Investigation of the effects of polymicrobial infection on the induction of otitis media

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A thesis submitted for the qualification of Doctor of Philosophy, Faculty of Science, Engineering and Health, Central Queensland University

August 28th, 2008

Certificate of authorship and originality of thesis

The work contained in this thesis has not been previously submitted either in whole or in part for a degree at Central Queensland University or any other tertiary institution. To the best of my knowledge and belief, the material presented in this thesis titled *"Investigation of the effects of polymicrobial infection on the induction of otitis media"* is original except where due reference is made in text.

Ajay Krishnamurthy
28th August 2008

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ACKNOWLEDGEMENTS

A PhD is a colossal task which cannot be achieved without the guidance and help from many people, to which I am very grateful for. Although my PhD started in the Univeristy of Canberra (UC), the majority of the work presented in this thesis was undertaken at the Central Queensland University (CQUniversity), Rockhampton. The scholarships from UC, which later got transferred to CQUinversity in 2006 has been extremely supportive in helping me to reach completion.

To begin with, I like to thank my supervisors Prof Jennelle Kyd and Prof Allan Cripps for their constant support, understanding, guidance and direction. *"Your enthusiasm and support have renewed my motivation countless times and allowed me to think and consider new ways of looking at different situations that came across during my candidature. The immense patience shown by you to refine my writing skills shall never be forgotten"*.

A special mention and thanks to Dr John McGrath and Ms Jessica Browne for assisting me during my animal experiments. As majority of my work involved animal experiments, this thesis would not have been possible without their support. *"John- your advice, explaining things to me in simpler terms and helping me understand different concepts (no matter how trivial it was) have made my PhD journey much easier"."Jess- your effort in managing the animal house, counting several of my white blood cell slides, doing the titrations and labelling was very critical"*. I thank you both for all your efforts during the many big days of animal experiments and am also glad to develop our friendship outside the lab. Before moving to CQUniversity, some of the animal work was also done in UC and I thank the research staff at the Gadi Research Centre (UC) - Gillian Nolen, Nancy Fisher, Claire Batum, Ray Ellet and Donna Easton for helping me with my prelimnary animal studies.

I was fortunate to be awarded the "Rita Colwell/Nancy Millis Postgraduate Travel award", that allowed me to present my work (Chapter 6) at the 108th American Society for Microbiology General Meeting in Boston, USA. This award also allowed me to perform real time monitoring of disease progression using bioluminescent bacteria (Chapter 5) at the Children's Research Institute, Nationwide Children's Hospital, Columbus, Ohio, USA headed by our collaborator Dr Lauren Bakaletz. I thank Dr Bakaletz and her research staffs- Laura Novotny, Joe Jurcisek, Glen McGillivary for providing such an exciting learning experience, and appreciate the effort from summer help students (Kara and Zac) who imaged the mice at certain days. A special thanks to Laura, Joe and Glen for helping with the split sections of mouse heads, titrations and capturing the images. *"You guys are absolute genious and thanks for making my trip to the US so memorable"*.

I also thank the research staffs at Griffith Health for allowing me to use their Bio-plex instrument and other resources that were critical for my data in Chapter 7. A special

thanks to Ms Pauline Low for scheduling me with the BioRad technical specialists that allowed me to learn about the Bio-plex and run my assay. The technical specialist, Mr Jackson Jones has been of good help and never hesitated to answer my queries anytime.

Being an international student I came here with all sorts of different feelings, however the friendship and support from my friends here have made this place a "second home" for me. *"Thanks for being there, guys"* A special mention to my ex-house mates, Lois Harris and Tanya Pegg for making my stay at Mills Avenue unforgettable. Lastly, I thank my parents and uncle for their love and support and also for giving me that extra bit of encouragement and motivation to face the outside world. Finally, I thank GOD for His blessings that helped me to reach completion.

LIST OF PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Krishnamurthy, A., McGrath, J., Cripps, A.W., Kyd, J.M. (2008). (Manuscript re-submitted) "The Incidence of *Streptococcus pneumoniae* otitis media is affected by the polymicrobial environment particularly *Moraxella catarrhalis* in a mouse nasal colonisation model". *Infect Immun*

Krishnamurthy, A., McGrath, J., Cripps, A.W., Kyd, J.M. (Manuscript in preparation) "Effect of bacterial concentration in the nasal inoculum on respiratory tract infections" (Chapter 3).

Krishnamurthy, A., Novotny, L., Jurcisek, J., McGillivary, G., Bakaletz, L.O., Cripps, A.W., Kyd, J.M. (Manuscript in preparation). "Real-time monitoring of disease progression: a novel approach to assess the incidence rate of otitis media in a polymicrobial environment" (Chapter 5).

Krishnamurthy, A., Cripps, A.W., Kyd, J.M. (Manuscript in preparation). One combined paper on bacterial adherence and cytokine responses *in vitro* to be submitted out of Chapters 6 and 7.

CONFERENCE PRESENTATIONS

Krishnamurthy, A., Kyd, J.M., Cripps, A.W. (2008)"Viral Infection of Respiratory Epithelial Surfaces Enhances Adherence of *Streptococcus pneumoniae* and *Moraxella catarrhalis* and Is Influenced by the Polymicrobial Environment" Poster paper, American Society for Microbiology General Meeting, Boston, United States.

