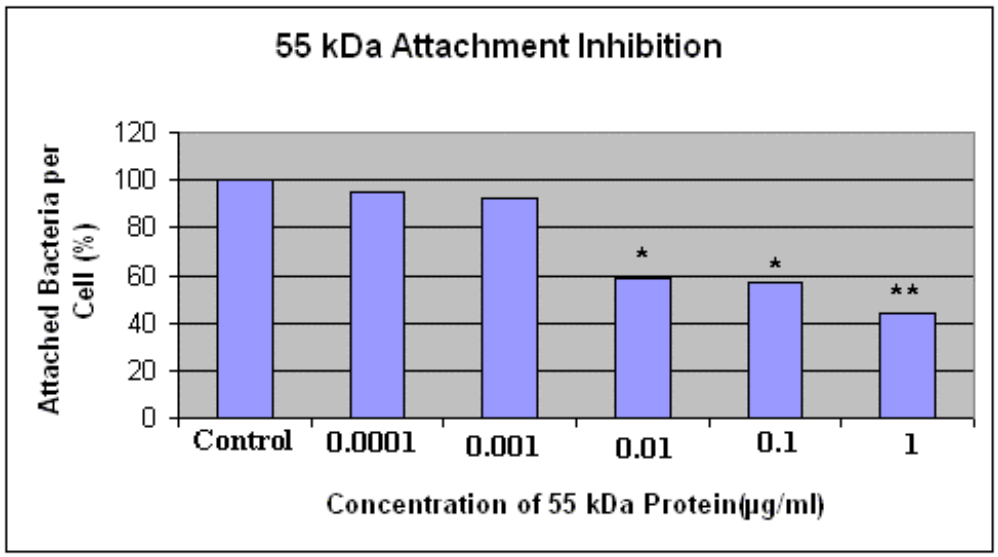

Figure 3.7: Results of attachment inhibition assay with 55 kDa protein. Each experiment was done in duplicate and the values are mean of the experiments. The attachment of bacteria are shown here as percentage of the control. At a dose of 0.0001, 0.001, 0.01,0.1 and 1 µg/ml, there was a 5%, 7.9%, 41.5%, 43.2% and 56.3% decrease of attachment when compared to the control, respectively.
* $p<0.05$, ** $p<0.005$

3.1.2 Attachment Inhibition Assay with mAb Against 55 kDa Protein:

When bacteria were treated with 5 , 0.5 µg/ml of mAb, there was a significant decrease of attachment compared to the control (Table 3.2). However, no significant decrease of attachment was observed when bacteria were treated with 0.1µg/ml of mAb. In this experiment, also we found a dose dependent effect between attachment inhibition and concentration of mAb as shown in Figure 3.8. As compared to the control (19.4 ± 14.8 bacteria/cell) there was no significant decrease of *M. catarrhalis* attachment, when bacteria were treated with 5 µg/ml of unrelated mouse IgM (28.3 ± 5.6 bacteria/cell).

Table3.2: Results of attachment inhibition by mAb against 55 kDa protein. Each experiment was done in duplicate and the values are expressed as mean ± SD. The values indicate number of bacteria attached per cell.

Dilution	Control	Experiment	No of Exp
0.1 µg/ml	28.3±10.5	27.9±6.5	3
0.5 µg/ml	30.6±4.1	14.8±11.5 *	5
5 µg/ml	33.1±2.9	10.2±3.1 **	3

*** p<0.05**

****p<0.01**

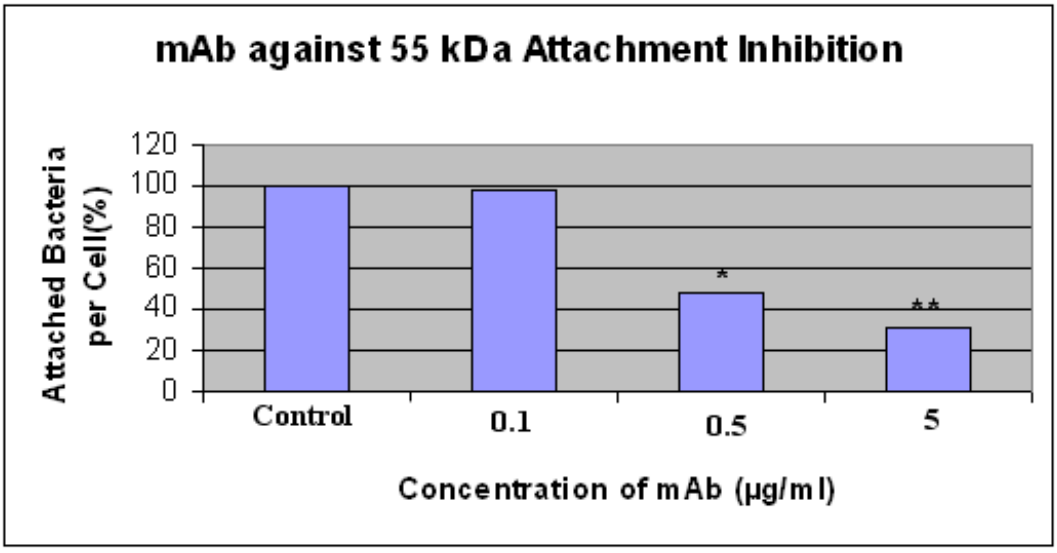

Figure 3.8: Results of attachment inhibition assay with mAb against 55kDa protein. Each experir attachment compared to the control, respectively.

*** p<0.05**

****p<0.01**

In addition, our mAb at a concentration of 5 µg/ml could inhibit the attachment of other 3 strains of *M. catarrhalis* significantly (Table 3.3).

Table3.3: Results of attachment inhibition of other strains by mAb. Each experiment was run in duplicate and the values are mean ± SD of at least three independent experiments. The values indicate number of bacteria attached per cell.

