EVIDENCE FOR A NOVEL BACTERIOPHAGE FROM *MORAXELLA CATARRHALIS*

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

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ABSTRACT

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Moraxella catarrhalis is one of the major causes of RTI and otitis media, which was known as a harmless inhabitant of upper respiratory tract until 1980s. The knowledge on virulence factors, pathogenesis of this bacterium is scarce and the reason/(s) for recent pathogenic conversion of *M. catarrhalis* is/are not known. Several examples demonstrate that bacteriophages control bacterial virulence in almost every step of pathogenesis. Number of bacteriophages are known to be solely responsible from bacterial virulence. We hypothesize that a bacteriophage may be responsible for pathogenic conversion of *M. catarrhalis*, and we investigated whether a bacteriophage is present in *M. catarrhalis*. In this study, evidence for a bacteriophage from *M.catarrhalis* is presented. Supernatants of *M.catarrhalis* broth cultures were shown to cause bacterial cell lysis on soft agar cultures, indicating presence of bacteriophages released from them. Two particles having different morphologies and different size ranges ($p<0.05$) were co-purified from these supernatants. One segment of dsDNA molecule and three segments of ssRNA molecules were extracted from these particles. The comparison between pathogenic and non-pathogenic strains of *M.catarrhalis* demonstrated marked differences in the quantity of these RNA and DNA molecules.Future studies are necessary to determine the origins of RNA and DNA molecules.

Keywords: Moraxella catarrhalis, bacteriophage, virulence.

ÖZET

MORAXELLA CATARRHALIS'DEN EDİNİLEN YENİ BİR BAKTERİYOFAJ'A DAİR BULGULAR

Gülçin Çakan
Moleküler Biyoloji ve Genetik, Yüksek Lisans
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1980'li yıllara kadar solunum yollarında yerleşik zararsız bir bakteri olarak bilinen *Moraxella catarrhalis*, solunum yolları enfeksiyonu ve otitis media'nın başlıca nedenlerinden biridir. Bu bakterinin virülens faktörleri ve patojenezi hakkındaki bilgiler az sayıda olup son zamanlarda gerçekleşen patojenik dönüşümünün ardında yatan nedenler bilinmemektedir. Bakteriyofajların bakteri virülensini, patojenezin hemen her aşamasında kontrol edebildiği örneklenmiştir. Bazı bakteriyofajlar ise bakteriyel virülensin tek sorumlusudurlar. *M. catarrhalis*'in yakın zamanda geçirdiği patojenik dönüşümün bakteriyofaj kaynaklı olduğunu varsayarak *M. catarrhalis*'in bakteriyofaj içerip içermediğini araştırdık. Bu çalışmada, *M. catarrhalis*'in bakteriyofaja sahip olduğuna dair kanıtlar sunulmaktadır. Varolan bakteriyofajların salındığını destekleyecek şekilde, *M. catarrhalis* sıvı (broth) kültürlerinden elde edilen süpernatantların, yumuşak agar bakteriyal kültürlerinin dağılmasına (lysis) yol açtığı gösterilmiştir. Bu süpernatantlardan farklı morfoloji ve boyutlara sahip olan iki ayrı yapı birlikte izole edilmiştir. Bu yapılardan biri dsDNA diger üçü ise ssRNA olan nükleik asit fragmanları elde edilmiştir. Patojenik ve patojenik-olmayan *M. catarrhalis* türleri arasinda yapılan karşılaştırmalar, bu iki grup arasında RNA ve DNA miktarlarında belirgin farklılıklar olduğunu göstermiştir. Elde edilen RNA ve DNA moleküllerinin kaynaklarının ortaya çıkarılması için araştırmaların sürdürülmesi gerekmektedir.

Anahtar sözcükler: *Moraxella catarrhalis*, bakteriyofaj, virülens.

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Abbreviations

BHI	Brain Hearth Infusion
cDNA	complementary Deoxyribonucleic Acid
CHIPS	Chemotaxis Inhibitory Protein
ddH_2O	distilled deionized water
DEPC	Diethylpyrocarbonate
dH_2O	distilled water
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetate
GAS	Group A *Streptococci*
GTPase	Guanidine triphosphatase
HMW	High Molecular Weight
Hfr	High Frequency of Recombination
ICTV	International Committee for Taxonomy of Viruses
LOS	Lipooligosaccharide
LRTI	Lower Respiratory Tract Infections
lt	liter
M	Molar
μg	Microgram
μl	Microliter
μM	Micromolar
MVs	Membrane Vesicles
mg	Milligram
ml	Milliliter
mM	Millimolar
MOPS	3-(N-Morpholino)propanesulfonic acid 4-Morpholinepropanesulfonic acid
OD	Optical Density

OMP	Outer Membrane Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PVL	Panton-Valentine Leukocidin
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonuclease
RTI	Respiratory Tract Infections
SM	Suspension Medium
SodC	Superoxide Dismutase
ssRNA	single-stranded RNA
TAE	Tris-Acetate-EDTA
tRNA	transfer RNA
RNase	Ribonuclease
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SCID	Severe Combined Immunodeficient
SDS	Sodium Dodecyl Sulfate
SM1	*Streptococcus mitis* Phage
SPI	*Salmonella* Pathogenicity Island
PAGE	Polyacrylamide Gel Elecrophoresis
TCP	Toxin Co-regulated Pilus
T_m	Melting Temperature
Usp	Ubiquitous Surface Protein
VPI	Vibrio Pathogenicity Island

Chapter 1

Introduction

1.1 *Moraxella catarrhalis*

Moraxella catarrhalis is a gram-negative, aerobic, diplococcus initially described in 1896 with the name *Mikrokokkus catarrhalis* (Frosch, 1896). The organism has also been known as *Micrococcus catarrhalis, Neisseria catarrhalis* (Enright & McKenzie, 1997), and *Branhamella catarrhalis* (Catlin ,1990). To date the argument over the nomenclature of this bacterium is unresolved, although most investigators now use *Moraxella catarrhalis.*

M. catarrhalis is frequently found as a harmless inhabitant of human respiratory tract, however since the first pathogenic strains of *M. catarrhalis* has been identified by Jonhson in 1981 the number of infections caused by this bacterium increased significantly (Johnson & Schilievert, 1981). Today, the organism is accepted as a common and important pathogen, although the factors responsible for its pathogenicity and the increase in the prevalence of *M. catarrhalis* infections are poorly understood (McGregor et al., 1998).

1.1.1 Infections

In infants, colonization of upper respiratory tract by *M. catarrhalis* is common; upto 78% of infants being colonized during the first two years of life. The number of colonized individuals decreases gradually as the children get older and finally only 1-5% of adults are colonized by this organism (McGregor et al., 1998).

In children, this bacterium is a major cause of otitis media preceeded by *H.influenzae* and *S. pneumoniae* (Catlin 1990, Murphy 1996, Christensen 1999). Various groups from Finland, Norway, United States, Japan and Spain demonstrated that about 15-20% of acute otitis media cases in children are caused by *M. catarrhalis* (Karalus & Campagnari, 2000). Acute otitis media is a common problem in infants and children, and recurrent episodes of infections are also common. This may result in hearing loss associated with learning and developmental problems as children reach school age (Murphy, 1996). In U.S. an estimated 3-4 million cases of otitis media are caused by *M. catarrhalis* alone (Murphy & Sethi, 1992) which cost an estimated 2 billion every year (Murphy, 1996).

M. catarrhalis is the third most common cause of respiratory tract infections (RTI), after *H.influenzae* and *S.pneumoniae*. *M. catarrhalis* can cause pneumonia (Wright 1990, West 1982), sinusitis (Brorson 1976), bronchitis, laryngitis and tracheistis (Verduin et al., 1992). In adults, *M. catarrhalis* infections are found in presence of predisposing factors such as chronic obstructive pulmonary disease (Wright 1990, Murphy, 1996), immunodeficient state (McNeely 1976, Diamond 1984), viral damage to respiratory tract epithelium (Arola 1990) or smoking (McGregor, 1998).

M. catarrhalis causes RTI predominantly in elderly (McGregor, 1998), and cause a significant number of morbidity in infected individuals. It is reported that 81% of the patients infected with *M. catarrhalis* are over 55 years old where 45% of elderly patients died within 3 months of acquiring *M. catarrhalis* (Wright et al., 1990). The reason for low rate of *M. catarrhalis* infections in healthy adults is thought to be the strong immune system in these individuals. Hence, children

whose immune system is immature and old people whose immune system is weak are more prone to *M. catarrhalis* infections (Verduin et al., 2002). In children , mostly under one year of age, it can cause lower respiratory tract infections (LRTI) in absence of predisposing factors (McGregor, 1998).

There have been sporadic reports of meningitis (Cochi 1968, Feign 1969, Doern 1981, Nagyi et al., 1988) and a case of fatal neonatal meningitis (Daoud et al., 1996) caused by *M. catarrhalis*. This bacterium is also associated with nosocomial infections and very rarely causes keratitis, endocarditis (Douer et al., 1977), urogenital tract infection (Elbasnier & Desphande, 1993) and septic arthritis (Craig et al., 1983).

1.1.2 Virulence Mechanisms and Factors

Our knowledge regarding the virulence factors and pathogenesis of *M. catarrhalis* is scarce. Although two decades has past since this organism was recognized as a pathogen, we do not yet know the mechanism/(s) of its pathogenic conversion.

M. catarrhalis appears to be a strict human pathogen, therefore a good animal model to investigate the pathogenesis does not exist. The best models available are the Murine Pulmonary Clearance Model (Unhanand et al., 1992) and the SCID (Severe Combined Immunodeficient) mouse model (Harkness et al., 1993). In the pulmonary clearance model, mice are challenged by introducing bacteria into their lungs and the rate of clearance is followed as a measure of the immune response. In SCID mouse model, bacteria are introduced by multiple routes including intranasal and intravenous, but the symptoms produced in these animals do not mimic those seen in human infection.

Fortunately identification of bacterial colonization factors responsible for attachment of *M. catarrhalis* to human cells is possible by doing attachment experiments. Since the attachment of bacteria to human cells is the first step for colonization and pathogenesis, adhesins are accepted as virulence factors.

Bacterial attachment is a complex process which might include more than one adhesin-receptor interaction. Fimbriae (Ahmed 1992a), hemagglutinins, UspA1 (Aebi 1998) and CD protein (Murphy 1993) are the adhesions; ganglioside GM2 (Ahmed & Matsumoto 1996) and asialoganglioside GM1 (Ahmed et al., 2002) are the receptors for *M. catarrhalis* identified so far.

1.1.2.1 Fimbriae

Fimbriae have been described on the surface of *M. catarrhalis* by electron microscopy (Ahmed et al., 1994). Recently, type IV pili expression by *M. catarrhalis* has been demonstrated by RT-PCR (Luke et al.,2004). Fimbriae are composed of repeating subunits; the part that interacts with the host cell is on the tip of the pilus (London & Kolenbrander, 1996). In *M. catarrhalis*, fimbriae are responsible for adherence and hemagglutination, therefore thought to be important virulence factors (Ahmed 1992a). It was also found that fimbriae are present on all fresh isolates of *M. catarrhalis*, and it decreases in number upon repeated *in vitro* passages (Ahmed et al., 1992). Since fimbriae have not been isolated, little information is available regarding the characterization of these structures.