Krishnamurthy, A., McGrath, J., Cripps, A.W., Kyd, J.M (2007)"The microbiology and inflammatory response in polymicrobial otitis media" Proffered paper, Australasian Society for Immunology (ASI) Annual Scientific Meeting, Sydney, Australia .

Krishnamurthy, A., McGrath, J., Cripps, A.W., Kyd, J.M (2007)"The inflammatory responses in polymicrobial otitis media infection model" Poster paper, Australasian Society for Immunology Annual Scientific Meeting (ASI) Sydney, Australia.

Krishnamurthy, A., McGrath, J., Cripps, A.W., Kyd, J.M (2007)"The microbiology and inflammatory response in polymicrobial otitis media" Proffered paper, Australian Society for Microbiology (ASM) Annual Scientific Meeting, Adelaide, Australia.

Kyd, J.M., Krishnamurthy, A., McGrath, J., Cripps, A.W. (2007), "Experimental evidence that the composition of the nasopharyngeal bacterial flora affects the incidence of otitis media" Plenary paper, International Symposium on Recent Advances in Otitis media, Florida, USA.

ABBREVIATIONS

AOM	acute otitis media
BAL	bronchoalveolar lavage
BHI	Brain-Heart Infusion
CBP	choline binding protein
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CFU	colony forming units
ChoP	phosphorylcholine
COM	chronic otitis media
COME	chronic otitis media with effusion
COPD	chronic obstructive pulmonary disease
CSOM	chronic suppuratives otitis media
eNOS	endothelial nitric oxide synthase
EPS	extra polysaccharide
ET	Eustachian tube
FCS	fetal calf serum
HMW	high molecular weight
ICAM	intracellular adhesion molecule
IFN	interferon
IgA	immunoglobulin A
IgD	immunoglobulin D
IL	interleukin
iNOS	inducible nitric oxide synthase
Lbp	lactoferrin binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LRTI	lower respiratory tract infection
Mcat	*Moraxella catarrhalis*
MCP	macrophage chemotactic protein
MEL	middle ear lavage
MSCRAMM	microbial surface components recognising adhesive matrix
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NFκβ	nuclear factor kappa beta
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
NOS	nitric oxide synthase
NTHi	nontypeable *Haemophilus influenzae*
OM	otitis media
OME	otitis media with effusion
OMPs	outer membrane proteins
PAF-r	platelet activating factor receptor

PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
PMNs	polymorphonuclear neutrophils
PRRs	pattern recognition receptors
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SPF	specific pathogen free
Spn	*Streptococcus pneumoniae*
Tbp	transferrin binding protein
TCID	tissue culture infective dose
TLRs	Toll-like receptors
TNF	tumor necrosis factor
URTI	upper respiratory tract infection
Usp A	ubiquitous surface protein A
VCAM	vascular cell adhesion molecule

ABSTRACT

Bacterial otitis media (OM) is a well known paediatric condition predominantly caused by *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*. The polymicrobial etiology and the mechanisms and pathogenesis of the disease caused by single bacterium, synergistic bacterial-viral interactions, and inflammatory processes have been studied using different animal models. The complexities associated with polymicrobial OM, mechanisms of host-bacterial relationships, inflammatory responses, and microbial interactions would be better understood in an experimental model comprising of the predominant bacteria involved. These issues were explored in this thesis in the context of investigating the effects of polybacterial infection on the incidence and severity of OM.

An experimental polymicrobial infection murine (BALB/c) model was established involving different combinations of the above mentioned bacteria along with a respiratory virus (Sendai). This model was used to demonstrate for the first time that the presence of *M. catarrhalis* as a co-colonising agent of the nasopharynx significantly exacerbates pneumococcal OM. Another significant finding was that the inflammatory response generated, was due to the synergistic bacterial-viral infection and not by the respiratory virus alone (Chapter 2). This model was next used to show that the incidence of lower respiratory and middle ear infections caused by *M. catarrhalis* and NTHi was not affected by bacterial dosage and pre-colonisation of the nasopharynx (Chapter 3 & 4 respectively). In contrast, pre-viral infection and increased bacterial numbers facilitated greater incidence of pneumococcal OM and lower respiratory tract infections (Chapter 3). The incidence of pneumococcal OM was also evident in the presence of a pre-coloniser (*M. catarrhalis*) in the nasopharynx (Chapter 4). The impact of the polymicrobial environment in the nasopharynx on the incidence of OM could be better understood with real-time monitoring of infection progression by biophotonic imaging. Following intranasal infection, co-colonisation patterns and its effect on OM of luminescent NTHi and *S. pneumoniae* were measured (Chapter 5). The data not only showed rapid ascension of *S. pneumoniae* when pre-infected with *M. catarrhalis* and co-infected with NTHi and *M. catarrhalis*, but also showed the ability of bacteria to colonise different niches.