Strain	Control	Experiment
B-87-69	31.5±5.3	9.2±1.5 *
B-87-94	11.8±4.0	3.0±2.3 *
F	10.6±2.7	6.3±2.2 **

*** p<0.05**

****p<0.01**

3.2 PCR Amplification of Partial Gene Encoding 55 kDa Protein

One way to sequence the gene encoding 55 kDa protein was to amplify it by PCR. Degenerate primers designed from the *N*-terminal amino acid sequence were employed. After optimizing the reaction conditions, we obtained a single band of 500bp (Figure 3.9). After sequencing the PCR product we couldn't find any homology with the *N*-terminal amino acid sequencing data.

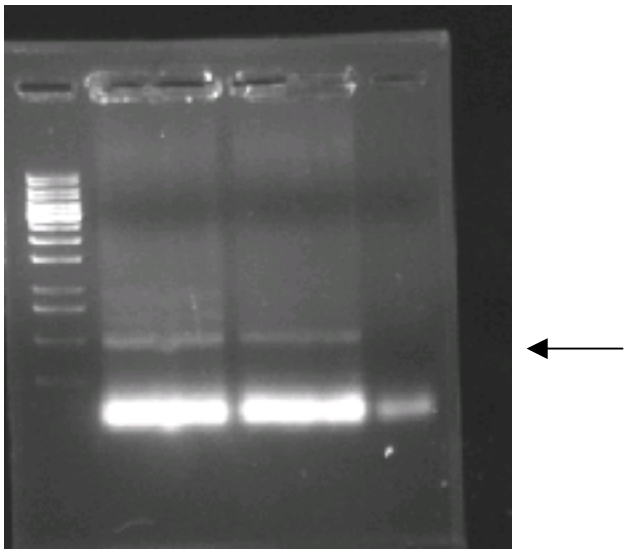

Figure 3.9: Agarose gel electrophoresis of PCR product. Lane 1: 1kb DNA ladder, lane 2&3: PCR product, lane 4: PCR reaction without template. The thick bands under the PCR product are excess primers.

3.3 Immunofluorescence

To check whether 55 kDa protein really binds with HPEC, we performed immunofluorescence experiment. 60% of the cells treated with 55 kDa protein gave positive reaction while only 33.3% of the cells treated with PBS gave positive reaction. This result indicates that 55 kDa protein could bind to the receptor on HPEC and thus inhibits the attachment of *M. catarhalis* when cells were treated with 55 kDa protein.

3.6 MOLECULAR CLONING EXPERIMENTS

In this part of our study, we attempted to clone and sequence the gene encoding the 55 kDa protein. We used two strategies: first one was to make genomic DNA library of *M. catarrhalis* in pQE-31 expression vector and screen this library with the monoclonal antibody against 55 kDa protein. Before preparing such a library, a dot blot was performed to find out the reactivity of mAb with *M. catarrhalis* and *E. coli*. Figure 3.10 shows that the monoclonal antibody reacted only with *M. catarhalis,* but not with *E. coli*.

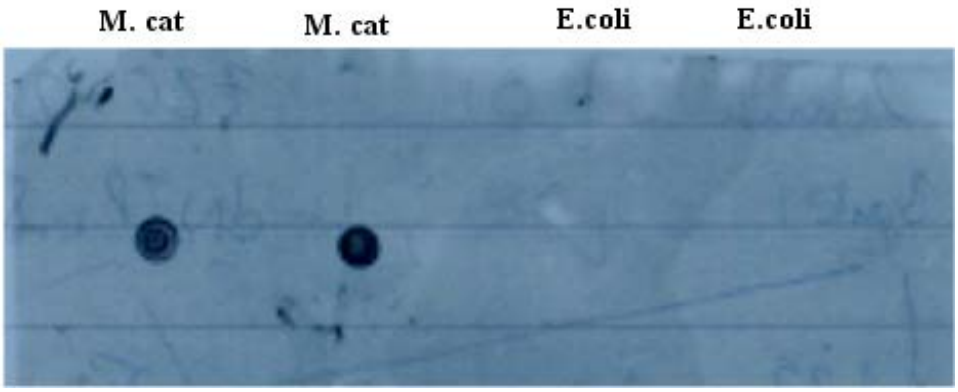

Figure3.10: Dot blot of *M. catarrhalis* and *E. coli* detected with monoclonal antibody against 55 kDa protein. *M. catarrhalis* gave positive signal while no signal was found in *E. coli* colonies.

3.6.1 Genomic Library in pQE-31 Expression Vector

The library was constructed by cutting the genomic DNA with *Hind*III and ligating the resulting fragments to linearized and dephosphorylated pQE-31 expression vector. The library was transformed into *E. coli* M15 strain and the expression was induced by IPTG. If 55 kDa protein was expressed on the surface of *E. coli,* it would be detected by the monoclonal antibody against 55 kDa protein. Finally, with colony lift we obtained 24 positive colonies after searching more than 4000 colonies. Next, we made dot blot to confirm the results, this time we got 10

positive clones reacted with the mAb. Then we sequenced these inserts and compared the results with the *N*-terminal amino acid sequence of 55 kDa protein. But we could not obtain any homology. However, the inserts were not sequenced up to the end, which remains to be done in future.

During the preparation of linearized plasmid DNA we encountered a problem that *Hind*III did not cut all the plasmid so that some uncut plasmid molecules remained. We solved the problem by excising the linear plasmid band from the gel and extracting the DNA from it. Figure 3.11 shows the linear and nonlinear forms of the pQE-31 vector.