1.1.2.2 Lipooligosaccharide(LOS)

Another prominent bacterial surface component of *M. catarrhalis* is the LOS. There are three major LOS serotypes in *M. catarrhalis*: A, B and C; these structures encompass 95% of all strains studied to date (Rahman & Holme, 1996). *M. catarrhalis* LOS shares homology with LOS of other gram-negative bacteria. These shared LOS epitopes have been implicated as virulence factors for *M. catarrhalis* i.e. resistance to bactericidal activity mediated by normal human serum (Zaleski et al., 2000).

Recently, it has been found that LOS is not an adhesin of *M. catarrhalis* however plays an important role in attachment by providing surface charge of the

bacteria (Akgulet al., 2005).

1.1.2.3 CD Protein

Outer Membrane Protein (OMP) CD is a heat-modifiable protein that was initially reported as OMP C and OMP D. Later it was realized that these two proteins were the two forms of a single antigen. This protein has homology with *Pseudomonas aeruginosa* porin protein (Murphy et al., 1993). Sequence analysis and RFLP studies on OMP CD show high degree of conservation among different strains of *M. catarrhalis*.

The antibody against OMP CD elicits complement-mediated bactericidal activity against various strains of *M. catarrhalis* which indicates that it may stimulate a protective immune response if used as a vaccine.

1.1.2.4 UspA1 and UspA2

Initially a ubiquitous protein in the outer membrane of *M. catarrhalis* was defined with the name High Molecular Weight OMP (HMW-OMP).Subsequently it was realized that the HMW-OMP was actually an oligomeric complex of two proteins and they were named as ubiquitous surface protein A1 and A2 (UspA1 and UspA2). They were initially characterized as a single protein not only because of a single band in SDS-PAGE, but also because they share a common epitope which is a 140-aminoacid region that is 93% identical (Cope 1999).

1.1.3 Antibiotic Resistance

M. catarrhalis is currently treated with following antibiotics: amoxicillin-clavulunic acid, second generation and third generation cephalosporins, trimethoprim-sulfamethaxole, fluoroquinolones and newer macrolides such as

azithromcycin or clarithromycin (Spach & Black, 1998). However the organism has acquired resistance to β-lactam antibiotics over the last 25-30 years. The first β-lactamase-positive clinical strain was isolated in 1977 β (Hoi-Dang et al., 1978). Subsequently, resistance to β-lactams has increased at a rate unprecedented for any bacterial species (Karalus & Campagnari, 2000), and currently more than 90% of clinical isolates are β-lactamase positive (Felmingham 1999). In North America and Europe almost 100% of the strains were reported as -lactamase producers (Clavo-Sanchez 1997). However, the ratio of β-lactamase producer strains varies from country to country; in Japan it is 94% (Martinez et al., 1998), in Kuwait it is 57% (Ahmed et al., 1999), in Uganda it is 100% (Yoshimine et al., 2001) and in Turkey it is 100% (Ozyilmaz et al., 2005). In a study conducted in five centers and four cities of Turkey, 81% of the *M. catarrhalis* isolates obtained respiratory tract specimens were found to produce β-lactamase. Rates of β-lactamase production varied from 38 to 90% in five centers (Gur et al., 2002).

The cause of the increase in number of β-lactamase producer strains is not known, but it is thought that increased use of antibiotics has contributed to this dramatic increase. However, such increase has not been demonstrated in other bacteria under the same antibiotic selective pressure (Felmingham & Washington, 1999).

Although *M. catarrhalis* is treatable with non-β-lactam antibiotics, β-lactamase production by this organism causes problems since *M. catarrhalis* can act as a co-pathogen, providing a source of β-lactamase that renders β-lactamase unstable drugs ineffective against β-lactamase-negative respiratory pathogens (Wardle 1986, Enright 1997).

The β-lactamase enzymes of *M. catarrhalis* are named BRO-1 and BRO-2, differ by a single amino acid substitution [BRO-2 contains a Gly residue at position 294 whereas BRO-1 contains Asp residue] (Bootsma , 1996). They confer resistance to penicillin, ampicillin and amoxycillin and are inactivated by β-lactamase inhibitors (Livermore 1995). The enzymes differed from other known β-lactamases of gram-negative bacteria by substrate profile and isoelectric point (Farmer 1982, Buu HoiDang 1978, Simpson 1983). BRO β-lactamases

have been identified only in 2 closely related Moraxella species (*Moraxella nonliquefaciens* and *Moraxella lacunata*) (Wallace , 1989), they are quite different from β-lactamases of other pathogenic bacteria, and the origin is unknown (Hoi-Dang 1978, Karalus 2000). They have been suggested to be lipoproteins originated from gram-positive bacteria (Bootsma, 1996), however this observation has not been yet confirmed with further studies.

Exceptional spread of BRO -lactamases suggests that these bacteria developed an efficient transfer mechanism (McGregor. 1998). The genetic heterogeneity seen on molecular typing of *M. catarrhalis* strains argues against clonal expansion as a mechanism of transferring β-lactamase enzymes (McGregor , 1998 his comment). Differences between β-lactamase producing strains were demonstrated by restriction endonuclease typing (Patterson, 1988; Patterson 1989). In addition, G+C content of the bro gene was shown to be 31% and was significantly lower than that of the flanking genes (47% and 50%),and overall G+C content of *M. catarrhalis* (41%) (Bootsma, 1996). These findings implicates horizontal gene transfer in the acquisition of the BRO β-lactamases (McGregor, 1998).

The emergence of β-lactamase-positive strains motivated scientists to find a vaccine against *M. catarrhalis*. But vaccine development for *M. catarrhalis* is only in the antigen identification stage (McMichael, 2001).

1.2 Role of Horizontal Gene Transfer in *M.catarrhalis* Virulence

Advances in the understanding of the molecular basis of microbial pathogenesis have led to the hypothesis that more virulent bacterial strains can emerge through recent acquisition of virulence factors. A virulent pathogenic bacterium may differ from its avirulent relative by only a small number of genes. These genes are called virulence genes and they are generally clustered together in pathogenicity islands (PIs) (Groisman 1996, Hacker 1997). A variety of pathogenicity

islands show evidence of recent acquisition by horizontal gene transfer. Mostly, the horizontal gene transfer is obvious since the virulence genes are encoded in mobile elements such as plasmids, transposons or bacteriophages. In some cases, although there is no evidence of currently functional transfer machinery the site of chromosomal integration of PIs indicates that they were acquired by horizontal gene transfer (Hacker 1997, Ochman 2000). For example, the pathogenicity islands often reside at tRNA and tRNA-like loci which appear to be common sites of foreign DNA integration (Hacker 1997, Ochman 2000). And frequently, the flanking sequences around the pathogenicity islands include short direct repeats reminiscent of those generated during mobile genetic element integration (Ochman 2000). In other cases, the difference between the nucleotide composition of virulence genes and the genes encoding highly conserved essential functions is a sign of horizontal transfer of the virulence genes (Miao 1999).

1.2.1 Mechanisms of Horizontal Gene Transfer

1.2.1.1 Conjugation

It is the most widespread mechanism of gene transfer between bacteria of same or different species. It requires cell to cell contact mediated by sex pili, for transfer of DNA. Transmission of DNA from donor to recipient can be mediated by a plasmid, which is self transmissible or mobilizable or which can integrate into the chromosome forming a high frequency of recombination (Hfr) strain (Buchanan-Wollaston 1987, Heinemann 1989). Conjugation may also take place through conjugative transposons which encode proteins required for excision, formation of a conjugative bridge and transposition into the recipient strain (Dutta 2002).

1.2.1.2 Transformation

With this mechanism naked DNA can be transmitted between related or unrelated bacterial species provided that the donor and recipient cells are present at the same place at the same time. Some species like *Bacillus subtilis* and *Streptococcus pneumoniae* can undergo high levels of transformation only when they reach specific physiological stages in their life-cycles (Dubnau 1999). But species like *Neiserria gonnhoroae* and *Haemophillus influenzae* are perpetually able to take up foreign DNA by transformation (Pifer 1985, Goodman 1988, Elkins 1991). Although transformation of *M. catarrhalis* is possible under experimental conditions where naked DNA is spotted on bacterial colonies (Skerker 2001), presence of an endogenous and very active endonuclease in *M. catarrhalis* (Kamme, 1984) suggests that natural transformation is difficult to occur in this bacterium.

1.2.1.3 Transduction

Transduction is the bacteriophage mediated transfer of genetic materials between organisms recognized by the phage (Schicklmaier 1995, Jiang 1998). In this process, phage proteins can promote the integration of the transferred DNA into host chromosome to protect from degradation by host restriction endonucleases (Dutta 2002). Two types of transduction may occur; specialized transduction and generalized transduction. Specialized transduction occurs when a prophage is imprecisely excised from the host and small segments of flanking bacterial DNA is co-packaged with the phage DNA and transferred to other bacteria in each infection. In the case of pathogenic bacteria these extra genes frequently encode important virulence factors like bacterial toxins (Baba 2002, Beres 2002, Smoot 2002). Generalized transduction occurs when a segment of bacterial DNA is packaged into phage particle instead of phage DNA and incorporated into the genome of next host. Phages such as *Salmonella* phage P22 or coliphage Mu occasionally commit the error to package even a head-full of bacterial DNA instead of phage DNA (Canchaya 2003).

Among the mechanisms for transfer of DNA, transduction by bacteriophages appears to be advantageous. Transduction is efficient, and in contrast to conjugative transfer of plasmids, does not require intimate contact between bacteria. Bacteriophages can carry large blocks of DNA and can survive harsh conditions that eliminate bacterial populations. Therefore, DNA important to a population can be preserved until a host for lysogenic conversion is reintroduced into an environmental niche. Bacteriophages can also spread DNA directly to an entire population of bacteria, eliminating the need for clonal expansion of a specific population. Bacteriophages encoding virulence factors can convert their bacterial host, in a process known as lysogenic conversion, from a non-pathogenic strain to a pathogenic strain or a strain with increased virulence (Boyd 2002).

1.3 Bacteriophage Control of Bacterial Virulence

Bacteriophages are viruses that infect bacteria, discovered at the beginning of 20th century. In 1896, Hankin reported that something which could pass through a very fine porcelain filter in the waters of the Ganges and Jamuna rivers in India had marked antibacterial action. Later in 1915, a British bacteriologist Frederick Twort, actually isolated filterable entities capable of destroying bacterial cultures and producing small cleared areas on *Micrococcus* cultures. Felix Hubert d'Herelle, a French Canadian microbiologist working at the Pasteur Institute in Paris, reported the lysis of *Shigella* cultures in broth in 1917. D'Herelle recognized the viral nature of his agent and named it 'bacteriophage' or bacteria-eater (from the Greek phago meaning to eat). He postulated the intracellular multiplication of viruses and introduced phage therapy of infectious diseases (Ackermann 2003).

Thousands of varieties of phage exist, each of which may infect only one type or a few types of bacteria. ICTV classified phages into 13 families, and 30 genera (Regenmortel 2000). Phage virions are tailed, filamentous, polyhedral or

pleomorphic (Ackermann 2003). Like all viruses, phages are simple organisms that consist of a core of genetic material surrounded by a protein capsid. The nucleic acid may be either DNA or RNA and may be double-stranded or single-stranded, linear or circular. RNA bearing bacteriophages may have segmented or unsegmented genomes.

