A STUDY ON

HUMAN ANTIBODY RESPONSE TO *MORAXELLA (BRANHAMELLA) CATARRHALIS* ANTIGENS DURING RESPIRATORY TRACT INFECTIONS

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ABBREVIATIONS

AIDS = Acquired immunodeficiency syndrome

ATCC = American Type Culture Collection

CFU = Colony forming unit(s)

CRP = C-reactive protein

DNA = Deoxyribonucleic acid

DNase = Deoxyribonuclease

EIA = Enzyme immunoassay

ELISA = Enzyme-linked immunosorbent assay

FCS = Fetal calf serum

g = Gram(s)

G+C = Guanine + Cytosine

h = Hour(s)

Ig = Immunoglobulin

LOS = Lipooligosaccharide

LPS = Lipopolysaccharide

µg = Microgram

µl = Microlitre

µm = Micrometre

mg = Milligram

min = Minute(s)

MHC = Major histocompatibility complex

ml = Millilitre

mm = Millimetre

NaCl = Sodium chloride

OD = Optical density

OMP = Outer membrane protein

PBP = Penicillin binding protein

SDS-PAGE = Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM = Standard error of the mean

ABSTRACT

In this study, *M. catarrhalis* was isolated from 68 of 200 (34.0%) sputum samples of patients with community-acquired pneumonia. It was also found in 56 (28.0%) of the nasopharynx of these patients. There were 42 (61.8%) males, and 26 (38.2%) females with pneumonia caused by *M. catarrhalis*. Fifty one of the 68 (75.0%) had chronic underlying diseases. Of the isolates from sputum 37 (54.4%) and 32 (57.1%) of the 56 nasopharyngeal isolates were β-lactamase positive. An ELISA was used to determine the antibody response of sera of patients with pneumonia caused by *M. catarrhalis* to the outer membrane proteins (OMPs) of the bacterium for both IgG-A-M and IgG3 levels in the sera. It was found that 40 of the 68 (58.8%) patients with pneumonia caused by this organism showed significant rise in the total immunoglobulin (IgG-A-M) level, and 43 of 68 (63.2%) in the IgG3 level in their convalescent sera. In the control sera only 1 of 30 (3.3%) patients had significant rise for IgG-A-M while none of them showed significant increase in titre for IgG3 ($P< 0.05$). Using lipopolysaccharide (LPS) antigens, only 16 of 68 (23.5%) paired sera showed significant increase in antibody titre for both IgG-A-M and IgG3 compared to 2 of 30 (6.7%) of controls ($P< 0.05$). Significant bactericidal activity of the convalescent-phase sera at a dilution of 60% was detected in 13 of 15 (86.7%) of the sera assayed. The same sera without complement were used as controls and none of the controls showed significant bactericidal activities ($P< 0.05$).

INTRODUCTION

M. catarrhalis is an aerobic Gram-negative diplococcus about 0.8 μm in diameter. It is a non-motile, non-spore forming, non-pigmented, non-fastidious, piliated organism often found in pairs with adjacent sides flattened. Occasionally, it is found in groups of four as a result of division in two successive planes at right angles to one another. In Gram-stained sputum specimens, it is found extracellularly and sometimes intracellularly (Hager *et al.*, 1987; Fallon and Young, 1989).

M. catarrhalis inhabits the nasopharynx of healthy individuals and is a frequent isolate from sputum specimens. Despite its pathogenic potentials, many microbiology laboratories often report it as "normal flora". A small percentage of healthy individuals acquire respiratory infections caused by *M. catarrhalis* but the majority of patients have chronic lung or systemic pre-existing infections. Respiratory tract infections caused by *M. catarrhalis* are common among both inpatients and outpatients. *M. catarrhalis* is the third commonest cause of acute otitis media and sinusitis in children (Hager *et al.*, 1987; Bourgeois *et al.*, 1993; Klein, 1994), and one of the three leading causes of pneumonia and bronchitis in adults following *Streptococcus pneumoniae* and *Haemophilus influenzae* (Doern, 1990; Sarubi *et al.*, 1990; McKenzie *et al.*, 1992; Seddon *et al.*, 1992). Less frequently *M. catarrhalis* may cause septicemia, keratitis, conjunctivitis, ophthalmia neonatorum, endocarditis, peritonitis, meningitis, urethritis (Naqvi *et al.*, 1988; Gray *et al.*, 1989; Saito *et al.*, 1991).

In mixed pulmonary infections, β-lactamase producing *M. catarrhalis* strains lead to the failure of penicillin treatment for pneumonia caused by *Strep. pneumoniae* (Hol *et al.*, 1994). Thus *M. catarrhalis* induces an indirect pathogenicity (Wardle, 1986).

In the study of virulence factors of *M. catarrhalis*, attention has been drawn to the antigens on the surface such as the outer membrane proteins (OMPs), lipopoly-saccharides (LPS), pili, and β-lactamase enzymes.

I. LITERATURE REVIEW

1.1. HISTORICAL BACKGROUND

M. catarrhalis was originally isolated and described in Germany in 1882 by Seifert. It was named as *"Mikrokokkus" catarrhalis* by R. Pfeiffer and *"Micrococcus" catarrhalis* in English. In 1896, Frosch and Kolle isolated it from sputa of patients with bronchitis and pneumonia (Riou and Guibourdenche, 1986). *M. catarrhalis* was isolated as a cause of respiratory tract infections either singly or combined with "influenza bacilli", staphylococci, pneumococci, streptococci and other organisms. In 1928-30 Sara E. Branham isolated a new *"Neisseria"* species from the cerebrospinal fluids of several individuals during an outbreak of meningitis in the U.S.A. and she called it *Neisseria flavescens* (Berger, 1984). The genus *Branhamella* was named in her honour.

Inspite of the findings of the authors cited above, *M. catarrhalis* was considered to be a commensal of the upper respiratory tract for almost a century. It is only in the last one or two

decades that it gained recognition as a cause of human infections (Collazos *et al.*, 1992).

1.2. TAXONOMY

The first attempt to classify the commensal species of Gram-negative aerobic diplococci occurring in man was made by Von Lingersheim in 1906. He differentiated two genera, *Micrococcus* and *Diplococcus.*

Several methods including serology, transformation experiments, DNA base composition, inter-specific DNA homologies, metabolic activity tests, enzyme profiles, electrophoretic patterns of soluble proteins and fatty acid contents have been used in the elucidation of taxonomic relationships in the genus *"Neisseria"*. Accumulated data from such studies have permitted division of the genus into true and false *Neisseria*. The true *Neisseria* consists of the pathogenic organisms *Neisseria gonorrhoea* and *Neisseria meninigitidis* as well as the non-pathogenic *Neisseria* species which have been designated as *N. canis, N. cinerea, N. cuniculi, N. lactamica, N. flava, N. perflava, N. subflava, N. sicca,* and *N. mucosa* (Johnson *et al.*, 1975). *N. cinerea* was misidentified as *Neisseria catarrhalis* because nitrate reduction was not used as a differential test for the classification of *Neisseria* species until 1961 (Knapp and Hook, 1988). The atypical *Neisseria* species consisted of *N. caviae, N. catarrhalis,* and *N. ovis. N. catarrhalis* has been redesignated as the sole representative of the newly created genus *Branhamella,* and has been assigned the name *Moraxella catarrhalis* (Murphy and Loeb, 1989). The two medically important genera of aerobic Gram-negative diplococci are *Neisseria* and *Branhamella. N. meningitidis* and *N. gonorrhoea* are important pathogens in the genus

Neisseria, and *Branhamella* is the only important member of the second genus. Both genera differ in their guanine plus cytosine (G+C) contents of the DNA which in *Neisseria* is higher than that of *M. catarrhalis*. They also differ on the basis of biochemical reactions. Enzymes like carbonic anhydrase, aspartase and some glycolytic enzymes are found in *Neisseria* species but not in *Branhamella.* Fatty acid composition of the cells was also used to distinguish the two genera (Berger, 1984). Studies on lipopolysaccharides and cell envelope proteins (Johnson *et al.*, 1975) also indicated differences in cellular compositions between species of *Neisseria* and *M. catarrhalis* (Eliasson, 1986).

1.3. NOMENCLATURE

In the early 1900's *Moraxella catarrhalis* was referred to as *Micrococcus catarrhalis.* However, because of its morphologic similarity to the *Neisseria* species, and its oxidase activity, it was renamed *Neisseria catarrhalis* in the 1960's. In 1970, based on analysis of G+C content, and genetic transformation experiments, Catlin proposed the genus *Branhamella* with a single species, *Branhamella catarrhalis* (Van Hare *et al.*, 1987). Then *Moraxella catarrhalis* was accepted in the 1974 edition of Bergey's Manual. As currently defined by Bergey's Manual of Systematic Bacteriology, the family *Neisseriaceae* contains the genera *Neisseria, Moraxella, Acinetobacter* and *Kingella* (Riou and Guibourdenche, 1986) and *Branhamella* (Ahmad *et al.*, 1987). In 1979, this organism was placed into the genus *Moraxella (Branhamella) catarrhalis* (Musher, 1994) and currently the nomenclature *Moraxella catarrhalis* is used (Bruckener and Colonna, 1995).

1.4. GENERAL CHARACTERISTICS OF *MORAXELLA CATARRHALIS*

1.4.1. PHYSICAL CHARACTERISTICS, STRUCTURE AND COMPOSITION OF *MORAXELLA CATARRHALIS*

M. catarrhalis consists of capsule, pili, cell wall, outer membrane and organelles similar to other Gram-negative bacteria.

1.4.1.1. CAPSULE

The surface of *M. catarrhalis* is composed of capsular material, proteins, lipopolysaccharides and phospholipids (Murphy, 1989; Jonsson *et al.*, 1994). The capsule is visible by electron microscopy in ultrathin sections of *M. catarrhalis* stained with magnesium uranyl acetate and lead citrate as a fuzzy or fibrillar coat. With ruthenium red staining, the polysaccharide nature of this fibrillar coat is seen as spicule-like material projecting from the outer membrane (Prost and Scavizzi, 1993).

1.4.1.2. PILI (FIMBRIAE)

M. catarrhalis strains have pili. The ordinary pili of *M. catarrhalis* which produce colonization antigens, serve as adhesins which enable the bacteria to bind to mucosal epithelial cells (Marrs and Weir, 1990; Owen *et al.*, 1991; McWilliam *et al.*, 1994).

Examination of *M. catarrhalis* strain by the electron microscopy revealed pili similar to type

4 (Ahmad *et al.*, 1987). Type 4 pili are present on various pathogenic bacteria such as *N. meningitidis, N. gonorrhoea, Vibrio cholerae* and *Pseudomonas aeruginosa. M. catarrhalis* has several of the phenotypic characteristics that are related to the presence of type 4 pili in other closely related *Moraxella* species including competence for DNA transformation, pellicle formation, autoagglutination, colony morphology and pitting of agar.

The degree of fimbriation of *M. catarrhalis* ranges from sparsely to densely fimbriated and these fimbriae are expressed during infections (Ahmed *et al.*, 1992).

1.4.1.3. THE CELL WALL

The cell wall of *M. catarrhalis* resembles that of *N. gonorrhoea.* It consists of an outer membrane located outside the peptidoglycan layer, two trilaminar structures between which lie a periplasmic space and a peptidoglycan layer. Nearly half of the outer membrane of Gram-negative bacteria is protein. The outer membrane allows the influx of nutrients and efflux of waste products (Nikoido and Vaara, 1985), resists lysozyme and leucocyte proteins, serves as a barrier to bile salts, digestive enzymes, slows down the diffusion of antibiotics into the cell and endows the bacterial surface with a strong hydrophobicity, which is important in evading phagocytosis, complement component resistance, and the capacity to avoid a specific immune attack by altering the constitution of surface antigens. These surface antigens, as well as the highly impermeable membrane layer, contain LPS, a characteristic component of the outer membrane (Nikoido and Vaara, 1985).

1.4.1.4. THE OUTER MEMBRANE PROTEINS (OMPs)

The protein composition of the outer membrane of *M. catarrhalis* is typical of that of other Gram-negative bacteria. Outer membrane proteins (OMPs) are released spontaneously as free endotoxin during growth. Blebs function as a packaging system for the distribution of endotoxin. The toxicity of the blebs is due to lipid A, the glycolipid component of LOS anchored in the outer membrane. In the laboratory, blebs are obtained by centrifugation of broth culture (Murphy and Loeb, 1989). Approximately 10-20 proteins are isolated with 8 major OMPs (A through H, with molecular weights of 98,000-21,000) predominating (Bartos and Murphy, 1988; Ejlertsen *et al.*, 1994; Fung *et al.*, 1994). Most Gram-negative bacteria contain one or more heat-modifiable proteins in their outer membrane (Berk, 1990). The outer membrane proteins of *M. catarrhalis* are antigenically conserved and homologous (Murphy, 1989). The OMPs are considered virulence determinants and are thought to facilitate infection (Owen *et al.*, 1991). Surface exposed epitope proteins of *M. catarrhalis* are expressed on its surface and have antigenic determinants responsible for immune responses to infection by this bacterium (Bhushan *et al.*, 1994; Klingman and Murphy, 1994; Sethi *et al.*, 1995). Thus, the OMP is a potent surface exposed vaccine candidate antigen and useful in the serological studies of *M. catarrhalis* (Doern, 1990; Murphy *et al.*, 1993; Christensen *et al.*, 1995).