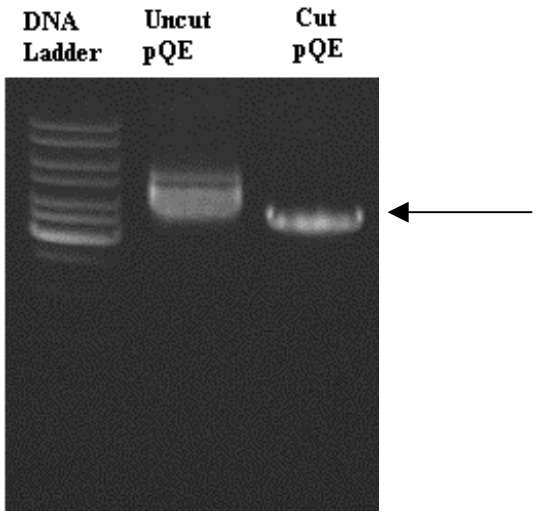

Figure 3.11: Agarose gel electrophoresis shows the pQE-31 plasmid vector after digested with *Hind*III and undigested form. DNA ladder molecular weight marker ranged from 10kb to 250 bp. The digested vector migrated at a size of 3.4 kb as expected. Arrow indicating the digested vector.

The inserts were prepared by digesting the genomic DNA of *M. catarrhalis* with *Hind*III. After running the digested DNA in agarose gel, the gel portion corresponding from 10 kb to 1 kb fragments was cut and purified by electroelution (Figure 3.12).

Figure 3.12: *Hind*III digested *M. catarrhalis* genomic DNA after electroelution. The fragments of the digested DNA were ranging between 1 kb and 10 kb.

After confirming the positive clones by dot blot, we isolated the plasmid DNA from recombinant *E. coli* and digested the plasmid with *Hind*III. After running the digest in agarose gel, different sizes of inserts were obtained e.g. 1 kb, 1.5 kb and 3 kb. An insert of ~3 kb can be seen in Figure 3.13.

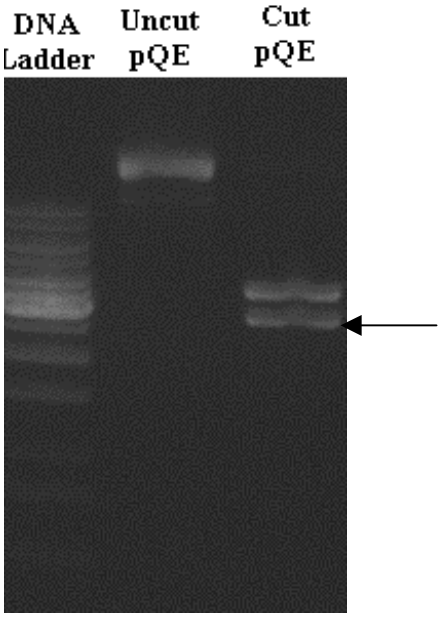

Figure 3.13: Agarose gel electrophoresis showing the plasmid (pQE-31) isolated from one of the putative clones. The insert was released upon digestion with *Hind*III is shown by the arrow.

3.6.2 Genomic Library in pBluescript SK Vector

As our second strategy, the library was prepared with pBluescript SK (pBSK) vector. Similarly, the genomic DNA of *M. catarrhalis* was cut with five different restriction enzymes (*Eco*RV, *Pst*I, *Hind*III, *Dra*II and *Bst*XI). Among these, *Eco*RV, *Pst*I and *Hind*III cut well while the restriction digestions obtained from *Dra*II and *Bst*XI were not satisfactory. Thus, *Eco*RV, *Pst*I and *Hind*III were used to construct the library. The fragments of the digested DNA were ligated into linearized and dephosphorylated pBluescript vector. Figure 3.13 and 3.14 show the inserts and the vector.

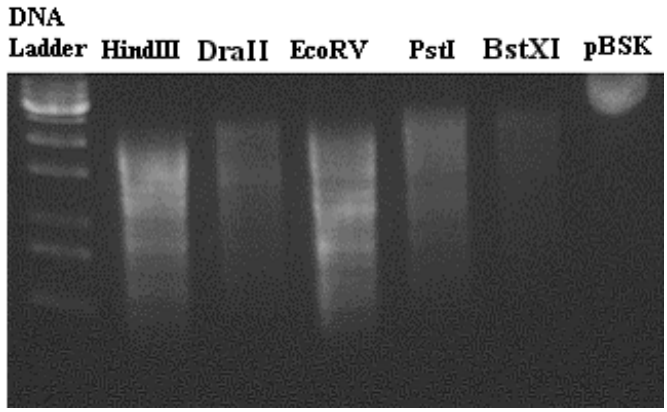

Figure 3.14: *M. catarrhalis* genomic DNA was digested by different restriction enzymes. pBluescript (pBSK) plasmid DNA was included for comparison of DNA concentrations.

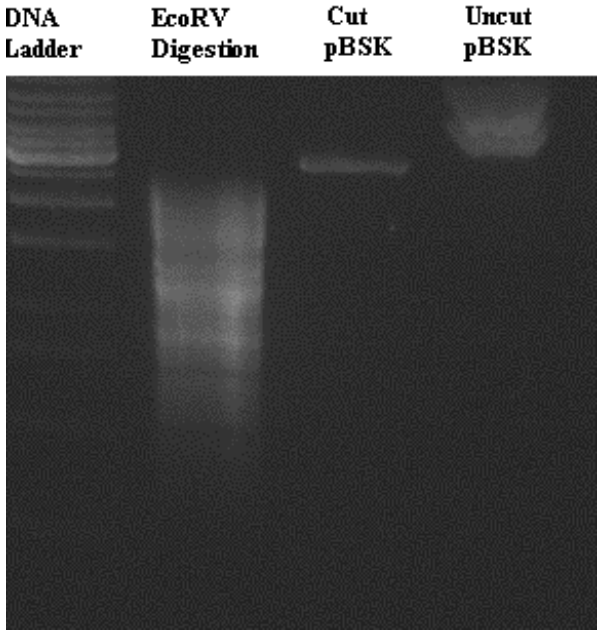

Figure 3.15: *M. catarrhalis* genomic DNA and pBluescript (pBSK) plasmid DNA were digested with EcoRV. Uncut plasmid is also shown.