During infection a phage attaches to a bacterium and inserts its genetic material into the cell. After this, a phage follows one of two life cycles, lytic (virulent) or lysogenic (temperate) (Madigan 2003). Lytic phages utilize the machinery of the cell to produce phage components and lyse the cell, releasing new phage particles. Lysogenic phages incorporate their nucleic acid into the chromosome of the host cell and replicate with it as a unit without destroying the cell. In this case, an induction event, such as stress to the host, can trigger a switch to lytic infection (Fuhrman, 1999).

A less well-defined type of phage-host interaction is termed pseudolysogeny, in which the phage nucleic acid may remain within a host cell for some time (possibly for a few generations) before lysis, or cell destruction, occurs (Lenski 1998). Following infection, bacteriophage can either enter a dormant intracellular phase or proceed with lytic infection (Wommack, 2000). In this respect, pseudolysogeny resembles true lysogeny. Unlike in true lysogeny, however, the phage genome does not integrate into host cellular replicons (Williamson et al., 2001).

Pseudolysogeny may be related to host starvation, in which the virus adopts an inactive state, unable to initiate viral gene expression owing to the low energy state of the cell; normal viral activity returns when the cell is fed (Ripp & Miller, 1997). Alternatively, pseudolysogeny may be regarded as a transient state of host immunity, apparently induced by an immunizing agent (perhaps a polysaccharide depolymerase) released from infected cells, and helping to foster coexistence of host and virus (Moebus 1996, Moebus 1997). Viruses or virus-like particles may also be involved in killing cells by mechanisms that do not result in virus reproduction (Chiura 1997) Pseudolysogens are often characterized by the sustained production of phage along with a thriving population of host cells (Ackermann, 1987). Another indicative characteristic of pseudolysogeny is the failure to induce

prophage into a lysis cycle through the introduction of a mutagenic agent, such as mitomycin C (Wommack & Colwell, 2000).This result is caused by the lack of chromosomal integration.

Bacteriophages' contribution to the bacterial pathogenicity began to be uncovered as early as 1927, when Frobisher and Brown discovered that nontoxigenic *streptococci* acquired the ability to produce scarlatinal toxin when exposed to filtered supernatants of toxigenic *streptococcal* cultures. Much later the scarlatinal toxin has been proven to be phage encoded (Zabriske 1964; Weeks 1984; Johnson 1984), and the list of phage-encoded virulence factors contributing bacterial pathogenicity has been growing since then. Today, we know that bacteriophages can alter the host bacterial properties relevant to all stages of infection; including adhesion, colonization, invasion, and spread of bacteria among human tissues; exotoxin production, resistance to antibiotics and immune defenses; and transmissibility among humans. (Patrick & Mathew, 2002).

1.3.1 Adhesion

One group of phage-encoded virulence factors are proteins involved in bacterial attachment to host cells.

Streptococcus mitis adheres to platelets, fibrin, and exposed valvular matrix components, giving rise to the vegetations that damage heart valves and provide a source of bacteremia and septic emboli in infective endocarditis. Recently, a bacterial loci encoding two surface proteins, PblA and PblB has been shown to be involved in platelet adherence (Bensing et al., 2001a). These genes which were initially found to have similar sequences with phage capsid and tail fiber genes, reside on an inducible prophage, SM1 (Bensing et al., 2001b), and encode proteins present in the SM1 phage particle. Whether and by what mechanisms the phage-particle-associated and/or the bacterial-membrane-bound fractions of PblA and PblB contribute directly to vegetation formation by *S. mitis* remain unknown(Patrick 2002).

In *Vibrio cholerae* the toxin co-regulated pilus (TCP) plays an essential role in colonizing the human host. The tcp gene cluster is required for biogenesis and regulation of TCP production, and was initially described as part of *Vibrio* pathogenicity island (VPI) (Kovach et al. 1996, Karaolis et al. 1998). However, it is now proposed that the 39.5kb VPI corresponds to the genome of an unusual filamentous phage, VP, and the major pilin protein TcpA is also the bacteriophage coat protein (Karaolis 1999).

1.3.2 Invasion

Some bacteria invade through human tissues by help of enzymes degrading extracellular matrix components, such as collagenases, hyaluronidases and hemolysins (Patrick 2002). The hyaluronidase of group A *streptococci* (GAS) is phage encoded (Hynes 1989) and the hyaluronidase activity is associated with the phage particles (Benchetrit 1977; Benchetrit 1978). This enzyme may function in aiding phage to penetrate the *streptococcal* capsule which is composed solely of hyaluronic acid; it may also have a role in spread of the *streptococci* through human connective tissue, which is suggested by presence of phage-encoded hyaluronidase in sera of GAS-infected patients (Halperin 1987). However, this hypothesis has not been tested yet.

Salmonella enterica serovar thyphimirium uses *Salmonella* pathogenicity island 1 (SPI1)-encoded type III secretion system, to inject effector proteins directly in to the cytoplasm of host cells aiding invasion (Hueck 1998). SopE is one of these secreted proteins, which activates human RhoGTPases and contributes to efficient entry of *Salmonella* into tissue culture cells (Hardt 1998a, Hardt 1998b, Wood 1996). The sopE gene is encoded in a temperate phage, SopE?. Thus lysogeny of this phage aids in adaptation of *Salmonella* to its mammalian host, by interaction of SPI1 and SopE? (Patrick 2002). Another early step during *Salmonella* infection is its localization to Peyer's patches, and the gene called gipA which is encoded by the phage Gifsy-1 is expressed specifically in the Peyer's pathces and is necessary for optimal survival in the patches (Stanley 2000).

1.3.3 Resistance to serum and phagocytes

Staphylococci produce a number of proteins involved in phagocyte invasion, two of which have been shown to be phage-encoded. The first one is a recently discovered chemotaxis inhibitory protein (CHIPS) that inhibits phagocytosis by binding to and attenuating the activity of neutrophil receptors for complement and formylated peptides (Veldkamp 2000). The second gene is the Panton-Valentine leukocidin (PVL), a cytotoxin with direct activity against human phagocytes (Kaneko 1997,Vijver 1972).

Some bacteria can reduce effectiveness of the toxic compounds released into the phagolysosome by producing enzymes such as catalase and superoxide dismutase that detoxify reactive forms of oxygen (Bacterial Pathogenesis, Farrant et al., 1997). Superoxide dismutase (SodC) of *S.enterica* is encoded by the functional phage Gifsy-2; isogenic *Salmonella* derivatives lacking Gifsy-2 were attenuated in a mouse model (Figueroa-Bossi & Bossi, 1999).

1.3.4 Exotoxin production

Exotoxins are the most widely recognized phage-encoded virulence factors; and exotoxin production is the major pathogenic mechanism for several bacteria. Since the discovery in 1951 that the *diptheria* toxin is encoded on the -phage genome of *Corynebacterium diptheriae*, bacteriophage encoded toxins have been found in a range of both gram-negative and gram-positive bacteria, including *Staphylococcus aureus* (Betley 1985), *Streptococcus pyogenes* (Goshorn & Schilievert, 1989), *Escherichia coli* (O'Brien 1984,Huang 1987), *Pseudomonas aeruginosa* (Nakayama et al., 1999), *Vibrio cholerae, Clostridium tetani, Clostridium botulinum* and *Shigella* spp. (Barksdale & Arden, 1974).

1.3.5 Susceptibility to antibiotics

Although no example of phage-encoded resistance gene is known yet, phages may play an important role in transduction of resistance genes encoded in plasmids of *Staphylococci* (Firth &Skurray, 2000) and *Streptococci* (Caparon, 2000). Resistance of *Streptococci* to tetracycline, chloramphenicol, macrolides, lincomycin, and clindamycin (Ubukata et al., 1975) and streptomycin (Hyder & Streitfeld, 1978) were shown to be transferred by *streptococcal* phages. This transfer occurs probably via generalized transduction of non-phage-encoded resistance genes.

1.3.6 Transmission of bacterial pathogens

Phage-encoded cholera toxin (Waldor & Mekalanos, 1996) up-regulates enterocyte adenylate cyclase activity, this leads to watery diarrhea (Finkelstein, 1992) which is widely assumed to contribute significantly to the fecal-oral transmission of *V. cholerae* (Mintz et al., 1994). This is an example of a bacteriophage contributing to the transmission of its bacterial host among humans.

1.4 Aim of the Study

Recent pathogenic conversion of *M.catarrhalis* and rapid spread of β-lactamase producer strains increased importance of this organism and motivated us to study the reasons behind pathogenicity of *M.catarrhalis*. Since bacteriophages have been shown to contribute bacterial pathogenesis at all stages of infection, and they are responsible for pathogenic conversion of several bacteria, we hypothesized that a bacteriophage may be found in *M.catarrhalis* which contributes to its pathogenicity.

The primary aim of the project was to investigate whether *M. catarrhalis* has any bacteriophages. Secondary aim of the project was to investigate whether a phage is responsible for the pathogenicity of the bacterium.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were of molecular biology grade and purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) except;

- Hydrogen Peroxide, 8M Sodium Hydroxide Solution and 2-Propanol were purchased from WAKO Pure Chemical Industries Ltd. (Osaka, Japan)
- NorthernMax Formaldehyde Load Dye,Agarose LE was purchased from Ambion Inc. (TX, USA)
- TRI LS reagent was purchased from Invitrogen Corporation (CA, USA).
- Tris, EDTA and sucrose were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

2.1.2 Bacterial Strains

In this study *M. catarrhalis* was used for all purposes. B-88-152, B-88-83, B-87-75, B-87-94, 34, 133 pathogenic strains, and B-91-06,B-91-07, B-91-09, B-91-22, B-91-25, B-91-26, B-91-27, B-91-28 non-pathogenic strains of *M. catarrhalis* were used. B-88-152 was used unless otherwise stated.

2.1.3 Enzymes

- DnaseI, Lysozyme, RNaseA were purchased from WAKO (Kyoto, Japan).
- ProteinaseK was purchased from MERCK (NJ, USA).
- RNaseIII was purchased from New England Biolabs (MA, USA).
- S1 Nuclease, T4 RNA ligase were purchased from Promega (WI, USA).
- BD Powerscript Reverse Transcriptase, KlenTaq LA polymerase mix were purchased from BD Biosciences (CA, USA).
- PCR Master Mix was purchased from Promega (WI, USA).

2.1.4 Oligonucleotides

All oligonucleotides were custom synthesized and purchased from Gene Net (Fukuoka,Japan)

- Primer A: 5'PO_4-AGGTCTCGTAGACCGTGCACC-NH_2-3'
- Primer B: 5'-GGTGCACGGTCTACGAGACCT-3'
- CapSwitch oligo (Clontech, CA, USA): 5'-TACGCTGCGAGAAGACGACAGAAGGG-3'
- 5'PCR primer: 5'-TACGCTGCGAGAAGACGACAGAA-3'

2.1.5 Commercially Available Kits

- RNaid Kit (Q-BioGene, CA, USA) was used to purify RNA from agarose gel and ligation reactions.
- Qiaquick PCR purification Kit (Qiagen, CA, USA) was used to purify PCR products.