1.4.1.5. THE LIPOPOLYSACCHARIDE

The Lipopolysaccharides (LPS) of *M. catarrhalis* consists of a short chain oligosaccharide attached to lipid A, but lacks the extended antigenic side chains consisting of repeating units

containing the O antigen which is found in enteric pathogens (Masoud *et al.*, 1994; Edebrink *et al.*, 1995). The absence of the O antigen increases the permeability of the outer membrane, resulting in enhanced susceptibility to hydrophobic agents. The LPS is similar in general structure to that of *Neisseria, Haemophilus,* and *Bordetella* species (Murphy and Bartos, 1989). The LPSs have apparent molecular weights of about 5500 as estimated by SDS-PAGE (Vaneechoutte *et al.*, 1990a). The LPS of *M. catarrhalis* is unique to this organism. Like the OMP, it is conserved, antigenically homogeneous, with only minor epitope differences observed between strains and does not vary (Doern, 1990).

Like most *Neisseria* species, *M. catarrhalis* secretes vesicles that modulate or deflect the immune system. These vesicles are thought to be important as virulence factors in infections with *M. catarrhalis* (Edebrink *et al.*, 1994). LPSs also help the bacteria to survive on the mucosa, and to attach to host cells.

1.4.1.6. THE CYTOPLASMIC MEMBRANE

The cytoplasmic membrane of *M. catarrhalis* contains penicillin binding proteins (PBPs) at its outer face. PBPs 1,2,3 have molecular weights of 77,000, 68,000, and 39,000 respectively. PBP-3 possesses a weak β-lactamase activity (Prost and Scavizzi, 1993).

1.4.1.7. THE DNA

The genome of *M. catarrhalis* contains approximately 2.3×10^6 nucleotide pairs. They encode

several thousand gene products (Prost and Scavizzi, 1993). It shows a low degree of DNA relatedness to *Neisseria* species which is shown by DNA-DNA hybridization. The DNA base composition (% G+C) of *M. catarrhalis* is 40-42 mole%, *N. gonorrhoea,* 48-50 mole%, *N. meningitidis,* 50-52 mole% (Duguid *et al.,* 1978), *N. cinerea,* 49 mole%, *N. caviae,* 46.5-47.5 mole% and *N. flavescens,* 44.5 mole% (Catlin, 1990).

1.4.1.8. FATTY ACID COMPOSITION

The fatty acid profile of the family *Neisseriaceae* has been determined, but caution is needed in comparing the results. The fatty acid content of *M. catarrhalis* is affected by the culture medium components and incubation temperature (Prost and Scavizzi, 1993).

1.4.2. NUTRITION AND GROWTH REQUIREMENTS

M. catarrhalis strains are able to utilize carbon sources with 16 to 95 carbon atoms (Christensen *et al.,* 1994). In addition, all strains of *M. catarrhalis* have unique requirements for arginine. *M. catarrhalis* hydrolyses the fat glycerol tributyrate and produces butyric acid. It fails to utilize sugars.

1.4.3. SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

M. catarrhalis appears to be more resistant to exposure to the environment than meningococci or gonococci. They survive for at least 3-4 weeks in sputum (Fallon and Young, 1989). They

were detected in settled culture media placed in wards where infections occur. In culture they remain viable for months at 20°C if prevented from drying (Catlin, 1990). For these reasons, they have been identified as causative agents of nosocomial infections.

1.4.4. BETA-LACTAMASE PRODUCTION AND DRUG RESISTANCE

The β-lactamases of Gram-negative bacteria are located in the periplasmic space. The enzymes may prolong infection by inactivating antibiotics or by maintaining β-lactamase producing *M. catarrhalis* strains after antibiotic sensitive strains have been destroyed.

β-Lactamase in *M. catarrhalis* was first reported in 1977 in France (Davies and Maesen, 1986; Wallace *et al.*, 1990; Fung *et al.*, 1994). The incidence of β-lactamase production by strains of *M. catarrhalis* is over 50% (Mannion, 1987; Deguchi *et al.*, 1995). β-Lactamases of *M. catarrhalis* are divided into three phenotypically related types by isoelectric focusing. The enzyme, BRO-1, named by Eliasson and Kamme (1985), is the most common β-lactamase in *M. catarrhalis* found in about 90% of the enzyme producers (Christensen *et al.*, 1990). A rarer type found in the remaining 10% is the BRO-2. Recently, a third type, BRO-3 has also been identified (Christensen *et al.*, 1991). The BRO enzymes are broad-spectrum in their activities hydrolysing penicillin, ampicillin, methicillin and other β-lactam antibiotics (Ahmad *et al.*, 1985). Isolates with BRO-1 pattern are more consistently resistant to ampicillin than those with BRO-2 pattern; correlating with the finding that the former enzyme is produced at double or triple the level of the latter (Fung *et al.*, 1994; Yeo and Livermore, 1994). The production of β-lactamase by *M. catarrhalis* is mediated by chromosomal determinants. Recently,

however, it has been shown that a plasmid-mediated enzyme does occur. Transfer of β-lactamase plasmids between strains of *Moraxella* as well as in *M. catarrhalis* has been reported. Rare isolates resistant to erythromycin, tetracyclines, chloramphenicol, or co-trimoxazole have been reported but little is known about the mechanisms of resistance (Jacoby, 1993). The presence of β-lactamses elaborated by various strains of *M. catarrhalis* can be detected by chromogenic, iodometric, acidimetric, and microbiological methods (Jones and Sommer, 1987). The chromogenic cephalosporin method detects most known β-lactamases.

1.4.5. IMMUNOLOGICAL RESPONSE TO *MORAXELLA CATARRHALIS*

The four subclasses of IgG in man differ in the primary sequence of the constant region of their heavy chains. They differ with respect to their biological properties, including the ability to activate effector mechanisms. The normal value of total IgG constitutes about 80% of all immunoglobulins in serum (Brooks *et al.*, 1991).

In mice actively immunized with OMPs of *M. catarrhalis* and passively with immune sera, serum antibody was raised and was able to clear *M. catarrhalis* strains from the lung (Helminen *et al.*, 1994). Elevated antibody titre have been detected by ELISA in convalescent-phase sera of patients with pulmonary infections (Chapman *et al.*, 1985; Black and Wilson, 1988) and otitis media and sinusitis (Leinonen *et al.*, 1981). Chi *et al.* (1990) detected a rise in the level of total IgG antibody to an outer membrane P-protein in 50% of adults with tracheo-bronchitis and pneumonia. Eliasson (1980) found a heat-stable P-antigen located on

the surface of *M. catarrhalis* which increased antibody titres in patient sera. Helminen *et al.* (1995) using whole bacterial cells as antigens observed a rise in EIA titre of convalescent-phase sera. IgG antibodies against the OMP were found more frequently in patient specimens with lower respiratory infections caused by *M. catarrhalis* (71%) than in patients without *M. catarrhalis* in lower respiratory tract specimens (28%) or healthy adult blood donors (22%). Jonsson *et al.* (1994) observed increases in antibody titre in 11 of 23 (47.8%) patients with lower respiratory tract infections caused by *M. catarrhalis* using whole bacterial cells in EIA as antigen.

Various factors may affect IgG subclass production. These include age of an individual, route of immunization, atopy, and previous exposure to antigen. Different types of antigens also lead to responses in which different IgG subclasses predominate. One factor is the ability of the antigen to recruit T-cell help. Thus T-cell independent antigens, (carbohydrate antigens and bacterial LPS) tend to induce IgG2 antibodies, whereas antibodies to T-cell dependent protein antigens are frequently IgG1, IgG3 (Madassery *et al.*, 1988) and IgG4 components (Falconer *et al.*, 1993). Pokeweed mitogen stimulates peripheral blood mononuclear cells to produce IgG1, IgG2, IgG3, IgG4. In addition to these, cytokines induce IgG subclasses. Among the inducing cytokines are IL-2, IL-4, IL-6, and IFN-γ *In vitro*, *M. catarrhalis* is a B cell mitogen and appears to stimulate an IgG3 response (Kawano *et al.*, 1995).

The IgG subclass specific response to infection is important since certain antigens induce a subclass restricted response (Yount *et al.*, 1968). Specific IgG1 and IgG2 begin to rise early in childhood below the age of 2 years, whereas specific IgG3 antibodies are detected above

the age of 4 years (Goldblatt *et al.,* 1990b). IgG3 concentrations are higher in adolescents and adult females than age matched males (Lee *et al.*, 1986). It appears early in ontogeny and primarily activates the complement cascade through the classical pathway, initiates opsonization, and enhances phagocytosis. A marked increase of IgG3 has been reported in responses to many bacterial infections, viral proteins and red cell antigens. On the other hand, deficiency of IgG3 is associated with increased susceptibility to recurrent respiratory tract infections, bronchial asthma, and diabetes mellitus. IgG3 but not IgG4 antibodies recognized the majority of outer membrane antigens of *M. catarrhalis* (Goldblatt *et al.,* 1990a; Carson *et al.*, 1994). In the sera of 160 patients with chronic and recurrent sinusitis, there was a highly significant correlation between the level of specific anti-*M. catarrhalis* IgG3 antibody and certain Gm allotypes (Goldblatt *et al.*, 1994). IgG4 tends to be associated with secondary responses to injected proteins.

For the investigations of bactericidal activities, serum from humans and animals are used as complement sources (Taylor, 1983). Various methods are employed in investigating antibody responses to *M. catarrhalis* infections (Chi *et al.,* 1990). In a study of the bactericidal effect of sera on *M. catarrhalis*, Chapman *et al.* (1985) found that 32% of acute-phase sera and 90% of convalescent-phase sera of patients with tracheo-bronchitis and pneumonia showed increased bactericidal activities against *M. catarrhalis.*

1.5. CARRIER STATE OF *MORAXELLA CATARRHALIS*

Adherence of *M. catarrhalis* to the epithelial cells of the oropharynx is mediated by lectins

located on the bacterial surface (Kellens *et al.*, 1995). Oropharyngeal colonization by *M. catarrhalis* starts early in childhood and is common in the upper respiratory tract of healthy children (Korppi *et al.*, 1992; Claesson and Leinonen, 1994). The carrier rate of *M. catarrhalis* is higher during respiratory infections than in healthy states (Lundegren and Ingvarsson, 1986). In healthy subjects it was isolated from 5.4% of adults, 50.8% of children, and 26.5% of elderly individuals above 60 years of age (Vaneechoutte *et al.*, 1990b). Bergogne-Berezin *et al.* (1994) isolated *M. catarrhalis* from the nasopharynx of 20% of 645 relatively healthy subjects. Ringertz *et al.* (1993) studied the pathogens of acute respiratory tract infection pathogens in children under 5 years in Ethiopia. They found the nasopharyngeal carrier rate of *M. catarrhalis* in these children was 83%. They also reported that out of the *M. catarrhalis* isolates 47% and 58% in two different rural communities and 69% in Addis Ababa City were positive for β-lactamase.

1.6. COMMON DISEASES CAUSED BY *MORAXELLA CATARRHALIS*

M. catarrhalis is predominantly a mucosal pathogen. It is primarily an opportunistic lung pathogen occurring mainly in patients with impaired pulmonary and systemic defence mechanisms including AIDS. *M. catarrhalis* was regarded as a commensal of the oropharynx and its presence was ignored in pulmonary secretions of patients with chronic obstructive pulmonary diseases, (COPD) (Wallace and Musher, 1986). It causes community and hospital-acquired infections.

1.6.1. PNEUMONIA

Pneumonia, community-acquired or nosocomial, is far more important in the elderly than in any other age group. It is common above the age of 60 years with a peak incidence during the seventh decade of life (Catlin, 1990; Wright *et al.*, 1990). The factors that determine the increased frequency of pneumonia in the elderly include social effects, immune changes, physiological changes in the lungs and increased frequencies of underlying chronic pulmonary diseases (Woodhead, 1994). Most patients with *M. catarrhalis* pneumonia have chronic lung diseases, lung cancer or are undergoing corticosteroid therapy (McLeod *et al.*, 1983). At the present time, pneumonia in the immunocompromised host is also an increasing problem in all age groups. Bacteraemic pneumonia caused by *M. catarrhalis* in neutropenic patients (Roson *et al.*, 1994) and in patients with immunoglobulin deficiencies has been reported (Diamond and Lober, 1984; Karnad *et al.*, 1986). On the other hand blood cultures of patients with pneumonia caused by *M. catarrhalis* show a lower rate of bacteraemia in immuocompetent individuals (Lode *et al.*, 1993).