The factors associated with microbial interactions, including adherence, with the host were identified using *in vitro* cell cultures as a model because of their ease of manipulation and cost-effectiveness (Chapters 6 and 7). This was performed using the above mentioned bacterial combinations and adenovirus on lung (A549) and bronchial (BEAS 2B) epithelial cell lines. When infected alone, adherence of *M. catarrhalis* and *S. pneumoniae* to adenovirus-infected BEAS-2B cells was greater than virus-infected A549 cells, but was reduced on BEAS-2B cells in a co-infection with *M. catarrhalis* and *S. pneumoniae*. In contrast, this co-infection increased the adherence to virus-infected A549 cells (Chapter 6). This observation supports the findings of the *in vivo* studies (Chapter 2) indicating a positive association between these two bacteria to cause infections. The pulmonary burden caused by bacterial products could be different in the various respiratory compartments, as seen with different adherence levels to epithelial

cells, suggesting the possibility of different cytokine responses. This was demonstrated for IL-6, with significant differences in the release of IL-6 by BEAS 2B and A549 cells in response to bacterial infection, and a synergistic effect of *M. catarrhalis* and *S. pneumoniae*, and, *S. pneumoniae* and NTHi co-infections on IL-6 production (Chapter 7).

The overall finding of this thesis was that the nasopharyngeal *M. catarrhalis* infection not only predisposes pneumococcal otitis media, but also promotes pneumococcal colonisation at a site distal from its initial inoculation site. Furthermore, this association also enhanced the adherence to alveolar epithelial cells releasing high levels of the inflammatory cytokine IL-6. This finding contributes to better understanding of the early onset of OM observed evidently at an alarming rate, particularly in Australian Aboriginal children, and will assist strategies to for preventing and managing infections.

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL FEATURES OF OTITIS MEDIA

1.1.1 Introduction

Upper respiratory tract infections (URTIs) are usually polymicrobial in nature and can have a number of serious clinical outcomes. Otitis media (OM) is one of the complicating sequelae of URTI and is a serious concern in childhood health care (Bakaletz 2002; McIntosh *et al.* 1993). OM is generally defined as inflammation of the middle ear due to viral or bacterial infection (Bantam 1981). However it is a broad term that includes clinical manifestations like myringitis, acute otitis media (AOM), otitis media with effusion (OME), chronic otitis media (COM) and chronic suppurative otitis media (CSOM) that are known to be caused by several bacteria (Faden 1997; Infante-Rivard & Fernandez 1993). AOM is usually manifested with the presence of middle ear effusion accompanied with the rapid onset of signs and symptoms of inflammation of the middle ear. OME is a condition recognised with the presence of middle ear fluid and without any signs and symptoms of acute ear infection. CSOM is a chronic inflammation of the middle ear along with a non-intact membrane and ear discharge (Gates *et al.* 2002).

1.1.2 Clinical presentation of otitis media

OM is a common childhood disease whose pathogenesis and immunology are still not completely understood. The clinical presentation of OM is often symptomatic, comprising of earache, fever and irritability with or without ear discharges (Infante-Rivard & Fernandez 1993). Contributors to susceptibility to OM are multifactorial: viral URTI, attendance at day care centres, gender, breast feeding, smoking, genetic background that are all well recognised (Paradise *et al.* 1997). The complications involved with untreated OM or recurrent OM with effusion may lead to hearing loss resulting in abnormalities in speech, language, behavioural and cognitive development (Teele *et al.* 1990).

1.1.3 The burden of disease

The presence and detection of bacteria in the middle ear effusions was first reported in 1958 (Senturia *et al.* 1958), during which, the treatment options were limited to purgatives, herbal remedies and surgical drainage. However, today OM remains the most frequently diagnosed childhood disease. The complications involved with untreated and recurrent episodes of OM include hearing impairment, with potential impact on learning, speech, language, and, cognitive abilities. In the United States, the number of visits to the doctor for the diagnosis of OM has significantly increased from approximately 10 million to 25 million over the period 1975-1990 (Schappert 1992). These visits have further added financial constraints to the families in the last decade (Alsarraf *et al.* 1999). Approximately 30% of infants and 80% of children have had at least one episode of AOM within 1 year and 3 years of age respectively, with peak incidences between 6 and 18 months of age (Teele *et al.* 1989). Moreover, 40% of children have had more than 6 episodes of AOM by 7 years of age. Due to its high prevalence, the disease has a significant impact on financial outcomes for both families with children and the health care system. In the United States, the annual total expenditure involved in the management of OM by the health care system is known to be approximately $5 billion (Bondy *et al.* 2000; Gates 1996). This estimate has increased and been shown to be a significant contributor to the health cost for families. The average number of days required for the antimicrobial therapy for OM was 41.9 days in first year and 48.6 days for children aged 2 years (Gates 1996), however, many studies now show that lowering antibiotic therapy does not result in significant loss of cure (Media 2004; Park *et al.* 2008; Pichichero & Brixner 2006). The costs include performance of surgical procedures such as myringotomy and insertion of tympanostomy tubes in approximately 1.8% of the 2253 infants aged less than 2 years in one study (Paradise *et al.* 1997).