2.1.6 DNA and RNA Size Markers

- M-VIII (Roche Applied Sciences, IN, USA), DNA-HindIII digest (TaKaRa, Shiga, Japan) were used as DNA size standards.
- RNA Millenium Markers (Ambion Inc. TX, USA) was used as single-stranded RNA size standards.

2.1.7 Apparatus and Equipment

- Horizontal Gel Electrophoresis Machines Mupid 2 and Mupid EX-U were purchased from Advance Co. (Tokyo, Japan).
- Thermal Cycler Perkin Elmer 9600 was purchased from GMI Inc. (MN, USA).
- Electron microscope JEM-1230 was purchased from Jeol (Tokyo, Japan).
- UV/Visible Spectrophotometer, Ultracentrifuge, Benchtop Ultracentrifuge were purchased from Beckman Coulter Inc (CA, USA).

2.1.8 Solutions and Buffers

PBS -

Working solution was prepared by dissolving 1 tablet (Dulbecco's PBS, Dainippon Pharmaceutical Co. Ltd.)in 100ml water. Solution was treated with 0.1% DEPC and autoclaved.

TAE

Stock solution (50XTAE) was prepared by adding 121g Tris, 18.6g EDTA, and 28.55ml glacial acetic acid. The total volume was brought to 500ml with ddH_2O. Working solution (1XTAE) was prepared by diluting 50XTAE by 50 times.

BHI Broth (1lt)

37g BHI broth powder (Difco) was dissolved in water. After preparation medium was sterilized by autoclaving.

BHI-Agar (1lt)

52g BHI agar powder (Difco) was dissolved in water. After preparation medium was sterilized by autoclaving.

Blood-Agar

7% rabbit blood was added into BHI agar.

Soft-Agar

0.8% BactoAgar was dissolved in BHI broth and sterilized by autoclaving.

30% Sucrose

300g sucrose was dissolved in 1lt 0.01M Tris by stirring.Sterilized by autoclaving.

Suspension Medium(SM) (1lt)

5.8gr NaCl, 100mg gelatin, 2g magnesium hepta hydrate was dissolved in 60ml 1M Tris (pH 7.5)and 945ml ddH_2O. Sterilized by autoclaving.

1M Tris (pH=8.0)

60.55g Tris was dissolved in ∼300ml ddH_2O, 21ml 37% HCl was added. pH was adjusted to 8.0 with HCl, and the total volume was brought to 500ml by adding ddH_2O.

0.5M EDTA (pH=8.0)

93.05g EDTA was dissolved in ∼300ml ddH_2O by adjusting the pH to 8.0 with NaOH. After pH is adjusted, total volume was brought to 500ml by adding ddH_2O.

MOPS Electrophoresis Buffer

10X stock solution was prepared by mixing 0.2M MOPS (pH 7.0), 20mM sodium acetate and 10mM EDTA (pH 8.0).

DnaseI Reaction Buffer

10X stock solution was prepared mixing 25mM $MgCl_2$, 5mM $CaCl_2$ and 0.1M Tris-HCl (pH 7.5) in ddH_2O.

2.2 Methods

2.2.1 Growth and Maintenance of Bacteria

M. catarrhalis strains were stocked in BHI broth containing 5% rabbit blood and stored at -80°C until use. For stocking, the bacteria were cultured on 7%

rabbit blood agar plates and the colonies were taken with a sterile cotton swab and transferred to the cryotube containing 1 ml of media. During experiments, the bacteria were cultured on BHI agar or BHI broth. The plates were incubated at 37°C in presence of 5% CO_2.

2.2.2 Bacteriophage Detection, Purification and Visualization

2.2.2.1 Plaque Assay

Phage suspension was prepared from 16 hr bacterial culture in broth.Bacterial cells were removed by 15min centrifugation at 3,000rpm at 4°C and subsequent filtration of supernatant through 0.45μm millipore filter.

675μl of 6 hr broth culture was mixed with 5ml soft agar and the mixture was overlayed on BHI agar plates. 10-20 μl of phage suspension was spotted on soft agar-bacteria mixture, and the plates were incubated overnight at 37 °C in presence of 5% CO_2.

2.2.2.2 Bacteriophage Purification

Purification from agar culture over 15% sucrose cushion

M.catarrhalis was cultured on BHI agar and grown at 37°C for 16 hr with 5% CO_2. Bacteria from surface of 40 agar plates were collected with sterile cotton swabs, and harvested in 15ml SM buffer. The suspension was aliquoted into 2 ml eppendorf tubes and mixed well after adding 3% (v/v) chloroform. The organic and aqueous phases were separated by centrifugation at 6000xg for 5 min and the aqueous phase was transferred into a sterile tube. The treatment with chloroform was repeated once more and the aqueous phase was added on 15% sucrose very

slowly by sterile pasteur pipette. Bacteriophage particles were precipitated by centrifugation at 100,000xg for 2 hr in Beckman Ultracentrifuge with rotor NVT 100. The supernatant was discarded and the pellet was resuspended in 150μl SM (Barbian 2000).

Standard Method (30% sucrose cushion)

3 ml of 6 hr starter culture was used to subculture into 300ml BHI broth which was incubated at 37°C in a shaking incubator for 16hr.Bacterial cells were pelleted by centrifugation at 3,000rpm for 15min and discarded. Bacteriophage particles were pelleted from supernatant at 30,000rpm in Beckman 45Ti rotor at 4°C for 1hr. Resulting pellet was suspended in PBS- and the Bacteriophage particles were purified further by centrifugation over 30% sucrose at 30,000rpm in Beckman SW 41 Ti rotor for 2hr at 4°C. The final pellet was suspended in PBS- and stored at -20°C until needed.

CsCl Isopycnic Centrifugation

30,000 rpm pellet obtained from 16hr bacterial supernatant was suspended in SM buffer containing cesium chloride in a density of 1.45g/cm. The suspension was centrifuged for 16 hr at 50,000 rpm in Beckman bench top ultracentrifuge rotor TLS 55, at 4°C. At the end of centrifugation a band and a precipitate was formed. The particles which formed the band, precipitate were collected separately. Also fractions from above and below of the band were collected to separate tubes. CsCl was removed by centrifugation at 50,000rpmn for 90 min. The pellets were suspended in PBS - , and spectrometric analysis was performed to find the nucleic acid containing fractions. Absorbance of each sample was measured in a scan from wavelength 220 to 350 nm the samples with highest absorbance at 260 nm wavelength were used for nucleic acid extraction.

30-60%Sucrose Gradient

Bacteriophage particles were pelleted from supernatant of 6 hr broth culture by at 30,000rpm for 1 hr, and suspended in PBS-. This suspension was centrifuged over a sucrose gradient formed by adding 2 ml of 30% sucrose over 2 ml of 60%

sucrose. Centrifugation was performed at 30000rpm for 90min, in a Beckman Ultracentrifuge using rotor SW41Ti . The pellet formed at the bottom of the tube and the band formed at 60%-30% boundary was diluted in PBS- (final volume 30 ml). The diluted samples were centrifuged at 30,000 rpm in rotor 60 Ti for 90 min in order to remove excess sucrose. The final pellets were suspended in 700μl PBS- and 500μl of the suspension was used to extract nucleic acid using phenol-chloroform extraction method.

60-80% Sucrose Gradient

Bacteriophage particles were pelleted and suspended in PBS- as described in the above section. Sucrose gradient was prepared by overlaying 80%, 70% and 60% sucrose solutions on top of each other, using 2 ml of each solution. Bacteriophage suspension was centrifuged as described in the above section and the bands formed at 80-70% boundary and in the 60% as well as the diffused band in the 70% region were diluted in PBS- and precipitated as described in the above section. Each pellet was suspended in 500μl PBS- and used for nucleic acid extraction using phenol-chloroform extraction method.

2.2.2.3 Electron Microscopy

One drop of phage suspension was applied on a cooper-grid and then stained with 3% uranyl acetate for 30 sec. Excess of uranyl acetate was removed with a filter paper tip. Specimens were examined with a JEM-1230 (JEOL Ltd., Tokyo, Japan) at a magnification of 100,000 times.

2.2.2.4 Morphometric Analysis

The sizes of each type of particle were determined by measuring the longest distance between two points on borders of the particles. The distances were measured on original pictures with a millimetric ruler. The sizes of hexagonal

and vesicle-like (pleopmorphic) particles were compared with Mann-Whitney test using Minitab 14.

2.2.3 Nucleic Acid Extraction

2.2.3.1 Phenol Extraction

500μl phenol:chloroform:isoamylalcohol (25:24:1) was added on 500μl phage suspension in PBS- and mixed by vortexing and hand mixing for a total of 3 min. The mixture was centrifuged at 12,000 rpm for 15 min at 25°C in a Beckman Avanti 30 centrifuge with rotor F 3602. The upper phase was transferred into a new tube and 0.7 volume 2-propanol was added. Nucleic acid was precipitated by centrifugation at 12,000 rpm at 4°C for 20 min. After the supernatant was discarded the pellet was washed with 70% ethanol at 12,000 rpm at 4°C for 15 min. The supernatant was discarded and the pellet was dried in MicroVac, the pellet was resuspended in 100μl water and stored at -80°C.

2.2.3.2 TRI LS Reagent Extraction

TRI LS Reagent was used according to manufacturer's instructions. 750μl TRI reagent was added to 250μl of phage suspension, and the phage was lysed by repeated pipeting. Homogenized samples were incubated at room temperature for 5 min. 200μl chloroform was added and the tubes were shaken vigorously in hand for 15min, the tubes were incubated at room temperature for 15 min. 500μl 2-propanol was added to the aqueous phase , mixed and incubated at room temperature for 10min and RNA was precipitated at 12,000xg for 10 min at 4°C. After removing the supernatant, RNA pellet was washed with 1ml of 75% ethanol and centrifugation at 7,500xg for 5 min at 4°C. The supernatant was discarded by a micropipette and the pellet was air dried/vacuum dried. The pellet was suspended in 100% formamide and stored at -80°C.

2.2.3.3 SDS-ProtK lysis

Bacteriophage particles were lysed with EDTA (final concentration: 20 mmol/L), proteinase K (final concentration: 50μg/ml) and SDS (final concentration: 0.5%) at 56C for 1hr. Equal volume of phenol at 60C was added to the lysate, mixed and incubated at 60C for 30min. The suspension was centrifuged at 5,000xg for 10 min and the aqueous phase was transferred to a sterile tube. Subsequent two extractions were performed using an equal volume of phenol:chloroform (1:1) and chloroform at same centrifugation conditions. Finally nucleic acids were precipitated overnight at -20C after adding 2 volumes of 95% ethanol and 0.1 volume of 3M sodium acetate. Nucleic acids were pelleted at 13,000 rpm for 15 min and the pellet was washed with 1 ml of 70% ethanol. The pellet was air-dried and dissolved in water (Oakey 2000).

2.2.4 Nucleic Acid Characterization

2.2.4.1 DnaseI Treatment

DnaseI was used according to manufacturer's instructions as follows; 0.5μg nucleic acid was treated with 2 units enzyme in a 10μl reaction mixture containing 1xDnaseI reaction buffer. Reaction was performed for 30 min at 37°C.