The four most common pathogens causing community-acquired pneumonia include *Strep. pneumoniae, H. influenzae, M. catarrhalis* and *Staph. aureus* (Vogel, 1993). The isolation rate of *M. catarrhalis* from sputa of patients with pneumonia and other respiratory tract infections ranges from 2 to 26% world wide (Van Hare *et al.*, 1987; Wallace and Oldfield, 1989). Brook (1986) isolated *M. catarrhalis* in 6 of 27 (22%) pneumonia patients. Maesen and Davies (1986) isolated it in 23% of cases, and Saito *et al.* (1986) in 29 of 71 (40.8%) cases from sputum of patients with respiratory tract infections. Nicolas *et al.* (1991) in 7 of 40 (17.5%)

sputum samples in patients with pneumonia. In W. Africa, Lagos, *M. catarrhalis* and commensal *Neisseria* species were isolated in 200 of 500 (40%) sputum samples from patients with lower respiratory tract infections. *M. catarrhalis* was isolated from 60 (12%) sputum samples. *M. catarrhalis* occurred in pure cultures in 15 (25%) of the 60 samples positive for this organism (Obi *et al.*, 1990). Hafiz *et al.* (1994) isolated *M. catarrhalis* from sputum of patients with pneumonia in 22.4% of cases.

1.6.2. ACUTE EXACERBATIONS OF CHRONIC BRONCHITIS

Chronic lung diseases are the major risk factors for *M. catarrhalis* respiratory tract infections. *M. catarrhalis* is recognized as one of the major causative agents of bronchitis (Takasugi, 1994) and pneumonia in patients with chronic pulmonary diseases (Murphy and Sethi, 1992). *H. influenzae*, and *Strep. pneumoniae* are also common in exacerbation of chronic pulmonary diseases (Rosen *et al.*, 1992; Davies, 1994) with *H. influenzae* causing more than half of all bacterial exacerbations of chronic bronchitis and *Strep. pneumoniae* and *M. catarrhalis* accounting for a further third (Ball *et al.*, 1995).

1.6.3. OTITIS MEDIA AND MAXILLARY SINUSITIS

Otitis media and sinusitis are among the most common diseases of childhood (Bluestone, 1986). The three most common causes of otitis media and sinusitis are *Strep. pneumoniae, H. influenzae*, and *M. catarrhalis*. There is a close correlation between nasopharyngeal colonization and pathogens isolated from otitis media fluid (Mbaki *et al.*, 1987; Faden *et al.*,

1992; Klein, 1994; Leach *et al.*, 1994). The incidence of *M. catarrhalis* in middle ear fluids has been reported to be 10-27% (Murphy, 1989). Restriction endonuclease analysis (REA) has shown that *M. catarrhalis* strains causing otitis media were similar to those found in the nasopharynx of children who develop the disease (Dickinson *et al.*, 1988).

Acute sinusitis is a common complication of upper respiratory tract infections in children. *M. catarrhalis* causes about 19% of the bacterial sinusitis (Bluestone, 1986).

1.6.4. OTHER DISEASES

Less common diseases caused by *M. catarrhalis* include meningitis, endocarditis, conjunctivitis, ophthalmia neonatorum, urethritis, tracheitis, laryngitis, wound infections, and tonsillitis. Rarely it may cause septicemia in immunocompetent individuals (Guthrie *et al.*, 1988). Infections associated with human and animal bites have also been reported (Brook, 1986).

1.7. CLINICAL FEATURES OF COMMON DISEASES CAUSED BY *MORAXELLA CATARRHALIS*

Pneumonia caused by *M. catarrhalis* is usually milder than that caused by other pathogens except in immunodeficient patients (Le Faou and Rio, 1983). An indication of its mildness is that *M. catarrhalis* is not usually found in blood cultures (Nicotra *et al.*, 1986; Catlin, 1990; Jordan and Berk, 1990; Wright *et al.*, 1990). Typical features of pneumonia caused by *M.*

catarrhalis include cough, fever, production of a moderate amount of sputum, dyspnea, and tachypnea (McLeod *et al.*, 1986). Physical examinations reveal consolidation of the lung with crepitations on auscultation. Leucocytosis is reported in most patients. The chest radiograph is considered the "gold standard" for the diagnosis of pneumonia (Marrie, 1994) which is shown by pulmonary infiltrations.

The clinical features of acute otitis media include fever, otalgia, otorrhea, and irritability. The tympanic membrane becomes opaque and the ear drum erythematous. Except for a low serum C-reactive protein (CRP) level, the clinical presentation, leucocyte count, and clinical course of otitis media caused by *M. catarrhalis,* is indistinguishable from that caused by *Strep. pneumoniae* and *H. influenzae* (Marchant, 1990).

The presenting features of acute sinusitis include cough, fever, and purulent nasal discharge (Goldblatt *et al.*, 1990a; Giebink, 1992).

1.8. PREDISPOSING FACTORS TO INFECTIONS

M. catarrhalis causes significant lower respiratory tract diseases in patients with antecedent compromised lung functions including emphysema, asthma, Goodpasture's syndrome, anthrasilicosis, bronchiectasis, chronic bronchitis, malignancies, immunosuppression, alcoholism, pneumoconiosis, and viral infections of the respiratory tract (Doern and Miller, 1981; Leedom, 1992; Abbasi *et al.*, 1994). Most patients with pneumonia caused by *M. catarrhalis* are elderly and malnourished (Musher, 1994). Advanced age above 60 years

appears to be a factor in *M. catarrhalis* infections, possibly as a result of the reductions of IgG and IgM (Catlin, 1990). Patients with chronic neurologic diseases and alcoholism are prone to aspiration of oropharyngeal secretions and acquire pneumonia (Ioannidis *et al.*, 1995). Patients with tuberculosis are commonly infected by *Strep. pneumoniae, H. influenzae,* and *M. catarrhalis* (Shishido *et al.*, 1990). Preceding viral infections of the respiratory tract increase the risk of both otitis media and pulmonary infections by altering eustachian tube function and host defences. These factors enable nasopharyngeal bacteria to ascend the eustachian tube and cause middle ear infection (Marchant, 1990). Damage to the respiratory tract by respiratory syncytial virus and influenza virus infection may promote invasion by *M. catarrhalis* (Alligood and Kenny, 1989). Boyle *et al.* (1991) found that *M. catarrhalis* isolates from patients with underlying predisposing factors were twice as likely to have pathogenic significance (51%) as those obtained from patients without such a predisposition (24%).

1.9. LABORATORY DIAGNOSIS OF *MORAXELLA CATARRHALIS*

1.9.1. BACTERIOLOGICAL DIAGNOSIS

A precise diagnosis of the microbial etiology of a disease is important because it guides the choice of antibiotics for specific therapy, excludes the need to investigate other causes of the disease, and contributes to improved survival of the patient.

Evaluation of sputum by Gram-stain has become mandatory in microbiology laboratories. This technique now allows the recognition of cases in which *M. catarrhalis* is serving as a potential pathogen (Kalin *et al.*, 1983; Wallace and Musher, 1986).

1.9.1.1. MORPHOLOGY AND STAINING

Expectorated sputum examination is a useful non-invasive approach provided respiratory secretions, and not saliva, are obtained. It is a reliable guide to direct initial antibiotic therapy in patients with community-acquired pneumonia (Thornley *et al.*, 1982; Glecokman *et al.*, 1988; Murphy, 1990). Sputum samples with numerous PMN and low numbers of squamous epithelial cells represent true lower respiratory tract bacterial infections (Pellegrino *et al.*, 1992). When the Gram-stain is made from such sputum specimens and shows numerous Gram-negative diplococci, *M. catarrhalis* will be identified in nearly 90% of the cases (Pollard *et al.*, 1986; Ainsworth *et al.*, 1990).

1.9.1.2. CULTURAL CHARACTERISTICS AND GROWTH OF *MORAXELLA CATARRHALIS*

Colonial morphology is very important in the primary identification of *M. catarrhalis*. *M. catarrhalis* grows on blood and chocolate agar 35-37°C in air and 10% CO_2 (Doern and Morse, 1980; Berger, 1984). Unlike the pathogenic *Neisseria* species, it also grows on nutrient agar at 22°C in air. Colonies on 5% sheep blood agar are non-haemolytic, non-pigmented (Table 1), circular, opaque, convex, greyish-white with a size of 1.5-2.0 mm diameter after 18-24 h of incubation at 37°C. Colonies are coherent, remain intact and slide across the agar when pushed with a wire loop. A well processed good quality sputum specimen yields *M. catarrhalis* in nearly pure culture (Wallace and Musher, 1986).

TABLE 1. Main morphological, bacteriological and growth characteristics helpful for the identification of *M. catarrhalis* (Berger, 1984; Riou & Guibourdenche, 1986)

Bacterial species	Morphology	Haemolysis on HBA	Pigment formation	Temp(°C) required
M. catarrhalis	Cocci	-	-	22,37
N. caviae	Cocci	-	-	22,37
N. ovis	Cocci	+	-	22,37
N. cuniculi	Cocci	-	-	22,37
N. flavescens	Cocci	-	Yellow	22,37
N. subflava	Cocci	-	Yellow	22,37
N. perflava	**Cocci**	**+**	**Yellow**	**22,37**
N. mucosa	Cocci	-	Yellow	22,37
N. lactamica	Cocci	-	Pale yellow	37
N. sicca	Cocci	V	Pale yellow	37
N. cinerea	Cocci	-	Pale yellow	37
N. canis	Cocci	-	Pale yellow	37
N. elongata	Rods	-	Pale yellow	37
N. gonorrhoea	Cocci	-	-	37
N. meningitidis	Cocci	-	-	37

Note. HBA = Horse blood agar; Temp(°C) = Temperature in degree Celsius; + = Positive; - = Negative; V = Variable.

Colony morphology and the oxidase test are useful to identify *M. catarrhalis* with a specificity of 95-99% (Jonsson *et al.*, 1990). *M. catarrhalis* fails to grow on MacConkey, crystal violet blood, modified Thayer-Martin (MTM) agar, and modified New York City (MNYC) medium. Since many individuals are colonized with different species of *Neisseria* (Knapp and Hook, 1988), the growth of *M. catarrhalis* and inhibition of other bacterial is best obtained on selective media containing the antibiotics - vancomycin (10 µg/ml), amphotericin B (2 µg/ml), trimethoprim (5 µg/ml), and acetazolamide (10 µg/ml). Incubation of cultures on this medium in air reduces the growth of *Neisseria* species because of the inhibition of the activity of carbonic anhydrase by *Neisseria* species but not by *M. catarrhalis*. Carbonic anhydrase catalyses the hydration of dissolved CO_2, as in red cells to form HCO^-_3 ions (Vaneechoutte

et al., 1988b; Nafi *et al.*, 1990). In respiratory specimens, *M. catarrhalis* may be present in pure culture in 50-61% of cases or may be co-cultured along with other bacteria including *H. influenzae, Strep. pneumoniae* or Gram-negative bacilli (Saito *et al.*, 1986; Sarubi *et al.*, 1990; Collazos *et al.*, 1992).

1.9.2. BIOCHEMICAL IDENTIFICATION OF *MORAXELLA CATARRHALIS*

Biochemical test are useful in differentiating *M. catarrhalis* from *Neisseria* species which are similar in morphology. *M. catarrhalis* is asaccharolytic failing to produce acid from various carbohydrates including glucose, lactose, maltose and sucrose in serum free broths containing indicators such as phenol-red and bromothymol blue (Doern and Morse, 1980; Christensen *et al.*, 1986; Richards, 1988). All strains of *M. catarrhalis* produce oxidase, an enzyme that catalyses the transport of electrons between electron donors in the bacteria and a redox dye tetramethyl-*p*-phenylene-diamine in the test solution. The dye is reduced to a deep purple colour when an enzyme producing bacterial colony is applied onto a filter paper wet with oxidase reagent. *M. catarrhalis* also produces catalase, an enzyme which catalyses the release of oxygen from hydrogen peroxide (H_2O_2). Production of gas bubbles from the colonies of *M. catarrhalis* after an application of 3% or 30% H_2O_2 indicates a positive reaction (Ahmad *et al.*, 1987; Fallon and Young, 1989). All strains of *M. catarrhalis* reduce nitrate to nitrite [Table 3]. A positive result is revealed by the development of a red colour within 1-2 min after the application of the reagent to bacterial growth in broth. The DNase test is highly specific in differentiating *M. catarrhalis* from the *Neisseria* species (Ahmad *et al.*, 1987; Brooks *et al.*, 1991).