1.1.4 Incidence of lower respiratory tract infections

Lower respiratory tract infections (LRTIs) have been one of the most frequent causes of mortality as stated by the World Health Organisation (Moellering 2002), with more than

3 million deaths to have been reported in children due to pneumonia (Leowski 1986). Prior to the introduction of vaccines, bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Klebsiella pneumoniae* were identified as major causative agents of bacterial pneumonia (Shann 1986). In certain developing countries like Ghana and South Africa, LRTI caused by *S. pneumoniae* has been reported to be as high as 60-90% in children less than 5 years of age, and 100 per 1000 population of adults per year and remains at equivalent rates a decade later (Cashat-Cruz *et al.* 2005; Obaro *et al.* 1996; Rudan *et al.* 2008). The epidemiology of pneumococcal infection in children has been previously reviewed (Greenwood 1999). In this review by Greenwood, B. (1999), the incidence of invasive pneumococcal pneumonia was reported and compared globally. In addition, the risk factors such as genetic factors, antecedent viral infections, socio-economic factors, age and seasonality likely to influence the high incidence of pneumococcal disease in children was also highlighted (Greenwood 1999). An epidemiological study conducted in the United States reported an overall incidence of pneumococcal disease to be highest in children less than 2 years of age and in adults aged 65 years and above (Robinson *et al.* 2001). In addition, the incidence of pneumococcal diseases was approximately 2.5 times higher in the black population in comparison with the white population. More recently, the incidence of pneumonia in children less than 5 years of age has been estimated to be higher in countries such as South-East Asia, Africa, Western Pacific countries, followed by the Americas and Europe (Rudan *et al.* 2008). This recent review (Rudan *et al.* 2008) has also predicted that the number of pneumonia cases to be high in countries such as India, China, Pakistan and Nigeria. In addition, bacteria such as *H. influenzae* and *M. catarrhalis* are also known to cause LRTIs such as infections associated with chronic obstructive pulmonary disease (COPD), bronchitis and cystic fibrosis (Bandi *et al.* 2001; Murphy *et al.* 2005(b); Murphy & Sethi 1992).

1.1.5 Risk factors for otitis media

The various risk factors for OM have been well described and include host associated factors such as gender, race, allergy, adenoids, and, genetic predisposition as well as

environmental factors such as other URTI, seasonal changes, day care centres, number of siblings, bottle feeding, exposure to tobacco smoke, breast feeding and use of pacifiers (Arola *et al.* 1990; Castagno & Lavinsky 2002; Froom & Culpepper 1991; Infante-Rivard & Fernandez 1993; Neto *et al.* 2006; Rovers *et al.* 2006; Stenstrom *et al.* 1993). The risk factors although not directly associated with the pathophysiology of OM, are known to increase the risk of the disease as they influence mechanisms that facilitate an infection advantage for the microbe.

Host associated factors such as age, gender and race are known to influence the structure and functional aspects of the ear canal. During infancy, the angle at which the ET is located and its length are major factors associated with infant susceptibility to OM. The shorter angle and length of ET in young infants is known to increase the ability of the pathogens to migrate from the nasopharynx to the middle ear. However during ET dysfunction, the fluid is not drained adequately and this results in bacterial proliferation accompanied by inflammation and pain (Harrington 2000; Wright & Meyerhoff 1994).

Amongst the environmental factors, the greater incidence of AOM and OME in large families with many siblings has been previously described and mentioned in certain reviews (Neto *et al.* 2006; Pukander & Karma 1988; Rovers *et al.* 2006). The greater number of siblings in Australian Aboriginal communities could be one of the reasons why young infants have high bacterial nasopharyngeal carriage rates from their siblings (Jacoby *et al.* 2008; Leach *et al.* 1994; Lehmann *et al.* 2008).

The incidence of OM parallel to upper respiratory infections, is usually higher during the winter months when compared to spring and summer months (Castagno & Lavinsky 2002). The exposure of tobacco smoke to young children is known to damage the epithelial lining of the upper respiratory tract and facilitate bacterial adherence and colonisation (Etzel *et al.* 1992; Jacoby *et al.* 2008). Also, certain allergy conditions like rhinitis have been suggested to predispose young children to OM by ET dysfunction caused by allergic reaction of nasal mucosa (Fireman 1997; Gungor & Corey 1997). A

damp environment is known to influence the growth of mold, dust mites, which are known allergens in the subtropical/tropical areas of the world (Li *et al.* 1994). However, the importance and the role of dampness in the pathogenesis of AOM are unclear. Breastfeeding seems to have some prophylactic effect on incidence of OM, as children who were breast fed until 6 months of age or longer failed to show any episodes of recurrent AOM (Saarinen 1982). In addition, the position at which the child is breast fed also seemed to affect the incidence of OM. It has been suggested that supine breast feeding was associated with early onset of chronic otitis media with effusion (COME) (Saarinen 1982). The use of pacifiers in infants has also been associated with increased incidence of OM (Niemela *et al.* 1994). The exact cause is unclear; however pacifiers may act as a fomite and carry different types of bacteria. Also, the pressure changes associated with sucking of the pacifier is suggested to facilitate the transfer of potential nasopharyngeal microbes into the middle ear via the ET, which may pose a risk of OM (Post & Goessier 2001).