2.2.4.2 S1 Nuclease Treatment

S1 nuclease was used according to manufacturer's instructions as follows; 0.5μg nucleic acid was treated with 50units enzyme in a 10μl reaction mixture containing 1xS1 nuclease reaction buffer. Reaction was performed for 10 and 30 min at 25°C.

2.2.4.3 RNAseIII Treatment

RNaseIII was used according to manufacturer's instructions as follows; 1μg nucleic acid was treated with 0.15unit enzyme a 10μl reaction mixture containing 1x RNaseIII reaction buffer. Reaction was performed for 20 min at 37°C.

2.2.4.4 Agarose Gel Electrophoresis

Phage nucleic acids were separated on 1.6 % agarose gel for routine use. Required amount of Agarose was completely dissolved in 1x TAE buffer by heating in microwave, and then ethidium bromide was added to a final concentration of 30ng/ml. The nucleic acid samples were mixed with 1/5 volume 6x loading buffer and loaded onto gels. The gel was run at room temperature in 1x TAE at 50 Volts.

2.2.4.5 Formaldehyde/Agarose Gel Electrophoresis

1.5% gel was used for separation of RNA fragments. 0.45 g agarose was dissolved in 21.6 ml DEPC-treated water by heating in a microwave oven. When the agarose was dissolved completely, it was cooled down to 60°C by incubating in a water bath. 3 ml 10xMOPS and 5.4 ml formaldehyde was added to the solution and the gel was poured and kept in a fume hood at least 1hr to solidify. RNA samples were mixed with 3 volumes of formaldehyde gel loading buffer and ethidium bromide was added to a final concentration of 0.05μg/μl. RNA millennium size marker was used according to the manufacturer's instructions. Samples were loaded to the gel and the gel was run at 50 volts at room temperature.

2.2.5 Preparation and Optimization for Sequencing of RNA

2.2.5.1 Gel Extraction

Isolated phage RNA was run on 1.2% Formaldehyde RNA gel and the separated segments were extracted from gel by RNAid kit as follows: RNA bands were excised from gel using a sterile RNAse-free scalpel and 0.3ml RNA binding salt (pH 5.0) was added per 0.1g of gel slice. Gel slice was melted by incubating at 37°C approximately 10min. When the gel was completely melted the vial was transferred to room temperature and 2μl RNA matrix per μg of RNA was added. RNA was allowed to bind RNA Matrix for 10min at room temperature. RNA/RNA Matrix complex was precipitated at 13000rpm for 1 min and the supernatant was removed. The pellet was suspended in 500μl RNA Wash Buffer and the mixture was centrifuged at 13000rpm for 1 min to wash the pellet. The washing step was repeated two times more. The pellet was suspended in 15μl DEPC-treated water and RNA was eluted by incubating at 70°C for 10min. The RNA matrix was removed by 2min of centrifugation at 13000rpm and RNA containing supernatant was transferred to a sterile vial for further experiments.

2.2.5.2 Linker Ligation

100ng of RNA was used to ligate Linker A to 3' end of the RNA. The ligation reaction contained 50 mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 5mM dithiothreitol, 1mM ATP, 100μM Linker A and 10 units of T4 RNA ligase. The reaction was performed in 25μl reaction mixture,at 16°C for 10 hr (Attoui 2000). The oligo-ligated RNA was recovered from the reaction using RNaid kit as follows: 75μl RNA binding salt was added to the ligation reaction mixture and mixed well. 5μl of RNA matrix was added to the mixture and incubated 10 min at room temperature to allow RNA binding to RNA Matrix. RNA/RNA Matrix complex was precipitated at 13,000 rpm for 1 min and the supernatant was removed. The pellet was suspended in 500μl RNA Wash Buffer and the mixture was centrifuged at 13,000 rpm for 1 min to wash the pellet. The washing step was repeated two times more. The pellet was resuspended in 15μl DEPC-treated water and RNA

was eluted by incubating at 50°C for 5 min. The RNA matrix was removed by 2 min of centrifugation at 13,000 rpm and RNA containing supernatant was transferred to a sterile vial for further experiments.

2.2.5.3 First Strand cDNA Synthesis

3μl RNA solution was mixed 1μM Capswitch oligo and 1μM primerB and heat denatured at 70°C for 5min. First strand cDNA was synthesized in a 10μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 1 mM of each dNTP, and 100Units BD-Powerscript Reverse Transcriptase (BD Biosciences, USA). Reaction was completed in 90 min at 42°C and stopped by incubating on ice (Attoui 2000). The optimum cDNA synthesis conditions were searched by changing the denaturation step (72°C for 2 min, 80°C for 10 min) and duration of synthesis reaction (60 min, 90 min).

2.2.5.4 Optimization of PCR Amplification

5'PCR primer and primer B were used to directly PCR amplify the cDNA, using KlenTaq polymerase mixture (BD Biosciences, USA). 100μl reaction mixture contained 2μl cDNA solution, 0.2 mM each dNTP, 0.2μM Primer B, 0.2μM 5'PCR primer, 10μl of 10x KlenTaq PCR buffer and 2μl of 50x advantage KlenTaq polymerase mix. PCR amplification was carried out using a cycle of denaturation at 95°C for 2 min and 35 cycles of incubation at 95°C for 15 sec and 68°C for 5 min (Attoui 2000). PCR optimization was performed by varying the extension time from 5 min to 3min using KlenTaq polymerase mixture. PCR amplification was also carried out with Taq polymerase (Promega Master Mix) using a cycle of initial denaturation at 95°C for 2 min and 35 cycles of incubation at 95°C for 30sec, 64/68°C for 45 sec and 72°C for 90/60sec followed by final extension of 5min at 72°C. The amplification product was analyzed at 1.5% agarose gel in 1x TAE. The PCR product was purified using Qiaquick PCR purification kit according to manufacturer's instructions.

Chapter 3

Results

3.1 Plaque Assay

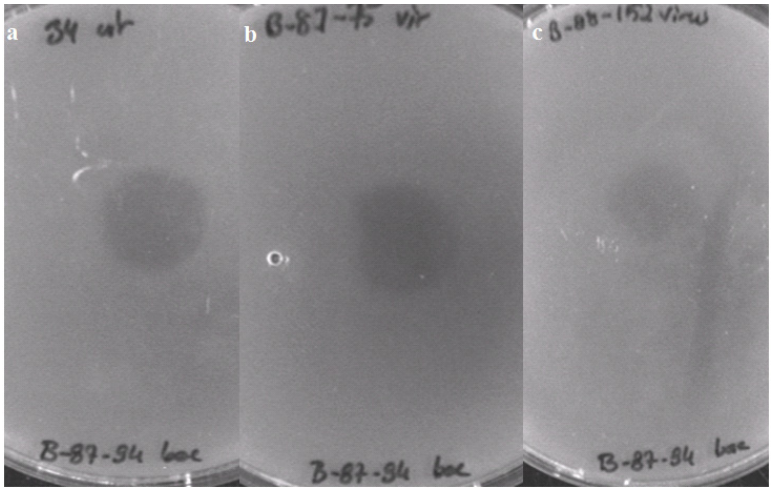


Figure 3.1: Representative pictures of plaque assay results. Supernatants from strain (a)34, (b)B-87-75, (c)B-88-152 caused cell lysis of strain B-87-94.

Phage suspensions of M. catarrhalis strains; 34, B-87-88, B-87-69, B-87-75, B-88-152, B-88-83 and F were used to induce plaque formation on soft agar cultures of same strains. Phage suspension of each strain was used to infect the same

strain and/or other strains (Figure 3.1). Plaque formation was observed on each plate except two. However all possible combinations were not performed and not all plaque assays were repeated.(Table 3.1)

Lytic effect of supernatant from:								
Strain	**B-87-133**	**B-88-83**	**34**	**B-87-69**	**B-87-75**	**B-87-94**	**B-88-152**	**F**
B-87-133	++	N/A	+++	+++	++	++	++	-
B-88-83	+++	+++	+++	++	+	+	++	+++
34	N/A	++	N/A	N/A	N/A	N/A	N/A	N/A
B-87-69	+++	++	N/A	+++	N/A	N/A	N/A	+++
B-87-75	++	++	+++	+++	++	+	++	++
B-87-94	+++	++	+++	+++	+++	++	+++	+++
B-88-152	+++	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F	-	++	++	+++	+	N/A	++	++

Table 3.1: Summary of plaque assay results. Lytic effects were measured by diameters of the clear zones produced on agar plates: +,< 15mm; ++, 15 to 18mm; +++,> 18mm; N/A, not available; -, no plaque formation.

3.2 Phage Isolation

The particles isolated over 15% sucrose cushion and 30% sucrose cushion were visualized by TEM. Several particles with different shapes and sizes were copurified when 15% sucrose cushion was used. Particles resembling fimbriae were co-purified with nonuniform vesicle-like particles and hexagonal particles were copurified (Figure 3.2A). When 30% sucrose cushion was used, only two types of particles were copurified. Hexagonal particles with varying sizes were co-purified with vesicle-like particles. However the vesicles had more uniform size and shape compared to the previous isolate. With this method very big vesicle-like particles and fimbriae-like particles were removed and the hexagonal particles were obtained with higher concentration. Aggregations of particles were observed frequently and the two types of particles were co-existed in these aggregations.