TABLE 2. Biochemical reactions which differentiate *M. catarrhalis* from some *Neisseria* species (Christensen *et al.*, 1986; Richards, 1988; Catlin, 1990)

Bacterial spp.	Acid formation from				Oxidase	Catalase	Nitrate	DNase	Tribut Hydrol
	Glu	Mal	Lac	Suc					
M. catarrhalis	-	-	-	-	+	+	+	+	+
N. gonorrhoea	+	-	-	-	+	+	-	-	-
N. meningitidis	+	+	-	-	+	-/+	-	-	-
N. cinerea	-	-	-	-	+	+	-	-	-
N. flavescens	-	-	-	-	+	+	-	-	-
N. sicca	+	+	-	+	+	+	+	-	-
N. lactamica	+	+	+	-	+	+	-	-	-
N. mucosa	+	+	-	+	+	+	+	-	-
N. subflava	+	+	-	V	+	+	-	-	-
N. cuniculi	-	-	-	-	+	+	-	-	-
N. ovis	-	-	-	-	+	+	+	-	-
N. canis	-	-	-	-	+	+	+	-	-
N. elongata	-	-	-	-	+	+	-	-	-

Note. Spp. = Species; Glu = Glucose; Mal = Maltose; Suc = Sucrose; Tribut. Hydrol. = Tributyrin hydrolysis;

- = Negative; + = Positive reactions; -/+ = Most strains negative; V = Variable.

It is produced by *M. catarrhalis* but not by most of the *Neisseria* species except *N. caviae*. *M. catarrhalis* and *N. caviae* are differentiated on the basis of their DNA-base compositions (Soto-Hernandez *et al.*, 1988). *M. catarrhalis* can be distinguished from *Neisseria* species by its ability to hydrolyse glycerol tributyrate to butyric acid, by the enzyme butyrate esterase which is not found in most *Neisseria* species (Vaneechoutte *et al.*, 1988a; Perez *et al.*, 1990).

1.9.3. STRAIN DIFFERENTIATION AND SEROLOGICAL IDENTIFICATION

1.9.3.1. SEROLOGICAL IDENTIFICATION OF ISOLATES

The serological methods have better increased possibility of diagnosing infections caused by *M. catarrhalis* than isolation alone (Burman *et al.*, 1994). Serological tests involving agglutination methods are unsuccessful because of the notorious spontaneous autoagglutination of *M. catarrhalis* in 0.85% NaCl solution (Eliasson, 1986).

Antibody cross-reactions between serotypes of *M. catarrhalis* have been reported indicating the presence of common epitopes among different serotypes (Vaneechoutte *et al.*, 1990a). Weak cross-reactions between *N. gonorrhoea* and *M. catarrhalis* were demonstrated using the complement fixation test. Jonsson *et al.* (1992) found an antigenic similarity among different strains of *M. catarrhalis* using an immunofluorescence test. *M. catarrhalis* antigens were species-specific and no cross-reactions with *Strep. pneumoniae*, and *H. influenzae* were detected by ELISA (Jonsson *et al.*, 1993). Serotyping of *M. catarrhalis* using LPS as antigen was reported in few studies. It was reported that 3 LPS serotypes were identified and 1 strain

was non-typeable. These included type A (60%), type B (30%) and type C (5%) accounting for 95% of the isolates. The remaining 5% were nontypeable (Vaneechoutte *et al.*, 1990a).

1.9.3.2. DNA-DNA HYBRIDIZATION

Christensen *et al.* (1994) using the dot method for DNA-DNA hybridization studied the genotypic and phenotypic relatedness of 80 strains of *M. catarrhalis* of world wide origin. They found that all isolates demonstrated homogeneity except for β-lactamase production.

1.9.3.3. RESTRICTION ENDONUCLEASE ANALYSIS

Restriction endonuclease analysis of DNA was used as a technique for distinguishing *M. catarrhalis* strains and confirming their identity (Soto-Hernandez *et al.*, 1988; Patterson *et al.*, 1989; Beaulieu *et al.*, 1993). On the other hand plasmid analysis was of little value in differentiating strains because plasmids are found infrequently in *M. catarrhalis*.

1.9.3.4. ELECTROPHORETIC PROFILES

The profiles of electrophoretic mobilities of soluble proteins are of potential value in identifying and differentiating *M. catarrhalis* strains. SDS-PAGE of OMPs from different strains of *M. catarrhalis* revealed a high degree of homogeneity. Murphy and Bartos (1989) also found that all strains from diverse sources showed similar OMP patterns by SDS-PAGE.

1.10. PATHOGENESIS, PATHOLOGY AND VIRULENCE

Life threatening infections, such as pneumonia caused by *M. catarrhalis*, are increasingly recognized especially in immunocompromised individuals. The virulence mechanisms of *M. catarrhalis* that are involved in producing pulmonary infections are not well studied (Unhanand *et al.*, 1992). It is believed that most pulmonary pathogens originate in the oropharyngeal flora and aspiration of these pathogens is the most common mechanism for initiating pneumonia (Levison, 1994). These organisms colonize the mucous membranes of the upper respiratory tract and penetrate into the lower respiratory tract by aspiration (Kayser, 1992). Middle ear infections too are caused by pathogens that originate in the oropharynx, traverse the eustachian tube, and invade the tympanic cavity. The process of infection starts with bacterial attachment to mucosal cells. The outcome depends on the number of bacteria and the nonspecific immune defences of the individual. Serum antibodies are important factors in halting infections (Taylor, 1983). β-Lactamases also confer an increased virulence of *M. catarrhalis* (Wardle *et al.*, 1982; Ahmad *et al.*, 1985; Ahmed *et al.*, 1994).

1.11. TREATMENT

The β-lactam antibiotics disorganize the cell wall structure of Gram-negative bacteria, thereby increasing permeability (Gotoh *et al.*, 1992). Most β-lactam agents diffuse through porin channels.

Effective strategies implemented to overcome β-lactamase mediated resistance include use of

non β-lactam drugs or β-lactamase inhibitors. Clavulanic acid is a potent enzyme inhibitor active against BRO-1 and BRO-2 β-lactamase types (Neu, 1985; Philippon *et al.*, 1986; Christensen *et al.*, 1990). Cefetamet pivoxil is very effective against *M. catarrhalis* and other pathogens irrespective of β-lactamase production (Schito *et al.*, 1994). Most isolates of *M. catarrhalis* are susceptible to the cephalosporins, chloramphenicol, clarithromycin, azithromycin, co-trimoxazole and erythromycin (Jacoby, 1993). The fluoroquinolones (ofloxacin) and cefaclor are also very effective against *M. catarrhalis* (Preston, 1993; Bourgeois and Bingen, 1994). Cefuroxime and cefotaxime have shown good efficacy in lower respiratory tract infections and are well tolerated by patients. These drugs are useful for patients infected with antibiotic resistant strains of *M. catarrhalis* and they should be reserved for life-threatening situations (Davies and Maesen, 1986; Fass, 1993; Vogel, 1993).

Community-acquired pneumonia caused by *M. catarrhalis* should be treated initially with either second or third generation cephalosporins, amoxicillin-clavulanate or co-trimoxazole (Barriero *et al.*, 1992; La Force, 1992; Leedom, 1992). Doxycycline 100 mg twice daily for 10 days is effective in the treatment of acute exacerbations of chronic bronchitis caused by *M. catarrhalis*. The first line antimicrobial therapy for uncomplicated acute otitis media and acute sinusitis cases caused by non-β-lactamase *M. catarrhalis* strains in children is amoxicillin. The drug is safe and effective against most of the causative bacterial pathogens as well (Eliasson *et al.*, 1990; Giebink, 1992). For β-lactamase producing strains a combination of amoxicillin/clavulanate, co-trimoxazole, or erythromycin/sulfisoxazole is recommended (Bluestone, 1992).

1.12. EPIDEMIOLOGY

M. catarrhalis infections have a world wide distribution. There is a striking seasonal variation in isolation of this organism from clinical specimens indicating a peak in late winter and a nadir in late summer (DiGiovanni *et al.*, 1987; Catlin, 1990; Musher, 1994). During the cold seasons, *M. catarrhalis* was recovered from clinical samples in about 20% of the cases (Wallace and Musher, 1986). A high average temperature is usually associated with low incidence, while the occurrence of lower respiratory tract infections are markedly higher when the average temperature is low (Mbaki *et al.*, 1987).

Prevalence rates for *M. catarrhalis* respiratory tract infections seem to be influenced by geographical and environmental differences. Davies and Maesen (1986) found *M. catarrhalis* in 26% of cases and Schonhyder and Ejlertsen (1989) isolated *M. catarrhalis* from sputum of patients with lower respiratory tract infections in 8.5% while Soto-Hernandez *et al.* (1988) isolated it from sputum in 15.5% of patients.

The upper respiratory tract appears to be the portal of entry of organisms associated with infection. The mode of spread is not well established, but person-to-person transmission is thought to be responsible for some hospital-acquired infections. Hand carriage has also been implicated in the nosocomial spread of the organism.

1.13. IMMUNITY

Resident pulmonary defence consists of aerodynamic defence, the mucociliary apparatus, alveolar macrophages, complement and surfactant. These non-specific immune mechanisms of defence are augmented by the development of specific immunity (Toews *et al.*, 1990; Lehnert, 1992).

Studies designed to investigate immunity to *M. catarrhalis* suggest that about half of the subjects manifested an antibody response following infection. Patients with pulmonary infections caused by *M. catarrhalis* produce convalescent IgG antibody which has bactericidal activity, which is thought to result from activation of the classical complement pathway (Faden *et al.*, 1994). Antibody to the OMP of *M. catarrhalis* is present in the sera of children over the age of 4 years (Musher, 1994). A prior study utilizing functional antibody tests detected a rise in bactericidal antibody to homologous organisms in 60% of adults with pulmonary infections caused by *M. catarrhalis* (Chapman *et al.*, 1985). Convalescent-phase but not acute-phase sera from patients with pneumonia caused by *M. catarrhalis* contained antibodies to this bacterial surface protein indicating that *M. catarrhalis* strains growing in vivo express this molecule (Helminen *et al.*, 1994). In mice actively immunized with OMPs of *M. catarrhalis* the serum antibody level was raised and homologous strains from the endobronchial tree were cleared more quickly than from control animals. In these animals antibodies were detected to both OMP and LPS antigens. Passive immunization of mice with immune sera resulted in enhanced pulmonary clearance of both homologous and heterologous strains of *M. catarrhalis* indicating the involvement of antibody in the clearance process

(Maciver *et al.*, 1993). Thus among the surface antigens of *M. catarrhalis* the OMPs are vaccine candidates (Helminen *et al.*, 1993; Bhushan *et al.*, 1994). Infections with *M. catarrhalis* are followed by the development of humoral and cellular defences. Both PMN recruitment and antibody production were observed in respiratory infections. The functions of PMN and natural killer cells is important in resolving *M. catarrhalis* infections (Harkness *et al.*, 1993; McWilliam *et al.*, 1994)). PMN were found to phagocytose *M. catarrhalis* (Marrs and Weir, 1990; Ahmed *et al.*, 1994).

II. AIMS OF THE STUDY

This study was performed with the following objectives:

1. To determine the level of the total immunoglobulins (IgG-A-M) in acute and convalescent sera of patients with acute *M. catarrhalis* respiratory infections (pneumonia) in response to the outer membrane proteins (OMP) of the bacterium using an ELISA technique.

2. To determine the rise in titre of the IgG3 subclass in response to *M. catarrhalis* outer membrane protein antigens in adult patients with pneumonia using an ELISA technique.

3. To determine the antibody response in acute and convalescent sera of adult patients with pneumonia to lipopolysaccharide (LPS) of *M. catarrhalis.*

4. To determine the bactericidal activities of convalescent-phase sera of pneumonia patients to viable cells of *M. catarrhalis.*

RELEVANCE OF THE STUDY

The results of this study would be applicable in clinical medicine and in research work. Since *M. catarrhalis* is usually considered a harmless commensal of the human pharynx by many laboratory personnel and clinicians, patients may not be properly diagnosed and treated. The possibility of a pathogenic role for *M. catarrhalis* should be more widely recognized. Serological methods to detect infection with *M. catarrhalis* may be of as great importance as bacteriological isolation since the bacterium is often isolated from healthy individuals as well. The study may stimulate research workers to acquire more knowledge about the antigens of *M. catarrhalis* because the antibody response to these antigens during infection is very important for the study of the pathogenesis and epidemiology of infections caused by *M. catarrhalis* and for vaccine production.

The results of this study would be of benefit to patients with respiratory tract infections since the methods of diagnosis of *M. catarrhalis* infections bacteriologically together with serology is more reliable than by isolation and identification alone, and treatment of serious infections caused by *M. catarrhalis* with appropriate drugs is beneficial to the patient. An exacerbation of chronic lung diseases due to infection with β-lactamase producing *M. catarrhalis* may be treated with an inappropriate antibiotic unless the microorganism is identified as a pathogen and antibiotic sensitivity and β-lactamase production is determined.

III. MATERIALS AND METHODS

3.1. STUDY AREA

The study was conducted on patients with community-acquired pneumonia during their attendance for medical examination and treatment in the outpatient Departments of Internal Medicine and Radiology in Tikur Anbessa Hospital, a teaching and referral Hospital in Addis Ababa.

3.2. SELECTION OF ADULT PATIENTS WITH LOWER RESPIRATORY TRACT INFECTIONS

The international ethical considerations were followed during the study according to the Helsinki Declaration. Informed consent was obtained from each patient during the first contact with the patient. A Medical history was obtained from the patient or his/her family.