The library was then transformed into *E. coli* DH5α strain. After screening the library as explained in Chapter 2, we obtained a total of 10 positive colonies 2 of which are shown in Figure 3.15. We made dot blot of all these 10 positive clones to confirm the results and 2 of these positive clones gave positive reaction again. Inserts from these clones were sequenced but there was no homology between the N-terminal amino acid sequence of 55 kDa protein and the 3-frame translations of the inserts.

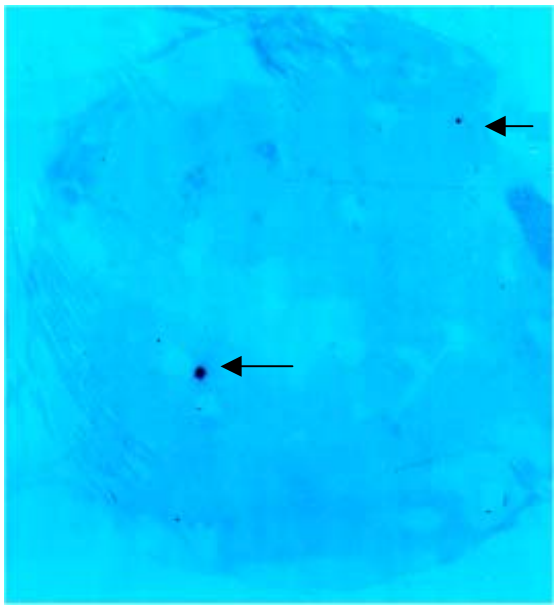

Figure 3.16: Screening of *Hind*III-pBluescript genomic library of *M. catarrhalis* with radiolabelled probe. The arrows indicated the positive colonies.

CHAPTER 4:DISCUSSION

Bacterial adhesion to host cells is the initial step of the colonization and subsequent infection. Although human body develop defense against bacterial attachment, there are some bacteria that can still attach to human host and can cause disease. Our body has developed several means to prevent attachment. For instance, presence of normal flora in the upper respiratory tract prevents attachment of several pathogenic bacteria by occupying space in the respiratory tract. In addition, IgA in our mucus prevent attachment of bacteria to the epithelial cells (Williams 1972). On the other hand, some bacteria secrete IgA protease, which degrades specifically IgA, and thus overcome this defense (Zakrzewski 2000). It is not certain whether *M. catarrhalis* also uses this protease as a virulence factor.

The bacterial surface constitutes the first line of defense against antimicrobial molecules and stress caused by the environment surrounding the bacterium. In the case of host-pathogen interactions, many bacterial surface molecules are virulent determinants. Thus, in order to understand the molecular basis for bacterium-host interactions, it is important to characterize the molecular architecture of the bacterial cell surface. In the present study we have isolated and characterized a surface protein from *M. catarrhalis*, which mediates attachment to human pharyngeal epithelial cells.

The isolation of surface proteins was done by “water extraction method”, which has been applied to *Campylobacter jejuni* (Pie 1988) and *H. influenzae* (Lu 1998) to extract surface proteins. Our initial findings showed that we could extract different types of proteins from *M. catarrhalis* by this method. This water extract was applied to celite chromatography in order to isolate a putative adhesin protein. Celite chromatography has been successfully used in the isolation of adhesin from *H. influenzae* (Lu 1998). In this technique, the hydrophobic interaction is thought to be involved between the proteins and the celite (Queiroz 2001).

Our attachment inhibition assay conclusively showed that 55 kDa protein is an adhesin of *M. catarrhalis.* When we treated HPEC with 55 kDa protein, the

attachment inhibition was achieved at a very low concentration of protein and there was a dose dependent effect also, yet, there was no effect when we treated cells with BSA. Similarly when we treated *M. catarrhalis* with mAb against 55 kDa protein, we achieved attachment inhibition at a low concentration of antibody. There was a dose dependent effect also in this antibody mediated attachment inhibition assay. On the other hand, unrelated mouse IgM had no effect on attachment inhibition. As a support to these data, our immunoflourescence results conclusively proved that 55 kDa protein indeed can bind with HPEC. We also showed that the mAb was effective in attachment inhibition of other strains of *M. catarrhalis*, which indicates that 55 kDa protein might have been conserved among different strains of *M. catarrhalis.* Therefore this protein may be designated as a virulence factor for *M. catarhalis* which is essential for attachment with host cells and for subsequent infection.

There are several research groups studying adherence mechanism of *M. catarrhalis*. However, cultured cells are used in those experiments (McMichael 1998). But cultured cells are transformed cells which might have several mutations in the genome that make them to express aberrant receptors (Barthelson 1998). However, we used normal epithelial cells taken freshly from a healthy adult. Therefore our method might more truly reflect the mechanism which occurs *in vivo*.

The concept of conserved nature of the protein and its immunogenicity get support from our experiments where we showed that antibodies against the 55 kDa protein is generated when we injected the whole cell *M. catarrhalis* into rabbit and also in sputum from patients infected with *M. catarrhalis.* The rabbit antiserum experiment also indicates that 55 kDa protein is indeed a surface protein, otherwise antibodies against this protein would not be generated by immunizing with whole cell *M. catarrhalis.* Fimbriae have been found as an adhesin in different types of bacteria as well as in *M. catarhalis.* The antiserum generated with a nonfimbriate strain also showed reactivity with 55 kDa protein. This indicates that our protein is not a fimbrial protein. Therefore it appears that a new adhesin has been found in this study.