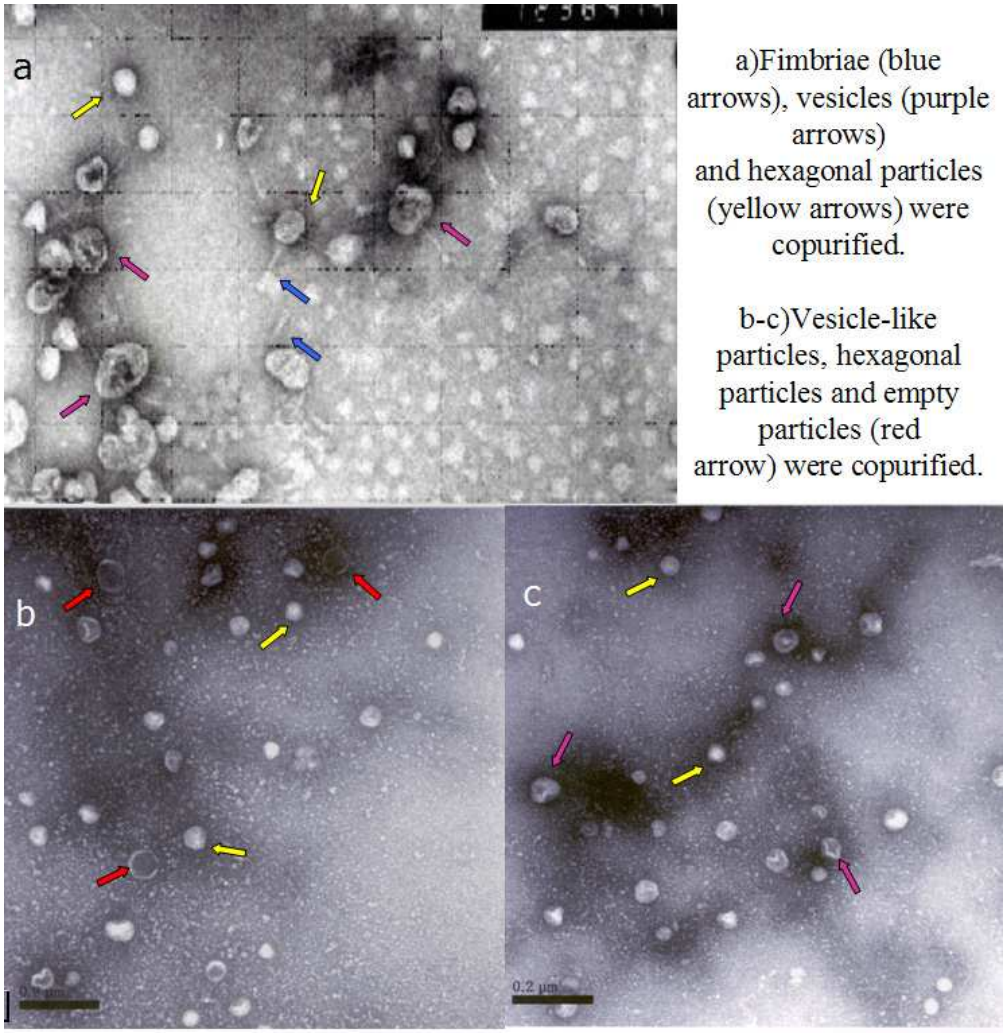
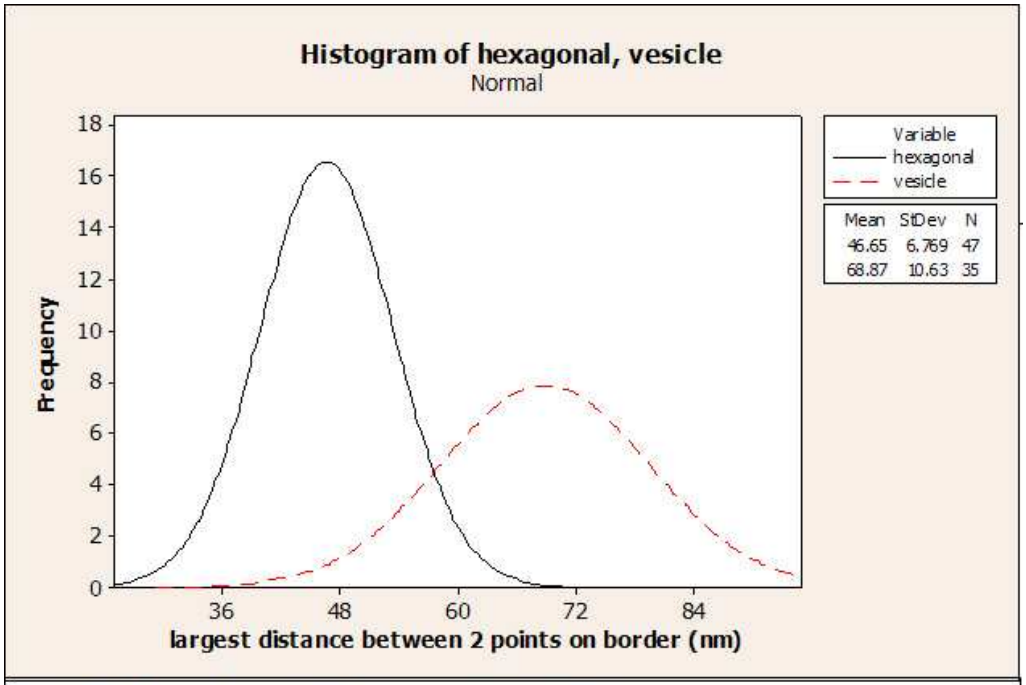


Figure 3.2: (a)Particles isolated over 15% sucrose.(b)Particles isolated over 30% sucrose by our standard method of phage isolation.

Longest distance measured on the hexagonal particles varied from 30 nm to 60 nm , while those of vesicle-like(pleomorphic) particles varied from 50 nm to 85 nm (Figure 3.2). Size distribution of these two particles were plotted into a histogram (Figure 3.3), and the sizes of two particles was found to be significantly different according to Mann-Whitney test.



Mann-Whitney Test and CI: hexagonal, vesicle-like

	N	Median
hexagonal	47	42.105
vesicle-like	35	63.158

Point estimate for ETA1-ETA2 is -21.052
95.1 Percent CI for ETA1-ETA2 is (-23.157,-21.054)
W = 1194.5
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.0000
The test is significant at 0.0000 (adjusted for ties)

Figure 3.3: Size variation of hexagonal and vesicle-like particles. Histogram was plotted on Minitab14.Sizes of two particles were found to be significantly different.

3.3 Nucleic Acid Extraction and Characterization

The nucleic acid was extracted with phenol:chloroform:isolamyl alcohol method and was separated on 1.6% and 0.8% agarose gel with 1 TAE and 3 bands of nucleic acid were observed. Dnase I, S1 Nuclease and RNaseIII treatments were performed and the reaction products were analyzed on 1 TAE agarose

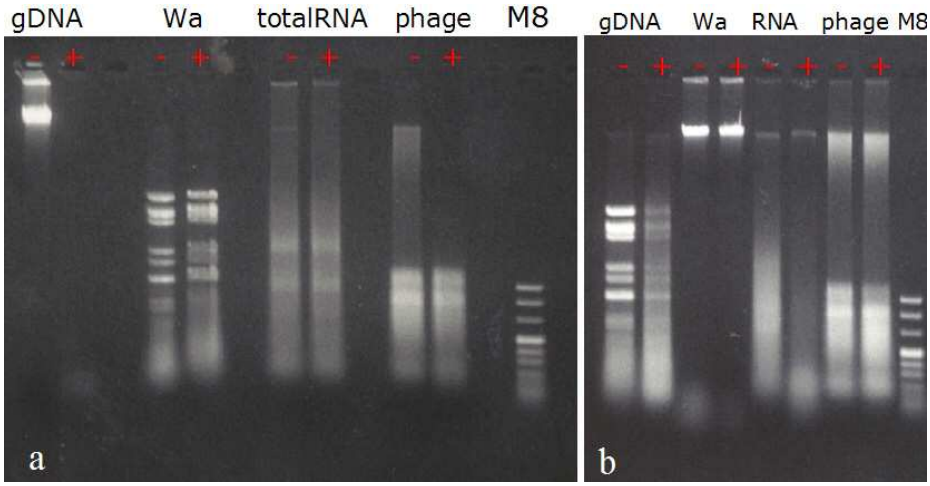


Figure 3.4: (a)DnaseI caused degradation of *M.catarrhalis* genomic DNA and high molecular weight nucleic acid extracted from phage particles.(b)RNaseIII caused degradation of rotavirus Wa genomic RNA(dsRNA) but phage RNA was resistant to RNaseIII

gel to understand the nature of nucleic acids isolated. High molecular weight (9 kb) nucleic acid was found to be double stranded DNA since it was sensitive to DNAseI (Figure 3.4A) and resistant to S1 (Figure 3.5) nuclease and RNAse III (Figure 3.4B). However the smaller bands were found to be single-stranded RNA since they were resistant to DNAse I and RNAse III treatment whereas they were digested completely by S1 nuclease.

Particles isolated with different methods we used, all contained double-stranded DNA together with single-stranded RNA with same mobility and banding pattern as visualized on agarose gels. Particles isolated from *M.catarrhalis* with different sucrose gradients also contained 3 bands of nucleic acid with the same mobilities (Figure 3.6). It was hard to tell the molecular size of the ssRNA bands by looking at their mobility in agarose gels since they do not have a consistent speed. The relative mobility of the RNA bands varied a lot depending on the concentration of the gel, run time and room temperature. When agarose gel was prepared free of contaminating ribonucleases three RNA segments with mobilities of 0.4kb, 0.7kb and 1.1kb were observed in every isolate (Figure 3.7 A).

TRI LS extracted RNA was visualized on 1.5% formaldehyde/agarose gels

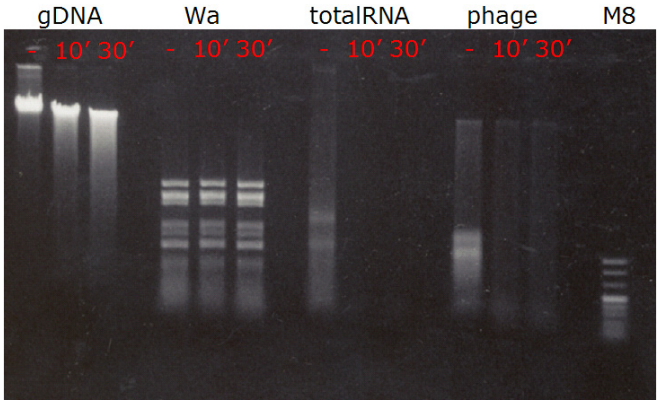


Figure 3.5: S1 nuclease caused degradation of RNA extracted from mouse spleen(totalRNA), and low molecular weight nucleic acids extracted from phage particles. gDNA:genomic DNA, Wa: Rotavirus Wa strain genomic RNA (dsRNA), totalRNA: total RNA extracted from mouse spleen, phage:nucleic acid extracted from isolated phage particles.

and 3 RNA segments with sizes of 0.5kb, 1.5kb and 2.4kb compared to RNA millennium markers were observed (Figure 3.7 B).

3.4 Ligation, Reverse Transcription and PCR

Ligation products were reverse transcribed with primer B in presence of cap-switch oligo, and PCR was performed with primerB and 5'PCR primer. PCR products were visualized on 1.5% agarose gel in 1TAE. All PCRs performed with KlenTaq polymerase mix yielded a smear, which did not contain any band/bands. Only in one condition banding was observed, the products were combined and cleaned with PCR purification kit. The purified product formed a smear on 1.5% agarose gel, and did not yield any bands. PCRs performed with Taq polymerase did not yield any smear or product , but only the primer dimers (Figure 3.8).

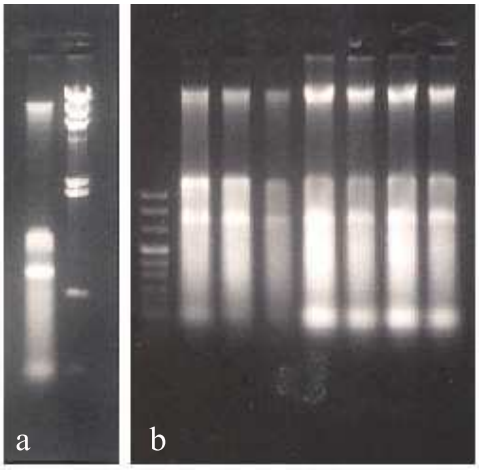


Figure 3.6: a)- Agarose gel in 1xTAE. lane1;Phage particles were isolated with standard method and nucleic acid was phenol extracted. lane2;HindIII digest was loaded as molecular size standard. b) lane1; M8 as molecular weight marker. lane2-7;Phage particles isolated from different fractions of sucrose gradients, nucleic acid was phenol extracted .

3.5 Comparison of Different Strains

Our standard method for purification of bacteriophage particles was used to isolate bacteriophage from other pathogenic and non-pathogenic strains we had. Although no visible pellet was obtained from non-pathogenic strains visible pellets were obtained from pathogenic strains, after centrifugation over 30% sucrose cushion. However the experiment was carried on as if pellets were visible in all strains, and nucleic acid was extracted and visualized on ribonuclease-free agarose gel. All strains yielded a high molecular weight band and 3 or more low molecular weight band as visualized on 1.6% agarose gel prepared free of contaminating ribonucleases (Figure 3.9).