Pneumonia was defined as an inflammation and consolidation of the lung tissue caused by an infectious agent (Marrie, 1994). A patient was considered to have pneumonia if he/she had signs and symptoms of acute lower respiratory tract infection such as productive cough of purulent sputum, chills, chest pain, fever, and auscultatory crepitations. In addition a patient was considered to have pneumonia if patchy infiltrates on the chest X-ray were observed.

Male and female adult patients of 15 years and above were enrolled in the study. To achieve the diagnosis of pneumonia caused by *M. catarrhalis*, 200 patients exhibiting clinical and

roentgenographic findings characteristic of pneumonia, have been studied from the bacteriological, biochemical and serological standpoints. For each patient the following clinical information was recorded when available: age of patient, history of underlying diseases, smoking, cough, sputum production, fever, chest roentgenographic findings, white blood cell counts, erythrocyte sedimentation rates, and use of antibiotics before coming to the hospital.

3.3. CONTROLS

Sera from adult healthy individuals, and surgical and other patients without pulmonary infections served as controls. None of them had a history of recent respiratory tract infections, and cultures for *M. catarrhalis* in their sputa and nasopharyngeal specimens were negative.

3.4. COLLECTION OF SPECIMENS FOR BACTERIOLOGICAL AND SEROLOGICAL STUDIES

Sputum and nasopharyngeal swab samples: These samples were collected from 200 adult patients with pneumonia. Samples were collected before antibiotic treatment was instituted in the hospital. Sputa were collected in wide mouthed, sterile plastic cups (Nunc, Denmark) in the laboratory. Nasopharyngeal samples were taken using sterile cotton tipped aluminium wires (Lab Design, Bio Hospital AB, Sweden). Specimens for the control group were also obtained in the same way.

Serum: Samples of five millilitres of blood were obtained aseptically with vacutainer in non-

citrated sterile test tubes from both pneumonia patients and controls. Paired serum samples were collected from each case and control. An acute-phase serum was drawn at the first visit of the patient to the hospital with onset of illness less than 10 days and a convalescent-phase serum 10-15 days after the acute sera was taken. Blood was taken from control individuals who had no history of recent respiratory tract infections.

3.5. HANDLING AND PROCESSING OF SPECIMENS

All specimens were processed immediatly in order to prevent drying. A purulent part of each sputum specimen was selected, processed (liquefied) with an equal volume of sputolysin, homogenized and then diluted 1:1000 in saline. A 10 µl loopful of the homogenized specimen was streaked onto 5% sheep blood agar, chocolate agar and selective medium immediately. The plates were incubated at 37°C overnight and checked for any bacterial growth. Then, Gram-stains of smears prepared from presumptive colonies of *M. catarrhalis* were prepared. Colonies identified as *M. catarrhalis* were subcultured onto blood agar and selective plates consisting of brucella agar supplemented with 5% sheep blood and antibiotics. The antibiotics added were vancomycin (10 µg/ml), amphotericin B (2 µg/ml), trimethoprim (5 µg/ml), and acetazolamide (10 µg/ml) (Vaneechoutte *et al.*, 1988b). Stock cultures were kept on nutrient broth with 15% glycerol in tubes and were stored at -70°C until used later (Jonsson *et al.*, 1990; Sarwar *et al.*, 1992).

Biochemical tests including carbohydrate fermentation, oxidase, catalase, nitrate reduction, and DNase tests were performed for each specimen collected.

England) with a heavy linear streak of bacterial colonies onto the plates and incubated at 37°C for 48 hours. The plates were flooded with 1 N HCl. Positive strains showed clear zones around the growth. *Staph. aureus* was used as a positive control (Richards, 1988).

Nitrate reduction was performed on broths consisting of 0.2 g of potassium nitrate, and 5 g peptone dissolved in 1 litre of distilled water. Bacterial isolates were inoculated into 5 ml of the broth and incubated at 37°C for 48 hours. Then an aliquot of 200 µl of test reagent was added to each test tube. A positive test for nitrites was revealed by the development of a red colour within 1-2 min.

The oxidase test was performed on filter papers placed in a clean petri dish. Two drops of tetramethyl-*p*-phenylene diamine solution, (Spot test, oxidase reagent; Difco Laboratories, Detroit, Mich., U.S.A) were placed on Whatman filter paper. A large inocula of the bacterial colonies was removed with a loop and smeared on the reagent saturated area. Positive reactions were identified by the development of dark purple colour within 10 seconds; the colour remained unchanged in negative tests.

Catalase tests were performed using a commercially available hydrogen peroxide solution. Bacterial scrapings from fresh colonies grown on nutrient agar were placed on clean microscope slides and 2 drops of 3% H_2O_2 solution was added. Positive isolates produced bubbles of gas within 5 s. Those which gave delayed ebulition of gas were considered negative.

M. catarrhalis isolates from sputum and nasopharyngeal swab specimens were tested for β-lactamase production by the cephalosporin method using commercially available nitrocefin discs as described later.

3.7. IMMUNOGLOBULIN CONJUGATES

Commercially available anti-human total immunoglobulin (IgG-A-M) and IgG3 subclass affinity purified peroxidase conjugates were used in the investigations for antibody response against *M. catarrhalis* OMP and LPS antigens.

3.8. *MORAXELLA CATARRHALIS* ANTIGEN PREPARATIONS

Outer membrane proteins (OMPs) antigens of *M. catarrhalis* strains designated as B26, B272 and B18284 were used in an ELISA. The OMPs were prepared by ultracentrifugation methods in Sweden and were available in 0.5 μg/ml solution and were kept at -20°C. LPS types A (ATCC 25238), B (B3292) and C (B26) were also available from the same source and were used as antigens. OMP and LPS B26 were used throughout the study, and ATCC 25238 OMP and LPS were used as standard antigens for comparisons.

3.9. ESTIMATION OF ANTIBODIES BY ELISA

OMP enzyme immunoassays. Ninety six well flat bottom immunoassay microtitration plates (Immulon 1, Dynatech Laboratories, Virginia, U.S.A) were coated with 100 μl of B26 OMP

antigens containing 0.5 µg/ml of protein in 0.05 M carbonate buffer (pH 9.6). The plates were kept overnight at room temperature. Then the plates were washed four times with washing solution [PBS solution containing 0.05% Tween 20 (pH 7.2)] with an ELISA washer (Nunc, Denmark). Sera were diluted in PBS T-20 at 1:1000 and 1:2000 for IgG-A-M and IgG3 respectively and 100 µl of each dilution were added into the respective wells of the plates. Then, the plates were incubated at 37°C for 2 hours. Commercially available biotinylated total immunoglobulin (IgG-A-M), and IgG3 (Binding Site, Birmingham, U.K.) were used as conjugates. After being washed 4 times with the washing solution, 100 µl of affinity purified horseradish peroxidase conjugated anti-human immunoglobulin isotypes and subclasses (IgG-A-M in dilutions of 1:4000 and IgG3 in dilutions of 1:2000) were added into the plates. Plates were incubated for 90 min at 37°C. After washing again with the washing solution, the reaction was developed with 100 µl of substrate (TMB) consisting of 3'5'3'5' tetramethyl benzidine (Merk, Germany) in dimethyl-sulfoxide (DMSO), (Sigma, U.S.A.) in 0.1 M sodium acetate (pH 6.0) and 0.002% (v/v) of 30% H_2O_2. The plate were incubated for 15 min at room temperature. Finally, the reaction was terminated by 50 µl/well of 2 M H_2SO_4 and the absorbence was measured in an ELISA plate reader (Bio Rad Microplate Reader, Japan) at an absorbance of 450 nm (Chin *et al.*, 1993).

All sera were run in duplicate wells and the mean value of the two readings was taken as the result. One known patient serum was included as a positive control in each plate in addition to sera from negative controls. The results were expressed as fold increase in antibody level of convalescent-phase/acute-phase sera.

LPS enzyme immunoassay. A solution containing 3 μg/ml of *M. catarrhalis* LPS type C strain from B26 in 0.05 M carbonate buffer was prepared. Each well of a microtitre plate was coated with 100 μl of the solution and kept at room temperature overnight. The serum dilution used for LPS antigens in this assay 1:200. The next day plates were processed in the same manner as for OMP procedure under similar conditions.

3.10. DETECTION OF BETA-LACTAMASE PRODUCTION

β-Lactamase production by *M. catarrhalis* was demonstrated using nitrocefin (cefinase) discs (PDM diagnostic disks AB, Sweden) which contained 12 μg of antibiotics/disc. Discs were dispensed into sterile empty petri plates. Each disc was moistened with 1 drop of sterile distilled water and with a sterile inoculating loop a colony of *M. catarrhalis* was smeared onto the disc surface. Then the disc was observed for colour change. A positive reaction was apparent when the disc turned from yellow to red in the area where bacteria were deposited. The test was considered negative when there was no colour change within 5 min (Leitch and Boonlayangoor, 1992).

3.11. DETECTION OF SERUM BACTERICIDAL ACTIVITIES

Determination of serum bactericidal activity involves exposure of a suspension of viable *M. catarrhalis* to a suitable concentration of antibody and complement, incubation at the optimum temperature for complement activity, and determination, after suitable periods of time, of the absolute concentration of surviving organisms by means of viable counting. In our studies the

following technique was used. *M. catarrhalis* species were grown overnight on blood agar at 37°C. The cells of *M. catarrhalis* were washed twice with PBS and centrifuged at 1700 g for 5 min at room temperature. Then the suspension was standardized in a spectrophotometer (Beckman, U.S.A) at an OD_{600} of 0.1. This standardized suspension contained approximately $1.0x10^5$ cfu/ml of cells. Cell supensions were well agitated and then streaked on blood agar and a plate count was done.

Test: For the test, 50 µl of serum from a healthy individual as a complement source plus 150 µl heat-inactivated patient test sera plus 50 µl *M. catarrhalis* suspension with a concentration of 10^5 cfu/ml were mixed in a sterile test tube.

Control: For the control assay, 50 µl *M. catarrhalis* suspension, 150 µl heat-inactivated test sera, and 50 µl PBS (instead of a complement source sera) were mixed in another test tube.

Both the test and control were placed in a shaking water bath at 37°C. At time 30, 60, and 90 min of incubation, aliquots of 25 µl from each tube were streaked and dispersed with a glass rod on 5% sheep blood agar plates and then incubated at 37°C overnight. On the next day, colonies were counted with the help of an electronic colony counter and the % killing effect determined. The percent (%) killing effect was calculated as the mean number of CFU at time 0 minus mean number of CFU at 60 minutes of incubation divided by the mean number of CFU at time 0 multiplied by 100 (Rubin *et al.*, 1991; Peters and Rubin, 1992; Takasugi, 1994). Bactericidal activity was defined as ≥50% killing after 60 min of incubation. At 0 time, $1.0x10^5$ was taken as constant in both the tests and controls.

3.12. STATISTICAL ANALYSIS

The student's *t*-test, and *z*-test were used to determine the significance of differences between patients and controls as appropriate, and P values of less than 0.05 were taken as being significant. The mean was expressed as plus or minus standard deviation (SD) or standard error of the mean (SEM).

IV. RESULTS

4.1. AGE AND SEX DISTRIBUTION OF PATIENTS WITH PNEUMONIA

For the study, 200 radiologically verified adult patients with community-acquired pneumonia were selected. There were 122 (61.0%) males and 78 (39.0%) females, a sex ratio of 1.56:1. The ages of the patients ranged between 18 and 85. The mean age of all pneumonia patients was 41.7. Of all the patients with pneumonia caused by *M. catarrhalis*, 42 (61.8%) were males, and 26 (38.2%) were females (Table 3).

TABLE 3. Distribution by age and sex of patients with pneumonia caused by *M. catarrhalis*

Age range	Sex and No (%) of patients		Total
	Male	Female	%
15 - 24	0 (0.0)	2 (2.9)	2.9
25 - 34	4 (5.9)	7 (10.3)	16.2
35 - 44	12 (17.6)	4 (5.9)	23.5
45 - 54	15 (22.1)	3 (4.4)	26.5
55 - 64	8 (11.8)	4 (5.9)	17.5
≥ 65	3 (4.4)	6 (8.8)	13.2
	42 (61.8)	26 (38.2)	100.0

The mean age of all pneumonia patients caused by *M. catarrhalis* was 45.1 ± 10.8, which was slightly higher than the total patients with pneumonia. The male to female sex ratio was 1.6:1.

4.2. PRE-EXISTING (UNDERLYING) DISEASES

Of the patients with pneumonia caused by *M. catarrhalis*, 51 of the 68 (75.0%) had either pulmonary or extrapulmonary pre-existing diseases (Table 4).