As *M. catarrhalis* is a mucosal pathogen, we were interested to see whether the antibody molecules against 55 kDa produced in sputum during *M. catarrhalis* infection. Although many other studies showed that antibodies against some

putative virulence factors are found in the serum of patients with *M. catarrhalis* infection, it is more important to detect antibody in the sputum since this is a mucosal pathogen. We showed that IgG, IgA and IgM are all produced against 55 kDa protein in sputa of these patients. Therefore whatever approach is taken for immunization, that is systemic or local, antibodies against 55 kDa protein can be induced in human. Various types of antibody have various types of function; therefore different isotypes of antibodies generated in patients against 55 kDa protein might have a role in *M. catarrhalis* infection. Taken this immunogenic potential in both animal and human, 55 kDa protein may be designated as a potential vaccine candidate for *M. catarhalis.*

Several very important points came out from the biochemical characterization of the protein in this study. This is a heat stable protein means its preservation is relatively easy which is an important criterion for a vaccine candidate. The protein was also unchanged after treatment with reducing agents indicates that any disulphide bond between cysteine residues is absent in this protein. This information might be helpful in future, to elucidate the structure function relationship of 55kDa protein.

Certainly the best result we obtained was the BLAST search performed using the *N*-terminal amino acid sequence of 55 kDa protein. The search found no significant homology with the available sequence of proteins, indicating 55 kDa protein is a novel one. Future study may be directed to find out if it is present only in *M. catarrhalis*. This will make a significant contribution to the clinical microbiology laboratories in the identification of *M. catarrhalis* for the benefit of patients.

We could not find homology between the sequence of PCR product and the sequence of *N*-terminal amino acids. The reason is most probably the inability of the sequencer to read the initial 60 bases of the PCR product. Therefore, possibly, *N*-terminal amino acid sequence corresponded to the region that could not be read. The solution to this problem is to clone the PCR product into a TA cloning vector, which enables the cloning of PCR products. Then by using the sequencing primers designed for this vector, we would be able to sequence the gene.

Sequencing all the inserts of pQE-31 vector did not match with the *N*-terminal amino acid sequence either. An interesting finding was that the

sequences of different inserts were not similar except for two of them. This raised the possibility that there were different fragments sharing the same epitope. Or the same gene was cut by *Hind*III in a way that there occurred more than one inserts containing the same epitope i.e. 1kb, 1.5 kb and 3 kb inserts containing the same gene segment. Another explanation is that we got false-positive results. However, this is of low probability because we confirmed the results by dot blot.

We did not search for open reading frame in the sequences of inserts because the sequencer could insert or remove one base or change one base. So if there were such an error it would change the frame completely. Instead we translated the sequence in all reading frames and compared them all with the *N*-terminal amino acid sequence even if there was a stop-codon. In future inserts of all the positive clones can be sequenced up to the end.

Sequencing of positive clones of colony hybridization did not give any homology with the *N*-terminal amino acid sequence and there was no homology with the oligonucleotide probe that was used to screen the library. However, we sequenced only 400 bp of the inserts, which were 750 bp, and 1200 bp in length. In future, the remaining part of the inserts should be sequenced, which might lead us to identify the whole gene of interest.

4.1 Conclusion

In this project a surface protein of *M. catarrhalis* has been isolated and characterized. It is demonstrated by two kinds of attachment inhibition assays that this protein is involved in the attachment process of *M. catarrhalis.* We also found from animal and human study that 55 kDa protein is immunogenic. Therefore, two conclusions can be drawn from this project: First, the protein is an adhesin and it has a therapeutic potential regarding the attachment process. Second, it is a surface protein and it is a potential vaccine candidate.

We used water extraction combined with celite chromatography to isolate the 55 kDa protein. This method is simple and efficient since plenty of protein can be isolated without any contamination. Therefore the method is suitable for mass production for future use.

We successfully constructed genomic libraries therefore in future the libraries can be used in future to identify the gene encoding 55 kDa protein or other gene of *M. catarhalis.*

4.2 Future Perspectives

In the continuation of this project, all the positive clones identified by antibody screening could be sequenced up to the end and checked whether they match with the *N*-terminal sequence. In future, it may also be possible to express 55 kDa gene on the surface of *E. coli* and determine the attachment ability compared to the wild type *E. coli* strain. Since *E. coli* is not a respiratory pathogen, the difference of attachment ability will further prove as to whether 55 kDa protein as an adhesin of *M. catarrhalis.*

In this study, we prepared genomic DNA libraries in pQE-31 and pBluescript SK vector. We confirmed that the library was constructed successfully by sequencing the positive inserts for pQE-31 library i.e. each colony in the library contains a different insert. In the case of pBSK libraries, we checked the quality of the libraries by X-gal (blue-white) selection. The libraries were highly efficient to be used in future to clone any gene of *M. catarrhalis.*

The immunogenic properties of a protein are important when considering it as a vaccine candidate. In this study, we have shown that 55 kDa protein is immunogenic in rabbit and in human. However, four other approaches should be taken in future to find out the significance of antibodies against 55 kDa in human:

1. Whether these antibodies against 55kDa protein decrease during convalescent or later period of infection
2. What other function these antibodies might have? (For example opsonophagocytic potential or complement induction, etc.)
3. Whether these antibodies protective in animal models of infection. However as stated before there is no good animal model for *M. catarrhalis* infection.
4. Whether the 55 kDa protein is conserved among different strains of *M. catarhalis*?

REFERENCES

Aebi C, LaFontaine ER, Cope LD, Latimer JL, Lumbley SL, McCraken GH, Hansen EJ. Phenotypic effect of isogenic uspA1 and uspA2 mutations on *Moraxella catarrhalis* strainO35E. *Infect Immun* 1998: 66; 3113-9

Ahmed K. 1992a. Fimbriae of *Branhamella catarrhalis* as possible mediator of adherence to pharyngeal epithelial cells. *Acta Pathol Microbiol Immunol Scand* 100:1066-1072

Ahmed K, Matsumato K. 1996. Attachment of *Moraxella catarrhalis* to pharyngeal epithelial cells is mediated by glycosphingolipid receptor. *FEMS Microbial. Lett.* 135: 305-309.