1.1.6 Causative agents of otitis media

Bacterial infection is regarded as the main etiological factor of AOM as they are cultured in approximately 44-84% of the middle ear effusions. The bacteriology of OM was different in the early 1900s, as bacteria such as β-haemolytic *Streptococci*, Group A *Streptococci* and Staphylococcal species were commonly isolated from the middle ear (Virolainen *et al.* 1994). However, the bacteriology of OM has changed significantly during the past 40 years. Bacteria such as *S. pneumoniae*, nontypeable *H. influenzae* (NTHi) and *M. catarrhalis* are now the dominant pathogens isolated from the middle ear. Bacteria such as β-haemolytic *Streptococci*, Group A *Streptococci* and Staphylococcal species which were frequently isolated in the pre-antibiotic era are now being infrequently isolated and are found in less than 3% of the middle ear effusions of AOM (Bluestone & Klein 1995; Virolainen *et al.* 1994). The reasons for this shift in the bacteriology of OM are unknown. The recovery rate of the dominant bacteria such as *S. pneumoniae*, NTHi and *M. catarrhalis* isolated from the middle ear are known to be approximately 35-50%, 20-60% and 5-15% respectively (Bulut *et al.* 2007; Casey &

Pichichero 2004; Faden 1997; Guven *et al.* 2006; Pichichero *et al.* 2008). Apart from these dominant pathogens, in late 1980s a new species of bacteria was isolated from the middle ear effusions of patients with OME and subsequently identified as *Alloiococcus otitidis* (Aguirre & Collins 1992; Faden & Dryja 1989). *A. otitidis* is a slow growing, gram-positive fastidious organism and has been detected using culture methods and PCR-based methods from the middle ear effusions (Harimaya *et al.* 2006; Hendolin *et al.* 1999; Hendolin *et al.* 1997). This gradual change in the bacteriology of OM during the past decades has demonstrated the need of continuous monitoring of the pathogens involved in OM, particularly with the introduction of the pneumococcal vaccine.

In URTI including OM, a preceding viral infection is well known to trigger secondary bacterial infections through mechanisms such as enhancing bacterial adherence, colonisation and translocation through the epithelial barrier [reviewed by (Hament *et al.* 1999)]. Over the last two decades, a considerable body of evidence has emerged indicating the crucial role of respiratory viruses in the development of AOM and has been summarised in several reviews (Hament *et al.* 1999; Heikkinen 2001; Ruuskanen *et al.* 1989). Many respiratory viruses like respiratory syncytial virus (RSV), coronavirus, parainfluenza virus 1 and 3, influenza virus A, adenovirus and rhinovirus have been found associated with AOM (Chonmaitree *et al.* 2008; Heikkinen *et al.* 1999), of which, RSV and rhinovirus are well known to frequently cause viral OM. Viruses have been detected in approximately 5-48% of children suffering from AOM (Arola *et al.* 1990; Bulut *et al.* 2007; Klein 1994; Nokso-Koivisto *et al.* 2006). Thus, the isolation of these viruses along with bacteria from the middle ear effusions of children with OM indicates a polymicrobial etiology of the disease.

1.2 BACTERIAL COLONISATION & PATHOGENESIS

1.2.1 Microbiology and pathogenesis of bacteria

Bacterial colonisation

In neonates, the commensal bacteria are known to colonise on the skin and other mucosal sites immediately after birth. Bacterial colonisation of the upper airways has been reported to increase gradually and peak between 2-3 years of life, and then decrease gradually until 15-16 years of age and eventually increase again in adults at 65 years of age or more (Leiberman *et al.* 1999; Syrjanen *et al.* 2001). Amongst the various commensals, *S. pneumoniae, H. influenzae* and *M. catarrhalis* have high rates of nasopharyngeal colonisation. The bacterial acquisition and carriage rates reported in studies from many countries have been reviewed (Garcia-Rodriguez & Martinez 2002) and the mean age of first acquisition of any of these bacteria was as early as 6 months of age. This review did not include studies on Australian infants. The relationship between nasopharyngeal colonisation and development of OM in children suggest that increased rate of colonisation to be one of the risk factors involved in OM (Faden *et al.* 1997). The nasopharyngeal colonisation rates with these bacteria were observed to be approximately 24%, 9% and 26% respectively during the first six months of life, which were then shown to increase to approximately 54%, 33% and 72% respectively by the end of one year (Faden 2001). An early onset and high levels of nasopharyngeal colonisation in the Australian Aboriginal children has caused serious concern. The median age for colonisation of *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* was reported to be 8, 10 and 20 days respectively, when compared to 200, 209 and 270 days in non-Aboriginal children (Leach *et al.* 1994). More recently, the nasopharyngeal carriage rates of *S. pneumoniae*, *M. catarrhalis* and NTHi in Aboriginal children have been reported as 49%, 50% and 41% respectively, compared with 25%, 25% and 11%, respectively, in non-Aboriginal children (Watson *et al.* 2006). The risk of developing OM was approximately four times higher when colonised with *S. pneumoniae* or NTHi, in comparison with *M. catarrhalis* (Faden *et al.* 1997; Faden *et al.* 1994). The dynamics

of bacterial colonisation has therefore provided an insight to the understanding of incidence rate of OM.