TABLE 4. Number of patients with pneumonia caused by *M. catarrhalis* who had chronic underlying diseases

Underlying disease	No. of patients	%
Chronic bronchitis	19	27.94
Tuberculosis	10	14.71
Bronchial asthma	8	11.77
Diabetes mellitus	5	7.35
AIDS	3	4.41
Chronic glomerulonephritis	2	2.94
Congestive heart failure	1	1.47
Emphysema	1	1.47
Sinusitis	1	1.47
Non-Hodgkin's lymphoma	1	1.47
T O T A L	51	75.0

Predisposing respiratory factors: Underlying pulmonary diseases were identified in 39 of the 68 (57.4%) patients with pneumonia. Nineteen patients suffered from bronchitis, 10 patients had been treated earlier for tuberculosis, 8 for bronchial asthma, 1 patient had emphysema, and another had sinusitis. Six of the 8 asthmatic patients were being treated with corticosteroids.

Other predisposing factors: The number of patients with extrapulmonary predisposing diseases and conditions was 12 (17.6%). The diseases included diabetes mellitus, AIDS, chronic glomerulonephritis, non-Hodgkin's lymphoma, and congestive heart failure.

Healthy hosts: Seventeen (25%) of the 68 cases of pneumonia caused by *M. catarrhalis* occurred in immunocompetent patients with no underlying disease.

4.3. CLINICAL FINDINGS

Of the 200 patients with community-acquired pneumonia, *M. catarrhalis* was isolated from 68 of them. Five of the 68 patients (2.9%) used antibiotics from private clinics and drug vendors prior to their arrival at Tikur Anbessa Hospital. Three of them used procaine penicillin and the remaining 2 patients used ampicillin but all claimed to have stopped taking medication 1 week before coming to the hospital. Seven patients had mucoid sputum and the other 61 produced purulent sputum, of whom 1 patient produced blood-tinged sputum. Seven of the 68 patients (10.3%) from whom *M. catarrhalis* was isolated were smokers or ex-smokers. All of the patients had pulmonary infiltrative changes suggestive of recent pneumonia. All of the patients with pneumonia caused by *M. catarrhalis* except 7 were febrile with a temperature of >38°C. Their leucocyte count ranged between 5,600 to 20,000 cells/mm^3 with a mean of 11,108 cells/mm^3. This result is near to previous results reviewed by Wright *et al.* (1990), which was 12,800 cells/mm^3. The erythrocyte sedimentation rate (ESR) ranged from 3 to 112 mm/h with a mean of 35.5 mm/h. There were 2 patients with pneumonia caused by *M. catarrhalis* which was accompanied by pleural effusion.

4.4. BACTERIOLOGY

The following bacterial agents of pneumonia were isolated from the sputum and nasopharynx of patients with pneumonia (Table 5).

TABLE 5. Bacterial etiologic agents of community-acquired pneumonia isolated from sputum and nasopharyngeal swabs of patients

Organism isolated	No (%)	
	Sputum	Nasopharynx
Strep. pneumoniae	82 (41.0)	87 (43.5)
M. catarrhalis	68 (34.0)	56 (28.0)
H. influenzae	21 (10.5)	25 (12.5)
DNase-negative Gram-negative cocci	21 (10.5)	14 (7.0)
Staph. aureus	5 (2.5)	11 (2.0)
Growth failure	1 (0.5)	4 (2.0)
Strep. pyogenes	2 (1.0)	3 (1.5)
T O T A L	200 (100)	200 (100)

Strep. pneumoniae was isolated from sputum of 82 (41.0%) of the patients and from 87 (43.5%) nasopharyngeal specimens. The second important bacterial agent isolated was *M.*

catarrhalis in which 68 (34.0%) were recoverd from sputum and 56 (28%) from the nasopharyngeal specimens. The third important pathogen was *H. influenzae*, isolated from 21 (10.5%) of sputum samples and from 25 (12.5%) of nasopharyngeal specimens. In general, organisms were isolated in 99.5 % of sputum and 98% of nasopharyngeal specimens. In several specimens multiple possible pathogens were isolated (Table 6).

TABLE 6. Isolation of *M. catarrhalis* from sputa and nasopharyngeal specimens in pure culture and mixed with other respiratory pathogens

Bacterial species isolated	Sputum		Nasopharyngeal swab	
	No.	%	No.	%
M. catarrhalis (Pure)	41	60.3	28	50.0
M. catarrhalis + Strep. pneumoniae	15	22.1	14	25.0
M. catarrhalis + H. influenzae	5	7.4	7	12.5
M. catarrhalis + Staph. aureus	2	2.9	5	8.9
M. catarrhalis + Strep. pyogenes	2	2.9	0	0.0
M. catarrhalis + Strep. pneumoniae + H. influenzae	2	2.9	2	3.6
M. catarrhalis + Strep. pneumoniae + Staph. aureus	1	1.5	0	0.0
TOTAL	68	100	56	100

Overall, the sputum samples screened, *M. catarrhalis* was isolated in pure cultures from 20.5% of the sputum samples. Of those cultures which allowed growth of *M. catarrhalis*, the organism was isolated in pure culture from 60.3% (Table 5). The nasopharyngeal specimens showed pure isolations in 14% of the total and in 50% of those specimens yielding *M. catarrhalis*.

4.5. BETA-LACTAMASE PRODUCTION

All the *M. catarrhalis* isolates collected from pneumonia patients were tested for the production of β-lactamase using nitrocefin discs. As illustrated below (Table 7), 37 (54.4%) of the 68 *M. catarrhalis* strains isolated from sputum and 32 (57.1%) of the 56 nasopharyngeal swab isolates produced β-lactamase detectable by the nitrocefin disc test.

TABLE 7. Beta-lactamase production by the *M. catarrhalis* isolates from sputa and nasopharyngeal swabs

Type of specimen	No. of isolates	β-lactamase positive	% positive
Sputum	68	37	54.4
Nasopharyngeal specimen	56	32	57.1

β-Lactamase was consistently detected on subcultures onto selective medium.

4.6. SERUM ANTIBODY RESPONSE TO THE OUTER MEMBRANE PROTEIN AND LIPOPOLYSACCHARIDE ANTIGENS OF *M. CATARRHALIS*

All the 68 sera obtained from patients with community-acquired pneumonia caused by *M. catarrhalis* were analysed by ELISA for antibody titres using IgG-A-M and IgG3 anti-human conjugated antibodies. Antibody titres of acute-phase and convalescent-phase sera were compared to see any increase in titre.

The ELISA test for antibody in acute-phase sera and convalescent-phase sera of the same

patient were done at the same time, in the same test. The results showing significant increases in antibody titres in convalescent-phase to acute-phase sera for to both IgG-A-M and IgG3 are shown in Table 8.

TABLE 8. IgG-A-M and IgG3 antibody response in paired-sera of patients with pneumonia caused by *M. catarrhalis* showing significant (≥ 1.5) fold increase in titre in homologous sera in both IgG-A-M and IgG3

Ser No	IgG-A-M	IgG3	Ser No	IgG-A-M	IgG3
1	1.7	1.5	21	1.5	1.8
2	1.5	1.7	22	1.6	2.7
3	1.8	1.8	23	1.6	1.6
4	1.5	2.5	24	1.5	1.5
5	1.6	2.4	25	2.1	2.0
6	1.5	2.7	26	2.5	1.7
7	1.5	2.0	27	1.6	1.5
8	1.5	1.8	28	1.6	1.7
9	1.5	2.1	29	1.6	2.3
10	1.6	1.6	30	1.8	1.8
11	2.2	3.4	31	1.7	1.9
12	1.9	2.4	32	1.5	2.9
13	2.1	2.2	33	1.5	1.6
14	1.8	1.5	34	1.6	1.9
15	1.8	1.5	35	1.8	2.3
16	2.6	1.5	36	1.8	1.8
17	3.7	2.3	37	1.5	3.5
18	1.7	1.6	38	1.8	2.3
19	1.6	1.8	39	2.2	1.6
20	1.5	2.2	40	1.7	1.8
			mean =	1.78	2.02

Note. Ser. No. = Serial number

An increase in titre of antibodies against the OMP of *M. catarrhalis* by ≥150% (≥1.5 fold increase in titre) for both IgG-A-M and IgG3 in convalsecent-phase sera compared to that of the acute-phase sera of patients with pneumonia caused by this organism was considered a significant fold increase.

In the analysis for the response of total immunoglobulins (IgG-A-M) to The OMPs of *M. catarrhalis*, 40 of the 68 paired sera (58.8%) showed significant increases in antibody titre for IgG-A-M (titre of or ≥ 1.5 fold increase) [Table 8]. In the the control group only 1 of 30 (3.3%) sera showed a significant fold increase of antibody titre in the convalescent-phase serum sample ($P < 0.05$) against the OMP antigen of *M. catarrhalis* using the IgG-A-M conjugates.

In the same way, in the determination of the IgG3 subclass response against the OMPsof *M. catarrhalis*, 43 of the 68 paired sera (63.2%) showed a significant fold increase in titre. In the control group none of the sera showed significant increase in antibody titre ($P < 0.05$) [Fig. 1].

All the sera which showed significant increase in titres for IgG-A-M were also significantly increased for IgG3. Antibody titres with ≥1.5 fold increases for both IgG-A-M as well as IgG3 were considered reliable increase in antibody.

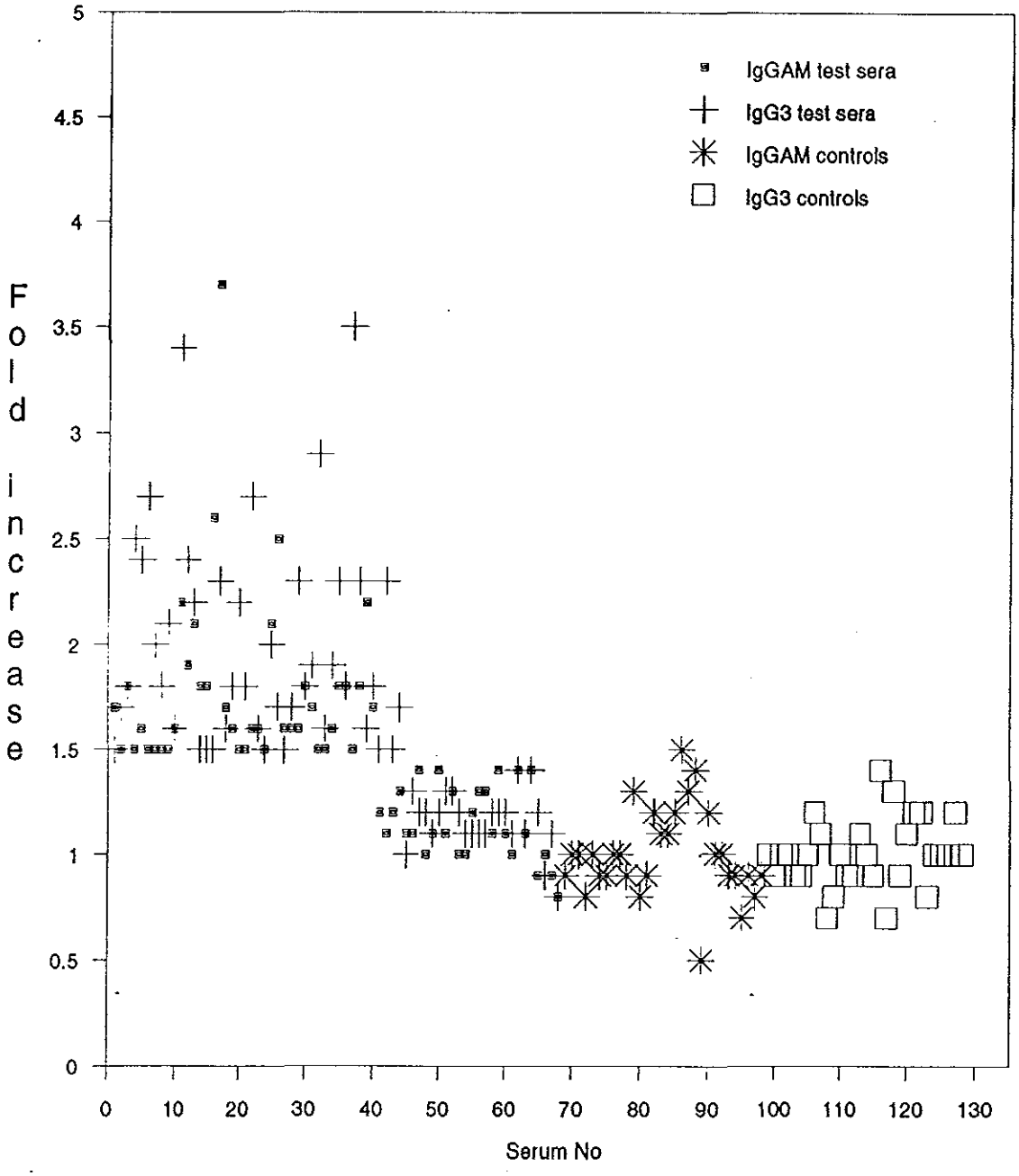

FIG.1 IgGAM & IgG3 levels in sera of pts with pneumonia (n=68) & controls (n=30) determined against the OMP of M. catarrh

In an ELISA, using 3 μg/ml of lipopolysaccharide (LPS) antigens obtained from *M. catarrhalis*, serum anti-LPS antibody response against these antigens was determined. As in the ELISA done for OMP antigens, both acute-phase sera and convalescent-phase sera of the same patient were run in different row of the same plate. Out of the 68 patients with community-acquired pneumonia positive for *M. catarrhalis* in their sputum, 16 (23.5%) of them showed significant anti-LPS antibody titre increase in their convalescent-phase sera to acute phase sera for IgG-A-M compared to 2 of 30 (6.7%) in the control group ($P< 0.05$) [Fig 2]. The remaining 52 (76.5%) patients with pneumonia had antibody titres of ⟨1.5 fold for convalescent-phase versus acute-phase sera.