Ahmed K, Nagawaka T, Nakano Y, Martinez G, Ichinose A, Zheng CH, Akaki M, Aikawa M, Nagatake T. 2000. Attachment of *Moraxella catarrhalis* occurs to the positively charged domains of pharyngeal epithelial cells. *Microb Pathog.* 28: 203-209

Ahmed K, Rikitomi N, Nagatake T, Matsumoto K. 1990. Electron microscopic observation of *Branhamella catarrhalis*. *Microbiol Immunol*. 34: 967-975

Ahmed K, Rikitomi N and Matsumoto K. 1992. Fimbriation, hemagglutination and adherence properties of fresh clinical isolates of *Branhamella catarrhalis. Microbiol Immunol.* 36:1009-1017

Ahmed K, Wilson S, Jamal WY, Martinez G, Oishi K, Nagatake T and Rotimi VO. 1999. Causative bacteria of respiratory tract infections in Kuwait by quantitative culture of sputum. *J Infect Chemother*. 5:217-219.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1998. Current protocols in molecular biology. Wiley &Sons press.

Bakaletz LO, White GJ, Post JC, Ehrlich GD. 1998. Blinded multiplex PCR analyses of middle ear and nasopharyngeal fluids from chinchilla models of single- and mixed-pathogen-induced otitis media. *Clin Diagn Lab Immunol*. 5: 219-224

Barthelson R, Mobasseri A, Zopf D and Simon P. 1998. Adherence of Streptococcus pneumoniae to respiratory epithelial cells is inhibited by sialylated oligosaccharide. *Infect Immun.* 66: 1439-1444

Bartos, L., C., and T. F. Murphy. 1988. Comparison of outer membrane proteins of 50 strains of Branhamella catarrhalis. *J Infect Dis.* 158:761-5

Braga PC. 2000. Bacterial adhesion to human cells. Scientifica press. Italy, pp6-34

Brorson JE, Axelsson A, Holm SE. 1976. Studies on *Branhamella catarrhalis* with special reference to maxillary sinusitis. *Scand J Infect Dis*. 8:151-155

Bovre K. 1979. Proposal to divide the genus *Moraxella* into two subgenera, subgenus *Moraxella* and subgenus *Branhamella*. *Int J Syst Bacteriol.*29: 403-406

Carter GL, Wilson L, Dunn-Coleman NS. 1994. Molecular characterization of chromosomes by pulsed-field electrophoresis. *Prog Industriel Microbiol.* 29:667-683

Catlin, BW. *Branhamella catarrhalis*: an organism gaining respect as a pathogen. *Clin Microbiol Rev* 1990: 3; 293-320

Chapman, A.J., Jr., Musher, D.M., Jonson, S., Clarridge, J.E., and Wallace, R.J., Jr. 1985. Development of bactericidal antibody during Branhamella Catarrhalis infection. *J. Infect. Dis.* 151: 878-882

Chen D, Barniak V, VanDerMeid KR, McMichael JC. 1999. The levels and Bactericidal Capacity of antibodies directed against the UspA1 and UspA2 outer membrane proteins of *Moraxella catarrhalis* in adults and children. Infe*ct Immun.* 67:1310-1316

Christensen, JJ. 1999. *Branhamella catarrhalis*: clinicalmicrobiological and immunological features in lower respiratory tract infections. *APMIS Suppl* 88:1-36

Cimolai N, Adderley RJ. 1989. *Branhamella catarrhalis* bacteremia in children. *Acta Pediatr. Scand.* 78:465-468

Clavo-Sanchez AZ, Giron-Gonzalez JA, Lopez-Prieto D, Canueto-Quintero J, Sanchez-Porto A, Vergara-Campos A, Marin-Casanova P, Cordoba-Dona JA. 1997. Multivariate analysis of risk factors for infectiondue to penicillin-resistant and multi-drug resistant *Streptococcus pneumoniae*: a multicenter study. *Clin Infect Dis.* 24: 1052-1059

Cope LD, Lafontaine RTSlaughter CA, HasemannJr CA, Aebi C, Henderson FW, Mc CrackenGH Jr, Hansen EJ. 1999. Characterization of the Moraxella catarrhalis UspA1 and UspA2 genes and their encoded products. *J Bacteriol* 181: 4026-4034

Craig DB, Wehrle PA. 1983. *Branhamella catarrhalis* septic arthritis. *J Rheumatol.* 10 :985-986

Del Beccaro MA, Mendelman PM, Inglis AF, Richardson MA, Duncan NO, Clausen CR, Stull TL. 1992. Bacteriology of acute otitis media: a new perspective. *J Pediatr* 120: 81-84

Doern GV. 1990. *Branhamella catarrhalis*: Phenotypic characteristics. *Am J Med.* 88 (suppl 5A):33S-35S

Douer D, Danziger Y, Pinkhas J.1977. *Neisseria catarrhalis* endocarditis. *Ann Intern Med*. 86 :116

Ejlertsen T. 1991. Pharyngeal carriage of *M. catarrhalis* in healthy adults. *Eur. J. Clin. Microbiol. Infect. Dis*. 10:89

Ejlertsen T, Thisted E, Ebbesen F, Olesen B, Renneberg J.1994. *Branhamella catarrhalis* in children and adults. A study of prevalence, time of colonisation and association with upper and lower respiratory tract infections. *J. Infect.* 29: 23-31

Enright, M. C., and H. McKenzie. 1997. *M. catarrhalis*-clinical and molecular aspects of a rediscovered pathogen. *J. Med. Microbiol.* 46:360-371

Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D and Tung Y. 1997. Relationship between nasopharyngealcolonization and development of otitis media in children. *J. Infect Dis.* 175: 1440-1445

Faden H, Harabuchi Y, Hong YY. 1994. Epidemiology of *Moraxella catarrhalis* in children during the first 2 years of life: relationship to otitis media. *J Infect Dis.* 169: 1312-1317

Felmingham D, Washington J. 1999. Trends in antimicrobial susceptibility of bacterial respiratory tract pathogens-findings of the Alexander Project. *J Chemother 11* Suppl 1: 5-21

Frosch P.W.1896.Kolle, Die Mikrokokken, in die Mikroorganism. Vol2 Flugge, Verlag von Vogel, Leipzig. 154-155

Harkness RE, Guimond MJ, McBey BA, Klein MH, Percy DH, Croy BA. 1993. *Branhamella catarrhalis* pathogenesis in SCID and SCID/ beige mice. Brief report. *APMIS* 101:805-810

Helminen ME, Maciver I, Latimer JL, Klesney-Tait J, Cope LD, Paris M, McCracken Jr GH, Hansen EJ. 1994. A large, antigenically conserved protein on the surface of *Moraxella catarrhalis* is a target for protective antibodies. *J Infect Dis*. 170: 867-872

Hendolin PH, Paulin L and Ylikoski J. 2000. Clinically applicable multiplex PCR for four middle ear pathoges. *J Clin Microbiol*. 38:125-132

Hol C, Van Dijke CC, Verhoef J, Fleer A, Van Dijk H. 1995. Complement resistance is a virulence factor for *Branhamella(Moraxella) catarrhalis. FEMS Immunol Med Microbiol.* 11: 207-211

Hoi-Dang AB, Brive-Le Bouguenec C, Barthelemy M, Labia R. 1978. Novel β - lactamase from *Branhamella catarrhalis*. *Ann Microbiol (Paris).* 129B:397-406

Hu W-G, Chen J, McMichael JC and Gu X-X. 2001. Functional characterization of a protective monoclonal antibody against serotype A and C lipooligosaccharides from M. catarrhalis. *Infect Immun* 69: 1358-1363

Johnson, M.A.,Drew, W.L. and Roberts, M. (1981) Branhamella catarrhalis a lower respiratory tract pathogen? *J. Clin. Microbiol.* 13:1066-9

Karalus R, Campagnari A. 2000. *M. Catarrhalis* :a review of an important human mucosal pathogen. *Microb Infect*. 2: 547-559

Kostiala AA, Honkanen T. 1989. *Branhamella catarrhalis* as a cause of acute purulent pericarditis. *J Infect.* 19 : 291-292

Lai E, Birren B. 1995. Use of secondary pulsed field gel electrophoresis in separation of large DNA. *Annal Biochem.*224:68-74

London J, Kolenbrander PE. 1996. Coaggregation: enhancing colonization in a fluctuating environment. In: Molecular and ecological diversity. Fletcher M(Ed.)Wiley-Liss, New York, pp249-279

Lu D, Boyd B and Lingwood CA. 1998. The expression and characterization of a putative adhesin B from *H. influenzae. FEMS Microbiol Letters.* 165:129-137

Martinez G, Ahmed K, Watanabe K, Tao M, and Nagatake T. 1998. Changes in antimicrobial susceptibility to *Moraxella catarrhalis* over a ten year period. *J Infect Chemother.* 4:139-141

Mbaki N, Rikitomi N, Nagatake T and Matsumoto K. 1987. Correlation between *Branhamella catarrhalis* adherence to oropharyngeal epithelial cells and seasonal incidence of lower respiratory tract infections. *Tohoku J Exp Med.* 153:111-121

McMichael JC, Fiske MJ, Fredenburg RA, Chakravarti DN, VanDerMeid KR, Barniak V, Caplan J, Bortell E, Baker S, Arumugham R, Chen D. 1998. Isolation and characterization of two proteins from *M. catarrhalis* that bear a common epitope. *Infect Immun.* 66:4374-4381

Mims CA, Dimmock NJ, Nash A, Stephen J. 1995. Mim's pathogenesis of infectious disease. Academic Press, London, p18

Molstad S, Eliasson I, Hovelius B, Kamme C, Schalen C. 1988. B- lactamase production in the upper respiaratory tract flora in relation to antibiotic consumption: A study in children attending day nurseries. *Scand J Infect Dis.* 20 : 329-334

Murphy TF, Bartos LC. 1989. Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarrhalis*. *Infect Immun.* 57:2938-2941

Murphy, TF. 1990. Studies of outer membrane proteins of *Moraxella catarrhalis*. *Am J. Med.* 88:Suppl5A:41-44

Murphy, TF. 1996. *Branhamella catarrhalis:* epidemiology, surface antigenic structure and immune response. *Microbiol Rev.* 60; 267-79

Murphy TF, Kirkham C, Lesse AJ. 1993. The major heat modifiable outer membrane protein CD is highly conserved amongstrains of *Moraxella catarrhalis*. *Mol Microbiol* 10; 87-97

Nagatake, T. 1985. Clinical significance of respiratory infection caused by Branhamella Catarrhalis with special reference to β-lactamase producing strains. *Tohoku J. Exp. Med*. 147: 1-13

Ndour CT, Ahmed K, Nakagawa T, Nakano Y, Ichinose A, Tarhan G, Aikawa M and Nagatake T. 2001. Modulating effects of mucoregulating drugs on the attachment of *Heamophilus influenzae. Microbial Pathogenesis.* 30:121-127

Oishi K, Tamaka H, Sonoda F, Boramn S, Ahmed K, Utsumomiya Y, Natanabe K, Nagatake T, Vaneechoutte M, Vercshraegen G and Matsumoto K. 1996 . A monoclonal antibody reactive with a common epitope of *Moraxella (Branhamella) catarrhalis* lipopolysaccharides. *Clin Diag Lab Immunol.* 3: 351-354