Bacterial pathogenesis

Many bacterial pathogens are still known to impose a major threat to human health despite making advances in the understanding of bacterial pathogenesis and infectious diseases using several molecular tools, and treating multi-drug resistant strains of different infectious agents (Morens *et al.* 2004; Waldvogel 2004). Koch's postulates have been one of the fundamentals in the understanding of the microbial pathogenesis. It has been well accepted that not all microbes are pathogenic, as different microbes are known to inhabit the mouth, gut, and on skin without causing any infection. Regardless of the nature of the disease, the attachment of bacteria to any mucosal surface is considered to be an important factor in the pathogenesis of most infectious diseases. In order to establish an infection, an infecting bacteria needs to avoid the host's defence mechanisms and compete with the normal flora to obtain essential nutrients for its growth and survival (Spitz *et al.* 1995). This process is regulated by microbial "virulence factors". Virulence is defined as the measure of the pathogenicity or ability of any pathogen to cause an infection. The term "virulence factor" comprises different microbial products such as secreted proteins, toxins, enzymes and polysaccharides, which enable them to colonise a host (Brogden *et al.* 2000). A selection of virulence factors for most gram-positive and gram-negative bacteria and the importance of their functions in pathogenesis of any disease are summarised in **Table 1.1**.

Table 1.1: Virulence factors which promote colonisation and survival of several bacteria [Adapted from (Salyers & Whitt 2002)]

Virulence factors	Function
pili & fimbriae	enables adhesion to mucosal surfaces
nonfimbrial adhesins	tight binding to host cells
invasins	force nonphagocytic cells to engulf bacteria
binding to M cells	portal of entry into body
sIgA proteases	prevent trapping of bacteria in mucin
siderophores, surface proteins like transferring, lactoferrin etc.	acquisition of iron
capsules	prevention of phagocytosis, complement inactivation
Lipopolysaccharide O antigen	inability to form membrane attack complex, serum resistance
toxins	killing of phagocytes
motility & chemotaxis	enables bacteria to reach mucosal surfaces

1.2.2 Streptococcus pneumoniae

Microbiology of *Streptococcus pneumoniae*

S. pneumoniae or pneumococcus is a gram-positive cocci, non-motile, facultative anaerobe that is known to occur in chains or in pairs. It belongs to the Family *Streptococceae* and is classified based upon its haemolytic capability and the presence of carbohydrate antigens located in the cell wall using the Lancefield system. The different types of hemolysis observed are alpha- incomplete hemolysis e.g.; *S. pneumoniae*, beta- complete hemolysis e.g.; *S. pyogenes*, and gamma, where no hemolysis is observed. Based on the capsular polysaccharides, the pneumococcus is further divided into more than 80 different capsular serotypes (Jensen *et al.* 1997b). The most commonly recognised serotypes of *S. pneumoniae* found in children are serotypes

6, 14, 19 and 23 (Bogaert *et al.* 2001; Coles *et al.* 2001; Varon *et al.* 2000). The involvement of polysaccharide capsule, various proteins and enzymes such as pneumococcal surface protein, choline binding protein, autolysin, hyaluronidase and neuraminidase in its pathogenicity have been discussed briefly in the subsequent sections of this review.

Pathogenesis of *Streptococcus pneumoniae*

The various pneumococcal virulence factors known to be associated with the pathogenesis of *S. pneumoniae* include; pneumolysin, autolysin, capsule and surface adhesins (AlonsoDeVelasco *et al.* 1995; Canvin *et al.* 1995). The other enzymes and surface proteins such as neuraminidase, SpxB (pyruvate oxidase), choline binding proteins CbpA are also known to play an important role in bacterial colonisation (Brooks-Walter *et al.* 1999). The characteristic functions of various proteins and enzymes involved in the pathogenesis of *S. pneumoniae* are summarised in **Table 1.2.**

Table 1.2: Virulence factors of *S. pneumoniae* and their functions. Compiled from (AlonsoDeVelasco *et al.* 1995)

Virulence factors	Functions
capsule	complement inactivation, inhibit phagocytosis
cell wall & its polysaccharides	induce inflammation and activate alternative pathway of complement, mediates attachment to endothelial cells
pneumolysin	cytolytic, cytotoxic at high and low concentrations respectively, inhibit ciliary movement, antibody synthesis, activates complement
PspA	complement inactivation

autolysin	release pneumolysin and cell wall products
neuraminidase	intracellular survival of bacteria
PsaA	transporting trace elements like manganese and zinc into the cytoplasm of bacteria
choline binding protein	adhesin
hyaluronidase	facilitates invasion