The control subjects were healthy individuals as well as patients without histories of respiratory infections. All tests were repeated and showed similar results.

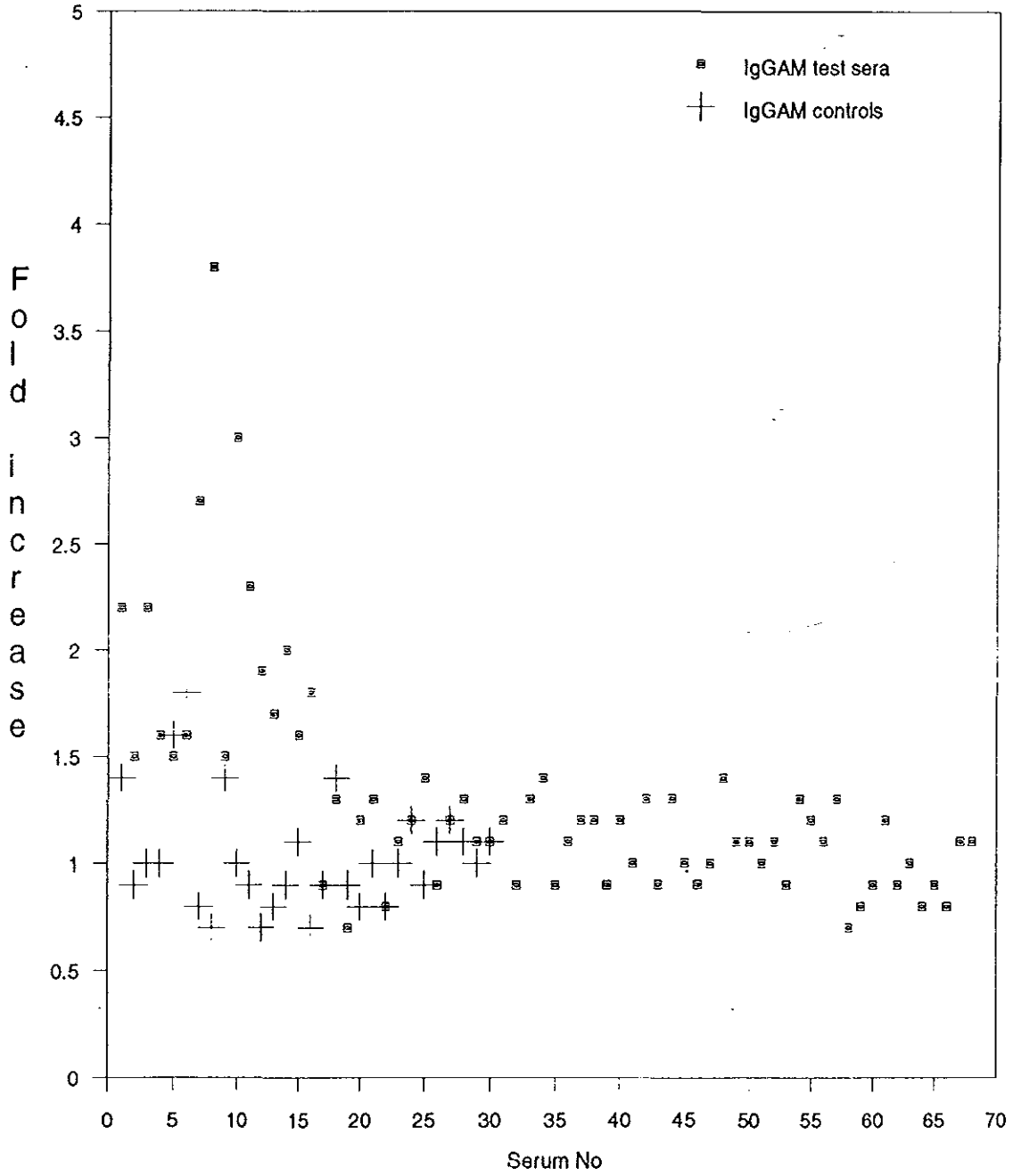

FIG.2 LPS response to convalescent/acute sera of patients with pneumonia (n=68) & controls (n=30) determined by ELISA

4.7. BACTERICIDAL ACTIVITIES OF SERA

Bactericidal assays were performed on convalescent-phase sera obtained from patients from whom *M. catarrhalis* was isolated from both sputum and nasopharyngeal swab samples. Sera from patients with pneumonia caused by *M. catarrhalis* were selected for measurement of their bactericidal activity against *M. catarrhalis* isolated from the same patient, on the basis of their antibody titres in the ELISA. Sera which had a low titre rise below 1.5 fold in either IgG-A-M or IgG3 or both (numbers 1-6), medium titre rise between 1.5-2.0 fold in either IgG-A-M or IgG3 or both (numbers 7-11), and high titre greater than 2.0 fold increase in either IgG-A-M or IgG3 or both (numbers 12-15). The normal human sera used as complement source in the test assay was obtained from a single healthy and well nourished individual without any history of lower respiratory tract infection as well as upper respiratory tract infection for at least one year. The results of these assays are shown in Table 9.

In this study, it was found that 13 of 15 (86.7%) convalescent-phase sera from patients with community-acquired pneumonia caused by *M. catarrhalis* showed significant bactericidal activities, i.e., ≥50% killing at serum dilutions of 60% against the patient's own infecting isolates (Table 9).

TABLE 9. Bactericidal activities of convalescent-phase sera from patients with pneumonia caused by *M. catarrhalis* showing the percentage of CFU/ml remaining after specific times of incubation

	Time of incubation (min) and % CFU					
	30		60		90	
Exp. No.	Test	Control	Test	Control	Test	Control
1	56.0	87.0	32.0	91.0	5.1	92.0
2	80.0	110.0	66.0	120.0	67.0	180.0
3	18.0	180.0	12.0	200.0	1.8	210.0
4	48.0	92.0	24.0	94.0	0.4	97.0
5	32.0	110.0	17.0	100.0	22.0	120.0
6	37.0	99.0	22.0	96.0	7.0	96.0
7	76.0	100.0	9.5	110.0	0.5	150.0
8	45.0	96.0	5.6	94.0	0.3	100.0
9	29.0	87.0	7.3	89.0	0.6	96.0
10	86.0	120.0	51.0	110.0	90.0	110.0
11	53.0	120.0	9.1	180.0	0.5	200.0
12	67.0	130.0	8.0	111.0	0.2	150.0
13	51.0	150.0	7.8	84.0	0.6	82.0
14	80.0	95.0	5.9	95.0	0.7	95.0
15	36.0	140.0	0.5	161.0	0.0	180.0
Mean	52.9	114.4	18.5	115.5	13.1	130.5
SD	± 20.97	± 26.11	± 18.4	± 35.5	± 27.3	± 43.8
SEM	± 5.42	± 6.75	± 4.75	± 9.17	± 7.05	± 11.3

Note. Exp. No. = Experiment number; min = minute; CFU/ml = colony forming units per millilitre; SD = standard deviation; SEM = standard error of the mean.

Only two sera (numbers 2 and 10, Table 9) failed to kill effectively the homologous bacterial isolates in which only 34% and 49% of the CFU were killed after 60 min of incubation at 37°C. These two sera had <1.5 and 1.5-2.0 fold increase in ELISA in convalescent-phase as compared to acute-phase sera. Of all the sera tested showing above two fold increase in antibody titre for convalescent-phase/acute-phase sera, all had significant bactericidal activities (numbers 12-15 in Table 9).

V. DISCUSSION AND CONCLUSION

DISCUSSION

Once considered as part of the oropharyngeal commensal, *M. catarrhalis* has been recognized as a significant pathogen of the respiratory tract during the last decade (Verghese and Berk, 1991). A better appreciation of its role in upper and lower respiratory tract infections, both in the community and in the hospital, has been mostly responsible for this change of attitude. The spectrum of disease caused by *M. catarrhalis* is diverse. In children, it is a major cause of acute otitis media with a pathogenic status equivalent to *H. influenzae*. It also causes maxillary sinusitis. It causes pneumonia, acute exacerbation of chronic bronchitis and bacterial tracheitis in adults.

The mean age (45.1 years) of patients with pneumonia found in this study is lower than that reported by Wright *et al.* (1990) which had mean ages of 64 years. We found that patients in their fourth and fifth decades of life showed a relatively higher percentage of infection with *M. catarrhalis* than younger and and older age groups. The reason could be that many elderly patients prefer not to come to hospitals for treatment. The average total IgG levels decrease significantly from the third through the sixth decade, and the average IgM concentrations decrease significantly by the sixth decade. In addition to these, predisposing pulmonary diseases increase with increasing age. However, some elderly patients develop pneumonia caused by *M. catarrhalis* not associated with COPD, immunoglobulin abnormality or corticosteroid therapy. Males and females are affected by the bacterium. In our study the sex ratio of patients with pneumonia caused by *M. catarrhalis* was 1:1.6 (F:M). A study by Wright

et al. (1990) also showed a slightly higher incidence in the males, 4:3 (M:F). This could be explained by increased tendencies of risk factors including alcoholism and smoking-associated bronchitis.

Correct identification of *M. catarrhalis* from respiratory tract specimens entails differentiation from *Neisseria* species, which are members of the upper respiratory tract flora and which sometimes causes confusion to laboratory personnel. Sputum Gram-stain is useful because results are immediately available, and bacteria not easily detected by routine culture methods could be identified. The Gram-stain of sputum is a reliable way of detecting lower respiratory tract infections caused by *M. catarrhalis* in clinical practice (Thornley *et al.,* 1982). Since Gram-stain should be routinely done in most laboratories, this technique would allow for the recognition of pulmonary infections caused by *M. catarrhalis* (Wallace and Musher, 1986; Kalin *et al.,* 1983). In sputum, nasopharyngeal samples as well as other body fluids, Gram staining, colony morphology and enzyme tests are important to identify *M. catarrhalis* with a specificity of 95%. New techniques have been developed that permit selective isolation and identification of *M. catarrhalis* including the use of antibiotics in culture media. It has been isolated in pure cultures from transtracheal and endotracheal aspirates, as well as sputum of patients with acute pulmonary infections. Occasionally, it has been isolated from blood cultures of patients suffering from pneumonia (Doern and Miller, 1981). The isolation of *M. catarrhalis* in sputum especially when isolated in pure culture from patients with acute lower respiratory tract infections is a good evidence of its pathogenicity (Wright *et al.,* 1990).

Oxidase and catalase tests are important preliminary tests in the identification of *M.*

catarrhalis. These enzymes are produced by *M. catarrhalis*. Confirmatory tests including the biochemical tests employing phenol red as indicator in carbohydrate fermentation tests are easy to use. Unlike many of the *Neisseria* species *M. catarrhalis* does not ferment sugars like glucose, lactose, maltose, sucrose, fructose and others. Two additional confirmatory tests useful in differentiating *M. catarrhalis* from the *Neisseria* species are the DNase and nitrate reduction tests. All *M. catarrhalis* isolates are able to produce DNase and reduce nitrate to nitrite. These tests are very helpful in the differentiation of *M. catarrhalis* from *Neisseria* species. They are easy to use and inexpensive.

Results of Gram-stained sputum samples when supported by specific antibody responses to *M. catarrhalis* is a good diagnostic method in pneumonia cases because anti-*M. catarrhalis* antibodies are usually increased in systemic infections more than in the case of nasopharyngeal carriage of the bacterium. In this study *Strep. pneumoniae* was the commenest cause of pneumonia isolated from 41% of all sputum samples screend. *M catarrhalis* was isolated in 68 of 200 sputum samples. The bacterium was isolated in 41 of 68 (60.3%) and 28 of 56 (50%) in pure culture (i.e., in 20% of the total sputum and 14% of the nasopharyngeal samples screened) in selective media. *M. catarrhalis* was also isolated in combination with other bacteria including *Strep. pneumoniae* (22.1%), *H. influenzae* (7.4%) and other bacteria each of which were isolated in less than 3%. On selective media, Slevin *et al.* (1984) and Black and Wilson (1988) isolated *M. catarrhalis* in pure cultures from the sputum of patients with pulmonary infections in 70.2% and 89% of cases respectively. Our result is similar to that reported by Hager *et al.* (1987), which was 61%. This result implies that *M. catarrhalis* is a common isolate in sputum; and an important cause of community-acquired pneumonia.