Omori A, Tsuchihashi Y, Takasugi M, Hirose H, Takahashi A, Watanabe K and Nagatake T. 1999. A nosocomial outbreak of *Moraxella catarrhalis* confirmed by restriction enzyme analysis. *J Jpn Assoc Infect Dis.* 157: 996-1001

Pie Z, Ellison III RT, Lewis RV and Blaser MJ. 1988. Purification and characterization of a family of high molecular weight surface-array proteins from *Campylobacter fetus*. *J Biol Chem.* 263:6416-6420

Prallet B, Lucht F, Alexandre C. 1991. Vertebral osteomyelitis due to *Branhamella catarrhalis* . *Rev Infect Dis.* 13:769

Queiroz JA, Tomaz CT and Cabral JMS. 2001. Hydrophobic interaction chromatography of proteins. *J Biotechnol.* 87: 143-159

Rahman M, Holme T. 1996. Antibody response in rabbits to serotype specific-determinants in lipopolysaccarides from *Moraxella catarrhalis* . *J Med Microbiol*. 44: 348-354

Reddy MS, Bernstein JM, Mutphy TF and Faden HS. 1996. Binding between outer membrane proteins of nontypeable Heamophilus influenzae and human nasopharyngeal mucin. *Infect Immun.* 64:1477-1479

Rikitomi Naoto et al.(1997) Moraxella catarrhalis Adherence to human bronchial and oropharyngeal cells : Role of adherence in Lower respiratory tract infections. *Microbiol. Immunol.* 41(6), 487-94

Rikitomi N, Mbaki N, Nagatake T. 1986 Adherence of *Branhamella catarrhalis* to human pharyngeal epithelial cells: relationship between adherence in vitro and

chronic respiratory tract infection. *Nihon Kyobu Shikkan Gakkai Zasshi*. 24: 633-638

Rotta AT, Asmar BI. 1994. *Moraxella catarrhalis* bacteremia and preseptal cellulitis. *South Med J.* 87:541-542

Sethi S, Hill SL, Murphy TF. 1995. Serum antibodies to outer membrane proteins (OMPs) of *Moraxella (Branhamella) catarrhalis* in patients with bronchiectasis: identification of OMP B1 as an important antigen. *Infect Immun.* 63:1516-1520

Shiimizu A, Koos WE. 1997. Pulse-field electrophoresis of Staphylococcus hyicus and Staphylococcus chromogens genomic DNA and its taxonomic, epidemiologic and ecologic applications in veterinary medicine. *J Vet Med Sci.* 59: 443-450

Tsujimoto, H. Gotoh, N., Nishino, T. 1999. Diffusion of macrolide antibiotics through the outer membrane of *Moraxella catarrhalis*. *J Infect Chemother*. 5; 196-200

Unhanand M, Maciver I, Ramilo O, Arencibia-Mireless O, Argyle JC, McCracken GH, Hansen EJ. 1992. Pulmonary clearance of *Moraxella catarrhalis* in an animal model. *J Infect dis.* 165: 644-650

Verduin CM, Hol C, Fleer A, Dijk H, Belkum A. 2002. *Moraxella catarrhalis*:from emerging to established pathogen. *Clin Micobiol Rev.* 15:125-144

Verghese A, Berk LS. 1990. Introduction: *Branhamella catarrhalis*; a microbiologic and clinical update. *Am J Med.* 88:1S

Wallace Jr RJ, Steingrube VA, Nash DR, Hollis DG, Flanagan C, Brown BA, Labidi A. 1989. BRO beta-lactamases of *Branhamella catarrhalis* and Moraxella subgenus Moraxella, including evidence for chromosomal beta-lactamase transfer by conjugation in *B. catarrhalis, M. nonliquefaciens* and *M. lacunata*. *Antimicrob Agents Chemother.* 33:1845-1854

Wallace RJ, Nash DR, Steingrube VA. 1990. Antibiotic susceptibilities in *Moraxella (Branhamella) catarrhalis*. *Am J Med.* 88: 46S.

Williams RC, Gibbons RJ. 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science.* 177: 697-699

Wilson R, Dowling RB, Jackson AD. 1996. The biology of bacterial colonization and invasion of the respiratory mucosa. *Eur Respir J.* 9:1523-1530

Yoshimine H, Oishi K, Mubiru F, Nalwoga H, Takahashi H, Amano H, Ombasi P, Watanabe K, Joloba M, Aisu T, Ahmed K, Shamada M, Muger WA and Nagatake T. 2001. Community acquired pneumonia in Ugandan adults: short-

term parenteral ampicillin therapy for bacterial pneumonia. *Am J Trop Med Hyg.* 64:171-177

Zakrzewski JL. 2000. The significance of IgA1 protease for infections of upper respiratory tract. *Infect Dis Rev.* 2:211-212

Zalacain R, Sobradillo V, Amilibia J, Barron J, Schotegui V, Pijoan JI, LlorenteJL. 1999. Predisposing factors to bacterial colonization in chronic obstructive pulmonary disease. *Eur Respir J.* 13: 343-348

Zaleski A, Scheffler NK, Densen P, Lee FK, Campagnari AA, Gibson BW, Apicella MA. 2000. Lipooligosaccharide P(k)(Galα1-4Galβ1-4Glc) epitope of *Moraxella catarrhalis* is a factor in resistance to bactericidal activity mediated by normal human serum. *Infect Immun.* 68: 5261-5268

Zheng CH, Ahmed K, Rikitomi N, Martinez G and Nagatake T. 1999. The effects of S-carboxymethylcysteine and N- acetylcysteine on the adherence of *M. catarrhalis* to human pharyngeal epithelial cells. *Microbiol Immunol.* 43:107-113