1.2.2.1 Adhesins involved in colonisation and adhesion

The pneumococcal capsule is a well known virulence factor in the pathogenesis of *S. pneumoniae* (Winkelstein 1984). Encapsulated strains of *S. pneumoniae* are known to be approximately 10^5 times more virulent than the noncapsulated ones (Watson & Musher 1990). In addition, the virulence of *S. pneumoniae* has been reported to be dependent on the amount of capsule produced by the pneumococcus (Kim & Weiser 1998). Phenotypic variation is an important mechanism by which bacteria adapt themselves to different host environments. The transparent variants of pneumococci are known to cause persistent nasopharyngeal colonisation, whereas the opaque variants cause systemic infections (Weiser *et al.* 1994). The transparent variants are also known to produce high amounts of teichoic acid containing phosphorylcholine, which serves as a binding site for several choline binding proteins. Therefore the components of the pneumococcus cell wall along with choline binding proteins facilitate bacterial adherence and colonisation on host tissues (Kim & Weiser 1998; Rosenow *et al.* 1997). There is evidence from animal studies using phenotypic variants that there is a requirement for the pneumococcal capsule for colonisation of the nasopharynx (Magee & Yother 2001). In addition to the capsule, certain encapsulated strains of *S. pneumoniae* are also known to possess pili which facilitate adhesion, induces inflammatory responses and contributes towards tissue invasion (Barocchi *et al.* 2006).

The choline binding proteins (CBPs) have a C-terminal choline binding module which is shown to bind to teichoic or lipoteichoic acid and functions as an anchoring device for

most gram-positive bacteria including *S. pneumoniae* (Wren 1991). Mutational studies identified that choline-binding proteins, such as CbpA, have virulence characteristics in animal models, and CbpA has a role as an adhesin on cytokine-activated human epithelial cells *in vitro* (Gosink *et al.* 2000; Rosenow *et al.* 1997). It is believed that there are approximately 10-15 different CBPs encoded by *S. pneumoniae* and include PspA, PspC and LytA [reviewed by (Bergmann & Hammerschmidt 2006)]. PspC has been shown to bind to human secretory IgA and may be involved in translocation of bacteria across the respiratory epithelium (Zhang *et al.* 2000).

After adherence of pneumococcus to a cell surface, further tissue invasion is known to be facilitated by enzymes such as hyaluronidase and neuraminidase. Hyaluronic acid is one of the most abundantly found glycans in the extracellular matrix of connective tissues (Yang *et al.* 1994). Hyaluronidases have the ability to degrade hyaluronic acid and facilitate tissue invasion of bacteria in infections such as pneumonia, meningitis and bacteremia (Meyer *et al.* 1941). Amongst the different types of hyaluronidase enzymes known to degrade hyaluronan, hyaluronate lyases produced by *S. pneumoniae* are involved with host tissue invasion (Humphrey 1948; Kreil 1995). The exact mechanism of its action was recently demonstrated with the help of structural studies (Li, S. *et al.* 2000). It is known to facilitate bacterial invasion into tissues, causing bacteremia and generating inflammatory responses in the lung [reviewed by (Jedrzejas 2001)]. Another enzyme, neuraminidase comprising of NanA and NanB, of which NanA has been shown to enhance intracellular survival and replication of the bacteria in the lung [reviewed by (Mitchell 2000)]. Moreover, the activities of NanA and NanB were shown to be pH dependent suggesting their involvement in different environments and in promoting pneumococcal colonisation (Berry *et al.* 1996). The exact mechanism of action of neuraminidase is unknown, however it is believed to cleave the sialic acid from glycans such as mucin, glycolipids present on host cells, and enhance bacterial colonisation [reviewed by (Jedrzejas 2001)].

S. pneumoniae has also been shown to bind to immobilised fibronectin (van der Flier *et al.* 1995) and the protein responsible was identified as PavA (Holmes *et al.* 2001). Recently, the involvement of PavA in modulating adherence and facilitating invasion associated with meningeal diseases was demonstrated in an experimental animal model (Pracht *et al.* 2005). There are various virulence factors involved in colonisation of both upper and lower respiratory tract and the role of these interactions with host's epithelial structures in causing disease has been recently reviewed by (Kadioglu *et al.* 2008).

1.2.2.2 Virulence factors involved in complement resistance

In addition to playing an important role in bacterial colonisation, pneumococcal capsule has also been shown to prevent opsonisation and phagocytosis of the pneumococcus by the host's defence systems (Winkelstein 1984). The pneumolysin protein of *S. pneumoniae* is released upon bacterial lysis under the influence of autolysin (Boulnois *et al.* 1991). Pneumolysin is an intracellular, major pore forming cytotoxin, known to cause lysis and activate the complement system, thereby contributing to the virulence of *S. pneumoniae* (Rubins *et al.* 1996). Upon its release, pneumolysin penetrates the physical defences of the host and disrupts the tight junctions and integrity of the ciliated bronchial epithelial cells (Rayner *et al.* 1995; Steinfort *et al.* 1989). This disruption was shown to be responsible for the reduction in the ability of bronchial cells to clear mucous, thereby creating an environment that facilitates the spread of bacteria potentially resulting in bacteremia [reviewed in (Jedrzejas 2001)]. PspA is reported to be a surface-exposed transmembrane protein and identified as a lactoferrin binding protein (Hammerschmidt *et al.* 1999; Talkington *et al.* 1992). Its mechanism of action has not been fully understood, however, it has been shown to inhibit complement activation (Tu *et al.* 1999).