H. influenzae was the third commonest isolate in the sputum of pneumonia patients. It was detected in 10.5% of sputum samples. Generally, organisms were isolated in 99.5 % of sputum and 98% of nasopharyngeal specimens. In the growth failure cases, the causative agents of pneumonia could be pathogens not amenable to cultivation by the media used. We did not attempt to exclude chlamydia, mycoplasma, and legionella infections since the radiographs did not show signs of atypical pneumonia. Oropharyngeal anaerobes were not also excluded due to their less importance and occurrences in community-acquired pneumonias.

Despite its predilection for the immunosuppressed and patients with chronic obstructive pulmonary diseases (COPD), *M. catarrhalis* also infects previously healthy people, sometimes with a fatal outcome despite aggressive care. Pulmonary diseases such as bronchitis, asthma, tuberculosis, bronchiectasis, prior viral infections of the respiratory tract as well as systemic disorders including diabetes mellitus, AIDS, malignancies, cardiac and renal diseases are important predisposing factors to infections caused by *M. catarrhalis*. Other precipitating conditions like alcoholism and smoking play a great role in pulmonary infections. In this study over 75% of the patients infected with *M. catarrhalis* had underlying diseases; only a small number of people without underlying diseases were infected. This suggests that prior damage to the respiratory epithelium by other infections as well as decreased antibody level due to systemic infections are important conditions for infection.

In this study, more than half of the *M. catarrhalis* isolates were β-lactamase producers. This indicates that it is very important to test *M. catarrhalis* isolates for β-lactamase production for successful treatment of the patient. The frequency of β-lactamase production by *M.*

catarrhalis isolated from sputum of pneumonia patients was 54.4% and those from the nasopharynx were 57.1%. These results are not much different from that of Mannion (1986) who found that 53% of *M. catarrhalis* isolates from patients with respiratory infections were β-lactamase producers. Although many β-lactamase producing strains appear to be susceptible to penicillin, ampicillin or amoxicillin *in vitro*, failures in treatment of patients have been reported when β-lactam drugs have been used for treatment of lower respiratory tract infections (Fung *et al.*, 1994). β-Lactamase production was reported earlier to be correlated with resistance to amoxicillin, penicillin, and ampicillin treatment of patients with respiratory tract infections from whom β-lactamase producing *M. catarrhalis* was isolated (McLeod *et al.*, 1983). In addition it is well known that β-lactamase producion by *M. catarrhalis* may protect other pathogens, notably, *Strep. pneumoniae* and *H. influenzae* from the actions of penicillin and ampicillin. The increasing frequency in β-lactamase producing strains is associated with the increase antibiotic use.

Because of the frequency with which *M. catarrhalis* is isolated from the upper respiratory tract and the difficulty presented by the presentation of the isolates, the analysis of total IgG as well as IgG subclass responses to this bacteria in convalescent-phase sera helps to identify its role in diseases (Goldblatt *et al.*, 1990b). In our study, 40 of 68 patients with pneumonia caused by *M. catarrhalis* showed significant increases in antibody titres to the OMPs of *M. catarrhalis* in the convalescent-phase sera. Patients without significant antibody responses had low titres in both the acute and convalescent-phase sera. These patients may have had pneumonia caused by other pathogens and later colonized by *M. catarrhalis*. It was found that more than half (58.8%) of the patients from whom *M. catarrhalis* was isolated showed

significant increases in IgG-A-M antibody titre and 63.2% in IgG3 in the convalescent-phase sera. The remaining 41.2% and 36.8% showed less than 1.5 fold antibody titre for IgG-A-M and IgG3 respectively. Leinonen *et al.* (1981) reported that 14 of 19 (52.6%) *M. catarrhalis* isolates showed increased IgG titre in the convalescent-phase sera. In another study, Jonsson *et al.* (1994) found a 48% increase in antibody titre. The relatively lower antibody titre increases seen in our patients could be explained by the presence of multiple pathogens, or because patients arrive at hospitals late when serum antibody levels have already increased and it is difficult to show a further significant increase. Some patients showed increased IgG-A-M titres but not in IgG3; other patients showed the reverse. This indicates that some of the patients may have been experiencing systemic illnesses caused by *M. catarrhalis* for the first time. In addition patients come to hospital at different stages of the illness and antibody responses are also different in different individuals.

The LPS of *M. catarrhalis* does not possess the O-antigen carrier side chain of enterobacteria and is similar to the O antigen of *N. gonorrhea* and *N. meningitidis*. The LPS of *M. catarrhalis* consists of different antigenic specificities (Vaneechoutte *et al.*, 1990a). Three major antigenic types of LPS designated as types A, B, and C exist in *M. catarrhalis*. Cross-reactions between these types is not uncommon.

Although the determination in ELISA of IgG-A-M as well as IgG3 subclass responses to *M. catarrhalis* antigens (OMP and LPS) in the sera of patient with pneumonia caused by this bacterium are important in confirming the diagnosis of *M. catarrhalis* infections, this technique takes more time, reagents are not easily availability and costy. It does not seem

likely that these tests would be performed routinely here.

The serum antibody response to the LPS of *M. catarrhalis* is lower even at higher LPS antigen concentrations than the antibody response to the OMP using the conjugated IgG-A-M and IgG3 antibodies. The LPS elicited no IgG3 response. A ≥ 1.5 fold increase in total immunoglobulins to LPS was observed in 16 of 68 patient sera (23.5%) and 2 of 30 (6.7%) of controls. The results indicate that the pathogen induces production of antibody against the LPS antigens in some infected patients. Unlike the OMP antigens, only a few of the patients in this study had significantly increased anti-LPS antibodies in their convalescent-phase sera compared to that of the acute-phase sera. This may be because only one LPS type was used in the study. But, generally, this low antibody response makes the use of LPS in ELISA to detect antibodies against *M. catarrhalis* less specific compared to that of the OMP (Vaneechoutte *et al.*, 1990a). The reason for the relatively reduced antibody responses is probably due to the weak immunogenic stimulus of the LPS of *M. catarrhalis* on the host. On the other hand, in mixed infections, one cannot be sure which bacterial species is more important in producing the illness and the amount of antibody produced. The small number of antibody titre increases seen in the controls could be a result of cross-reactions to the LPS of other respiratory pathogens.

OMP and LPS based vaccines are currently being studied as potential vaccines against infections caused by group B meningococci (Verheul *et al.*, 1995). From our studies it would seem that, at least the OMPs of *M. catarrhalis* would be good candidates to use for vaccine development. Different components of the host defence act in concert after challenge by

bacteria. The bactericidal activity is facilitated by the complement system. Complement-mediated humoral killing is of greater importance in extracellular bacterial infections. Adequate amounts of antibodies are necessary to provide complement-antibody activity. The bactericidal action of the antibody-complement systems plays a role in the pathogenesis of infections caused by *M. catarrhalis*. Human host defence mechanisms against *M. catarrhalis* remanied to be elucidated. There seems to be a marked variation of susceptibility among clinical isolates to the bactericidal effects of human serum. Up to 95% of isolates were identified to be resistant to normal human serum (Chapman *et al.*, 1985).

Human host defence mechanisms against *M. catarrhalis* have remained largely unstudied. Convalescent-phase sera has bactericidal activity against homologous strains of *M. catarrhalis* at higher concentrations. The normal human sera used as a complement source throughout the study was able to induce the killing effect in our study. In the control group which were devoid of complement, minimal or no bactericidal activity was observed. This shows that specific antibodies produced against *M. catarrhalis* are able to protect against infection. In this study, different concentrations from 20-60% of sera were performed and good bactericidal activities were observed at 60% concentrations. Significant bactericidal activity was obtained in 86.7% of the sera. This shows that *M. catarrhalis* is able to induce the production of bactericidal antibodies. The control assay did not show significant bactericidal activity compared to that of the test assays ($P< 0.05$). This finding is in agreement with that reported by Chapman *et al.* (1985) which was 90%. Two sera failed to kill effectively the homologous bacterial isolates. These *M. catarrhalis* isolates may carry resistant phenotypic characteristics which renders them able to resist killing by human serum. Such phenotypic characteristics are

possible virulence factors. In addition, other antibodies may block bactericidal activities of serum by combining with antigens that are the sites that recognize bactericidal antibody, thereby preventing the attachment of these antibodies and preventing deposition of C5, C6-C9 which complete the membrane attack system.

In suitable *in vitro* systems, the complement-mediated reactions often proceed at an impressive rate. On the other hand various structures such as outer membranes, LPS and other cell envelope polymers are implicated as mediators of resistance to activated complement components.

Before any β-lactam antibiotic is prescribed for the treatment of a patient with bronchopulmonary infection, antibiotic susceptibility tests should be done. On the basis of the susceptibility test results, the first line antimicrobial therapy recommended for uncomplicated cases of acute otitis media and acute sinusitis caused by non-β-lactamase *M. catarrhalis* strains in children is amoxicillin. The drug is safe and effective against most of the causative bacterial pathogens as well (Eliasson *et al.*, 1990; Giebink, 1992). Antibiotics like ampicillin, erythromycine, amoxicillin, and tetracycline are effective in the treatment of patients with pneumonia caused by non β-lactamase producing *M. catarrhalis* strains. For patients with pneumonia caused by β-lactamase producing *M. catarrhalis* strains a combination of amoxicillin/clavulanate (augumentin), co-trimoxazole (bactrim), and erythromycin/sulfisoxazole are recommended (Bluestone, 1992). These antibiotics combinations are easily available, inexpensive and well tolrated by the patient.

CONCLUSION

M. catarrhalis, either singly or in combination with other pathogens, is a common cause of community-acquired pneumonia especially in patients with underlying diseases. The full spectrum of respiratory diseases caused by *M. catarrhalis* has not been delineated. We know little about the pathogenicity factors that the organism possesses, the nature of important surface antigens, and the host response to infection. But we do know that identification of *M. catarrhalis* in sputum needs biochemical confirmation because many similar non-pathogenic *Neisseria* species mislead laboratory personnel and clinicians. The Gram-stain technique is simple, inexpensive that should be employed primarily. When supported by clinical, radiological, biochemical and serological findings, it helps the clinician to decide on the choice of antibiotics. Despite the limited numbers of serological studies, it is becoming evident that *M. catarrhalis* has clinical significance in lower respiratory tract infections. There is a good IgG3 response to the OMPs of *M. catarrhalis*. On the other hand *M. catarrhalis* does not seem to induce a good antibody response to its LPS antigens. Serum antibodies to *M. catarrhalis* in the presence of complement kill the organisms in vitro very effectively. The bactericidal assays show that the antibodies induced by the bacterial OMPs together with complement components are able to kill *M. catarrhalis in vitro*. Our findings support the view that isolation of *M. catarrhalis* from nasopharyngeal and sputa samples is of value for the etiological diagnosis of lower respiratory tract infections, and that sensitivity testing is important for the choice of chemotherapeutic agents.

Our studies show that *M. catarrhalis* is an important pathogen of the lower respiratory tract

which should be isolated, identified, and reported. The β-lactamase production by *M. catarrhalis* in our patients is high. Screening of the isolates for β-lactamase is important for the appropriate drug administration and management of infections. Otherwise, treatment with inappropriate antibiotics may result in increased morbidity or mortality especially in patients with pneumonia and chronic obstructive pulmonary diseases (COPD). Because of the above problems for serious infections enzyme inhibitors such as clavulanic acid and sulbactam may provide a promising approach in the treatment of *M. catarrhalis* infections. The second and third generation cephalosporins are also effective against the bacterium.

The high antibody titres against the outer membrane proteins shows that serology is an important diagnostic method in the diagnosis of systemic *M. catarrhalis* infections. However, base-line values helpful to separate naturally occurring antibody titres from those resulting from present acute infections are not known and should be further studied.

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APPENDIX

***Moraxella (Branhamella) catarrhalis* study**

Pt's. name _____ Sex _____

Age _____ Date _____

Hosp. No._____ Address _____

Date of onset of illness _____

Inclusion criteria

All adult patients living in Addis Ababa with community-acquired pneumonia visiting the out patient department.

Underlying diseases ?

Chronic bronchitis _____ Tuberculosis _____

Diabetes mellitus _____ Known HIV _____

Cancer _____ Other ? _____

Smoking ?

Yes _____ No _____

Antibiotics in the last 2 weeks ?

Yes _____ No _____

If yes, which antibiotics ? _____

Date of last dose _____

Symptoms

Fever >38°C ____ Cough _____ Purulent sputa ___

Diarrhoea ? _____ Vomiting ____ Meningitis _____

Others ? Specify _____

Treatment given in the hospital

Antibiotics given _____

For how many days? _____

Sample collection

Day of 1st visit: Sputum culture _____

Nasopharyngeal swab culture _____

Serum No. 1 _____

Day of 2nd visit (2 weeks after the first serum was taken) :

serum No. 2:_____

Laboratory and X-ray results

ESR _____

WBC _____

X-ray _____

Other, Specify _____