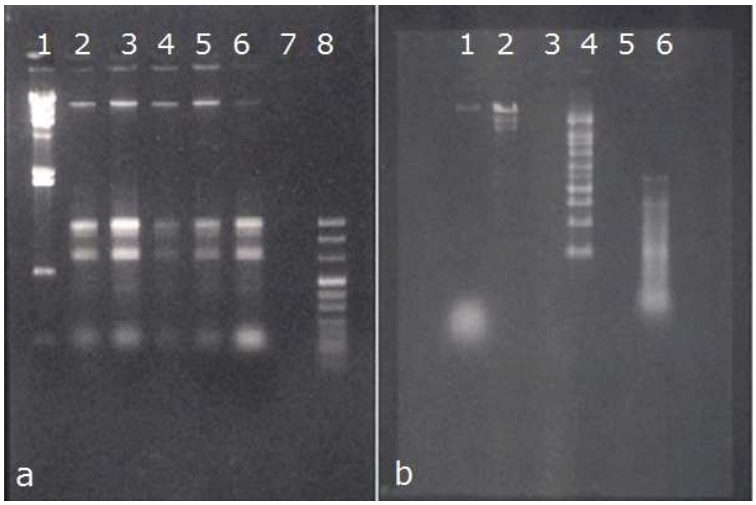


Figure 3.7: a)Rnase free agarose gel. lane1; HindIII digest. 2-6; Particles were isolated with CsCl isopycnic centrifugation and nucleic acids were phenol extracted.8; M8 as molecular size standard. b) 1.5% Formaldehyde/Agarose Gel.lane1; *M.catarrhalis* genomic DNA.2;HindIII digest.4;RNA millennium markers.6;TRI extracted phage RNA.

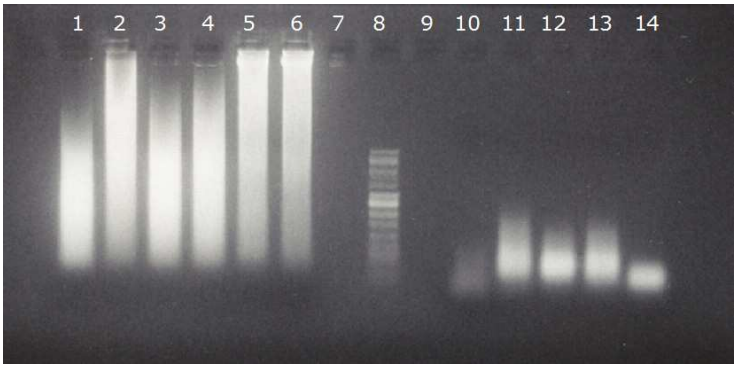


Figure 3.8: PCR products lanes 2-6; by KlenTaq polymerase. 11-14; by Taq polymerase.8; Molecular Weight Marker MVIII.1,10; negative controls

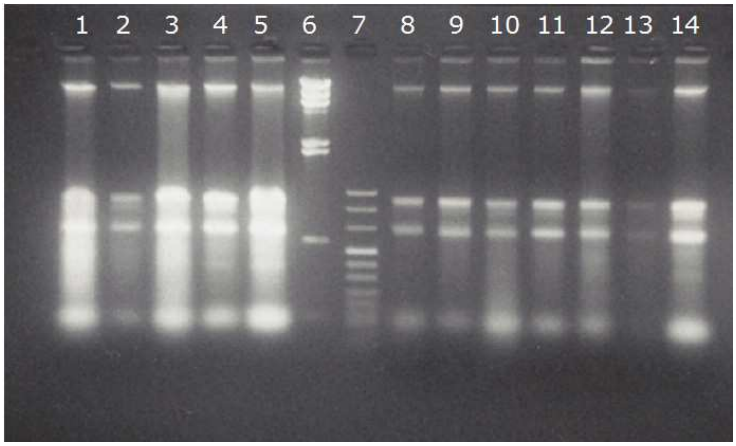


Figure 3.9: Nucleic acids isolated from phage and vesicles of pathogenic and nonpathogenic strains were loaded to the gel.Lanes (1-5): 34,B-87-75, B-88-83, B-87-94, B-88-133; (8-14): B-91-6, B-91-7, B-91-9, B-91-22, B-91-25, B-91-26, B-91-27 (Samples were diluted 10-60 times).Lanes (6-7): HindIII Digest, MVIII as molecular weight markers.

Chapter 4

Discussion

Bacteriophages are the most abundant life forms in the biosphere. Thousands of varieties of phage exist, each of which may infect only one type or a few types of bacteria. Even though no bacteriophage from *M.catarrhalis* has been yet defined, it is very unlikely that it would not have one. In this study, evidence for a bacteriophage from *M.catarrhalis* is presented.

Supernatants of*M.catarrhalis* broth cultures were shown to cause bacterial cell lysis on soft agar cultures, indicating presence of bacteriophages in them. Two particles having different morphologies (hexagonal and pleopmorphic(vesicle-like) and different size ranges ($p<0.\ 05$) were co-purified from these supernatants. Two methods for isolation of bacteriophage particles were used. The 15% sucrose cushion method yielded an impure isolate containing low number of hexagonal phage particles, however the 30% sucrose cushion method yielded purer isolate containing larger number of hexagonal phage particles due to increased sucrose concentration and usage of broth culture as starting material. The sizes of the two particles were represented by the largest distance between two points on these particles. However this may not reflect the real sizes of the particles, and the measurement should be improved by including multiple distances on each particle and combining those in a more complex analysis.

One segment of dsDNA molecule and three segments of ssRNA molecules were extracted from the isolated particles. Since several attempts to separate two particles from each other failed, it was not possible to determine the origins of RNA and DNA molecules. These two particles are believed to attract each other and form aggregates of different sizes, since small and large aggregates containing the two particles together were visualized under electron microscope. Most likely, the aggregates of various sizes have different densities therefore they are collected from different fractions of the sucrose and CsCl gradients. Usage of another buffer during phage isolation (instead of PBS- or SM) which can block the attraction between the hexagonal and pleomorphic(vesicle-like) particles will resolve this problem.

Optimization of RNA sequencing reactions also are performed. There might be several reasons causing failure of RNA sequencing trials. Ligation of linker to template can be improved by using phosphatases to prevent self ligation and concatenation of the RNA template, and also by changing linker/template ratio, incubation time and temperature. Moreover the cDNA synthesis and PCR amplification should be optimized.

The nucleic acid content particles isolated from pathogenic and non-pathogenic strains also indicated differences in quantity suggesting possible involvement of the identified particles in the pathogenesis, which has to be investigated further.

Although sufficient information to determine the classification of the phages isolated in this study is not yet available, apparently the morphology and nucleic acids of these particles are not extraordinary. Currently, International Committee for taxonomy of viruses (ICTV) classifies phages into 13 families, and 30 genera (Regenmortel et al., 2000). Phage families are chiefly defined by particle morphology and nature of nucleic acid. Phages with different morphologies have been discovered including tailed, filamentous, polyhedral or pleomorphic phages (Ackermann, 2003). In this study polyhedral (hexagonal) particles and vesicle-like pleomorphic particles were co-purified. Polyhedral phages which have been identified until now contain ss/dsDNA in circular, linear or supercoiled form;

single-stranded linear RNA; or double-stranded segmented linear RNA (Ackermann, 2003).(Table 4.1) The polyhedral phages have been classified into 5 families consist of corticoviridae, leviviridae, microviridae, podoviridae and tectiviridae(Figure 4.1) .

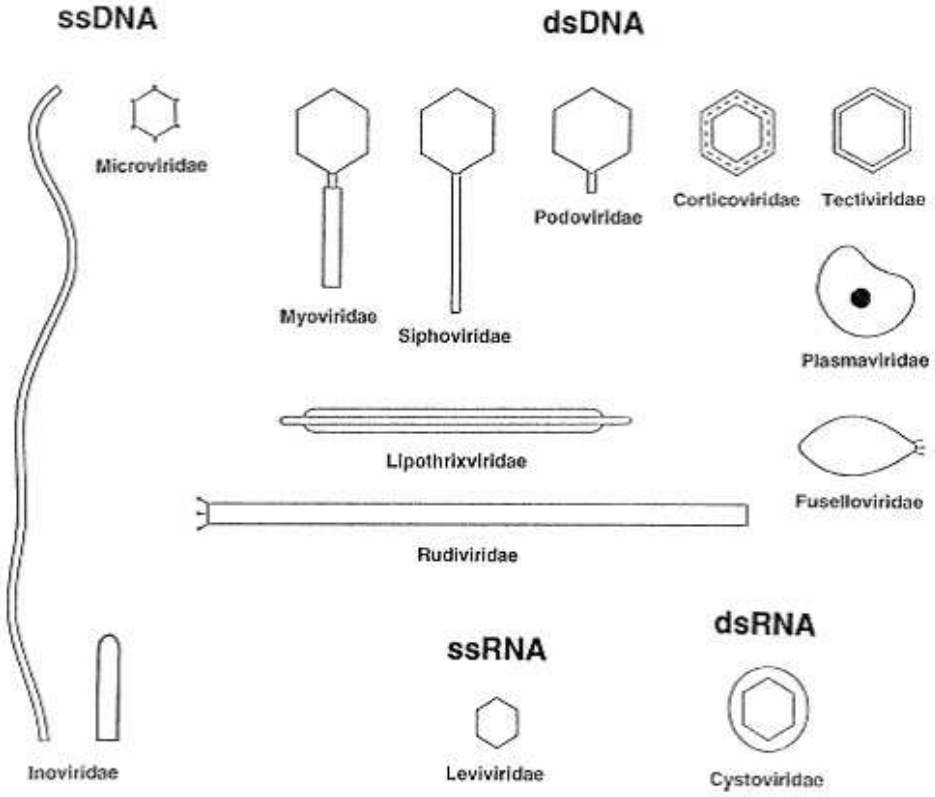


Figure 4.1: Schematic Representation of Major Phage Groups (Ackermann 2003).

In order to determine whether the polyhedral bacteriophages released from *M.catarrhalis* belong to one of these families, these particles need to be isolated and the nature of nucleic acid in them needs to be defined. Pleomorphic bacteriophages yet discovered all contain ds, supercoiled or circular DNA and they are grouped into two families being plasmaviridae and fuselloviridae. Plasmaviridae virions are enveloped, their shapes are from spherical to pleomorphic; and they are 50 to 125 nm in diameter with a loose fitting membrane (baggy membrane). A regular capsid structure is not detectable (ICTVdB descriptions). The vesicle-like particles isolated in this study resemble plasmaviridae since they have

Classification and basic properties of bacteriophages [a]

Shape	Nucleic acid	Order and families	Genera	Examples	Members	Characteristics
Tailed	DNA, ds, L	*Caudovirales*	15		4950	
		Myoviridae	6	T4	1243	Tail contractile
		Siphoviridae	6	λ	3011	Tail long, noncontractile
		Podoviridae	3	T7	696	Tail short
Polyhedral	DNA, ss, C	*Microviridae*	4	ϕX174	40	
	ds, C, T	*Corticoviridae*	1	PM2	3?	Complex capsid, lipids
	ds, L	*Tectiviridae*	1	PRD1	18	Internal lipoprotein vesicle
	RNA, ss, L	*Leviviridae*	2	MS2	39	
	ds, L, S	*Cystoviridae*	1	ϕ6	1	Envelope, lipids
Filamentous	DNA, ss, C	*Inoviridae*	2	fd	57	Filaments or rods
	ds, L	*Lipothrixviridae*	1	TTV1	6?	Envelope, lipids
	ds, L	*Rudiviridae*	1	SIRV1	2	Resembles TMV
Pleomorphic	DNA, ds, C, T	*Plasmaviridae*	1	L2	6	Envelope, lipids, no capsid
	ds, C, T	*Fuselloviridae*	1	SSV1	8?	Spindle-shaped, no capsid

[a]Modified from reference [4]. With permission of John Libbey-Eurotext. Phage numbers are from reference [3]. C, circular; L, linear; S, segmented; T, superhelical; 1, single-stranded; 2, double-stranded.

Table 4.1: Classification of Bacteriophages (Ackermann 2003)

similar shapes and sizes (from 50 to 80nm). Vesicle-like particles we isolated might also be the membrane vesicles (also called blebs) that are released spontaneously from *M. catarrhalis* during growth (Johnson 1976, Murphy 1989, Russell 1976). Many gram-negative bacteria including *Pseudomonas aeruginosa* (Kadurugamuwa 1995), *Neisseria gonorrhoea* (Dorward et al., 1989), *Actinobacillus actinomycetemcomitans* (Nowotony 1982), *Bacteroides fragilis* (Patrick 1996), and *Haemophilus influenzae* (Kahn et al.,1983), *Escherichia coli* (Kolling & Mathews, 1999) produce membrane vesicles. Studies during last two decades have presented strong evidence supporting importance of membrane vesicles (MVs).

MVs are found emanating from gram-negative bacteria growing in the planktonic or biofilm mode (Beveridge 1997), on solid or liquid media (Kadurugamuwa 1997), as swarming cultures (Fuhrman, 1999), and in natural environments (Beveridge 1997). Typical vesicles released from the surfaces of gram-negative bacteria are spherical, bilayered, membranous structures, from 50 to 250 nm in diameter (Beveridge 1999). They are made up of outer membrane and encapsulated periplasmic components (Beveridge & Kadurugamuwa 1996, Kolling & Mathews 1999), may contain LOS, periplasmic proteins, outer membrane proteins (OMPs), phospholipids, and other factors associated with the virulence of the producing bacteria (Dorward et al., 1989, Kadurugamuwa 1995, Patrick et al., 1996).

Interestingly, vesicles have been shown to carry DNA and deliver it to other bacteria (Kadurugamuwa 1995, Patrick 1996). Specific DNA-binding peptides were reported to be present in vesicle lysates of *H. influenzae* vesicles (Kahn 1982, Kahn 1983) and to be associated with vesicles from *Neisseria gonorrhoea* (Dorward et al., 1989). Dorward et al. has shown that vesicles released by *N. gonorrhoea* harbor both linear and circular DNA, including 4.2- and 7.1-kb plasmids; and MV-mediated transfer of an antibiotic resistance plasmid can occur between two strains of *N. gonorrhoea* (Dorward et al.,1989).Vesicles produced by *Pseudomonas aeruginosa* were reported to contain DNA (Kadurugamuwa 1995).

Chromosomal and bacteriophage-associated virulence genes were detected in *Escherichia coli* O157:H7 vesicles (Kolling & Mathews 1999). Yaron et. al. demonstrated that *E. coli* O157:H7 vesicles contain genes of chromosomal, plasmid and phage origin; and vesicles mediate the transfer of these virulence genes, which are subsequently expressed by recipient enteric bacteria (E. coli JM109 and *Salmonella* serovar Enteritidis) (Yaron et al., 2000). Incubation of MVs from *E. coli* O157 : H7, which carried ampicillin resistance (AmpR) and green fluorescent protein genes on a plasmid (pGFP), with *E. coli* lacking this plasmid resulted in fluorescent transformants, which also suggests that *E. coli* MVs act as vectors of DNA transport (Yaron et al., 2000).

There are certain advantages for DNA to encapsulate itself in MVs. DNA would be protected from exonucleases. Furthermore, MVs should also enhance the efficiency of DNA delivery and uptake into a recipient cell as they fuse into the outer membrane (OM). Because naturally competent bacteria are genetically programmed to permit the efficient uptake of macromolecular DNA (Dubnau, 1999) and non-competent bacteria lack such a mechanism, MVs could possibly overcome such genetic barriers.

MVs of *M.catarrhalis* were not previously investigated for presence of DNA in them; however our findings support this possibility. If the DNA molecule we isolated is actually encapsulated in blebs, this might have important function in horizontal transfer of virulence genes. Three main mechanisms of gene transfer have been identified in bacteria; transformation, conjugation and transduction (as

explained in introduction). Possibly, membrane vesicles constitute an alternative mode of gene transfer among bacteria (Yaron et al., 2000). MVs of *M.catarrhalis* may aid in transformation of this organism and spread of virulence genes such as bro β-lactamases.

Several findings (as explained in introduction) implicate horizontal gene transfer in the acquisition of the *bro* β-lactamases (McGregor, 1998). The suspected location of the *bro* genes on the chromosome and the high efficiency of transfer tend to support theory of a conjugal transposon as a method for spread of resistance (Wallace1989). Although there are indications suggesting presence of conjugative process in transfer of *bro* genes, transfer factors required for conjugation have not been reported in this organism (McGregor 1998). Transformation has been suggested as a mechanism for interbacterial transfer of bro genes (Catlin,1990). Transformation of streptomycin resistance is known to occur between *M. catarrhalis* and *M. nonliquefaciens* strains (Catlin 1964). Transformation by naked DNA is unlikely to occur between *M. catarrhalis* strains since this organism secretes very active endonuclease to its environment (Kamme et al., 1984). Transformation mediated by MVs containing DNA would protect the DNA from endogenous and exogenous nucleases.

MVs have been shown to be important in bacterial pathogenesis in various ways, therefore the MVs of *M. catarrhalis* should be investigated in detail to elucidate their role in pathogenesis of this organism. Since the knowledge regarding pathogenic mechanisms and virulence factors of this organism is scarce, the information on MVs might become very significant.

Vesicles facilitate pathogenesis of bacteria by improving attachment to eukaryotic cells. Grenier and Mayrand (1987) demonstrated that *B. gingivalis* vesicles adhere to epithelial cells and act as an intermediate for the attachment of bacteria to host cells. Vesicles produced by *A. actinomycetemcomitans* were reported to promote the adherence of the parent cell to epithelial cells (Meyer 1993). Contribution of vesicles to the bacterial pathogenesis is proposed to be more than improving adherence only. Since vesicles have ability to integrate into the membranes of foreign bacteria and to adhere to or be engulfed by eukaryotic cells and

integrate into the membranes, they are believed to function as secretory vesicles involved in delivery of virulence factors to heterologous and homologous bacteria or to host cells (Beveridge 1999, Kadurugamuwa 1999). In fact, virulence factors associated with the parent strain, including proteases, phospholipases, autolysin, hemolysins, and Shiga toxins, have been isolated from vesicles (Beveridge 1999, Kadurugamuwa &Beveridge 1995, Kolling & Mathews 1999, Li 1998). The MVs of *P. aeruginosa, Proteus mirabilis*, and *Serratia marcescens* package phospholipase C, proteases, proelastase, and hemolysins (Beveridge 1999). It is probable that MVs from *Borrelia*, enterotoxigenic *E. coli, Haemophilus, Neisseria,* and *Vibrio* species also contribute to the virulence of these other pathogens (Beveridge 1999). Research suggests that vesicles released by other pathogens possess enzymatic and toxic activity towards prokaryotic and eukaryotic cells (Patrick et al. 1996, Wai et al. 1995). Vesicles released from *P. aeruginosa* are able to fuse with the membranes of gram-negative and gram-positive organisms, whereupon they release autolysins, resulting in cell lysis of the targeted organism (Kadurugamuwa 1996, Li 1996). Lysis of competing bacteria, for instance, would provide nutrients for the vesicle-producing strain and limit competition from other bacteria.

Recently, it has been discovered that a chromosome-encoded β-lactamase in *P. aeruginosa* can also be packaged into MVs (Ciofu et al., 2000). This raises the intriguing possibility that β-lactamase-containing MVs could be discharged from pathogens at their infection sites in tissue to increase the breakdown of β-lactam antibiotics in the local tissue environment. Because MVs contain porins as part of their OMP complement, the antibiotic readily diffuses into these MVs and is inactivated. This could be a general strategy for reducing antibiotic concentrations at infection sites, and it could also be one of the ways in which chronic, hard-to-treat infections (such as cystic fibrosis) are maintained by the infecting bacterium (Ciofu et al., 2000). Since MVs can readily fuse to the outer membranes of a wide range of other gram-negative bacterial pathogens (Kadurugamuwa 1999), thereby emptying their luminal contents into the periplasm of recipient cells, β-lactamase (and other antibiotic-inactivating enzymes) could be physically transferred from a donor cell to a non-β-lactamase-producing recipient (e.g., following the cystic

fibrosis analogy, from *P. aeruginosa* [donor] to *Burkholderia cepacia* [recipient]). In this way, a varied group of gram-negative non-producers in close proximity to a donor cell could withstand antibiotic treatment and contribute to the infection without these same bacteria having the genetic capacity to produce the inactivating enzyme themselves (Beverigde 1999). Transportation of the active β-lactamase to β-lactamase sensitive strains may explain the high rate of β-lactamase producer *M. catarrhalis* strains found in clinical studies.

Chapter 5

Future Perspectives

Here we presented evidence for a novel bacteriophage from *M. catarrhalis*, however investigation should continue in order to demonstrate the presence of this phage. Number of questions have to be answered, with new sets of experiments.

First of all, the two types of particles have to be isolated, and the nucleic acid content of each has to be determined.

Second of all, the DNA and RNA have to be sequenced. If our current method of RNA sequencing will not yield results, reverse transcription with random primers will be used to form a cDNA library of the RNA. This cDNA library can be sequenced with conventional methods, and the complete sequence can be retrieved by alignment of the partial sequences. For sequencing of DNA, a genomic library has to be formed using at least two different restriction enzymes cutting the DNA in sufficient number of locations. Once the genomic library is formed, each clone will be sequenced and the complete sequence will be obtained by alignment of all partial sequences. Sequences of the RNA and DNA are valuable to determine the nature of the particles we isolated. Presence of phage genes on these nucleic acids will ultimately prove the presence of bacteriophage in *M.catarrhalis*. Moreover, the sequence homology searches and phylogenetic tree analysis will let us comment on classification of the phage(s). The sequence data

will let us understand the nature of the vesicle-like particles. If the vesicle-like particles are the MVs, and they contain the DNA; this DNA most likely carries important virulence genes. The proteins and enzymes carried on or in these MVs are also candidate virulence factors of *M.catarrhalis*, and one can investigate the importance of them. In addition, the biological properties of the phage such as burst size, growth curve, life cycle have to be studied. Potential of phage in phage therapy has to be considered in the future.

Chapter 6

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