1.2.2.3 Virulence factors involved in inflammation

Autolysins are well recognised enzymes that can degrade peptidoglycan present in the cell wall of bacteria resulting in cell lysis (Rogers *et al.* 1980). In addition, various cell

wall degradation products such as peptidoglycan, teichoic acid, and toxins like pneumolysin have also been shown to be released following autolysis and induce inflammatory responses in the host (Lock *et al.* 1992; Tuomanen 1999). Autolysin is also triggered by human lysozyme, which is released upon infection and inflammation, thereby inducing pneumococcal lysis and enhanced inflammation (Bruyn *et al.* 1992). In *S. pneumoniae*, N-acetylmuramoyl-L-alanine amidase (LytA amidase) is one of the well characterised autolysins. Its role in the release of various components from the cell wall and therefore its contribution to inflammation has been documented in a review (Tuomanen 1999).

S. pneumoniae being a nasopharyngeal commensal, gains access to the ET and lung via the nasopharynx, and causes middle ear infection and pneumonia, respectively (Tuomanen 2001). During this process, there is a massive influx of neutrophils driven by pro-inflammatory cytokines, such as IL-6, IL-1 and TNF-α. Pneumococcal OM is often characterised by profound inflammation and the importance of the pneumococcal cell wall components in facilitating inflammation is well known (Carlsen *et al.* 1992). The progression to pneumonia is associated with a viral infection which can enhance the adherence of *S. pneumoniae* to the respiratory epithelia (Hakansson *et al.* 1994). This has been thought to be via interactions between phosphorylcholine of the pneumococcus cell wall and platelet-activating factor receptors (PAF-r), believed to be up-regulated by cytokine stimulation (Cundell *et al.* 1995).

The various actions of pneumolysin such as: induction of cytokines like TNF-α and IL-1β; disruption of the integrity of epithelial cells; decrease bactericidal activity; inhibit migration of neutrophils; and inhibition of lymphocyte proliferation and antibody synthesis, indicates its important role in the pathogenesis of *S. pneumoniae* [reviewed by (AlonsoDeVelasco *et al.* 1995)].

1.2.3 Haemophilus influenzae

Microbiology of *Haemophilus influenzae*

H. influenzae is a well known pleomorphic, fastidious, gram-negative coccobacilli and a normal inhabitant of the human respiratory tract. It is non-motile, often capsulated and is a facultative anaerobe requiring growth factors such as X (hemin) and V (NAD, NADP) *in vitro*. Depending on the presence of capsular polysaccharides, *H. influenzae* is divided into encapsulated and non-capsulated forms. The encapsulated forms are known to express one of the six different capsular polysaccharides a-f (Jensen *et al.* 1997a). The non-capsulated forms are unable to react with the antisera against the recognised polysaccharide capsule, do not have the capsule genes and are referred to as nontypeable *Haemophilus influenzae* (NTHi). NTHi is one of the major pathogens involved in OM and is shown to initiate infections by colonising the upper respiratory tract and accounting for approximately 40% of the disease (Rayner *et al.* 1998). It is also known to cause both upper and lower respiratory tract infections especially in the immunocompromised host and in children below the age of four (Jensen *et al.* 1997a).

Pathogenesis of *Haemophilus influenzae*

Various virulence factors involved in the pathogenesis of *H. influenzae* include; lipooligosaccharide (LOS), high molecular weight proteins (HMW), Hia protein, IgA protease, Hap protein, Oap A protein and protein D (Dawid *et al.* 1999; DeMaria *et al.* 1997; Hendrixson & Geme 1998; St. Geme & Cutter 2000; Swords *et al.* 2000; Weiser *et al.* 1995).

1.2.3.1 Adhesins in NTHi pathogenesis

The pathogenesis of NTHi OM begins with its colonisation of the host's mucosal surface with the help of different adhesins, including haemagglutinin pili. The different types of adhesins involved include the high molecular weight proteins 1 and 2 (HMW1 and HMW2), Hia and Hap proteins.

Certain strains of NTHi have been known to express pili extending up to 450 nm long (St Geme *et al.* 1996). These pili are known to promote bacterial adherence to human respiratory epithelial cells *in vitro* (Farley *et al.* 1990; Read *et al.* 1991). In addition to pili, the majority of NTHi strains have surface exposed high molecular weight proteins, designated as HMW1 and HMW2, that can function as adhesins to promote bacterial adherence (Barenkamp & St. Geme 1996). Another adhesin, Hia adhesin was first identified in NTHi strain 11 (St. Geme *et al.* 1996) and was shown to be highly conserved in the strains of NTHi lacking the HMW adhesins. This adhesin is known to share homology with the Hsf adhesin present in *H. influenzae* type b strain (St. Geme *et al.* 1996) and has been shown to interact with the same receptor molecule, stabilise bacterial adherence and promote persistent colonisation (Laarmann *et al.* 2002).

The Hap protein was first identified from a NTHi strain N187 and was shown to promote adherence *in vitro* to human epithelial cells (St. Geme *et al.* 1994). In addition to being an adhesin, it is also involved with bacterial aggregation, formation of microcolonies and interactions with extracellular matrix proteins such as fibronectin, laminin and collagen IV, allowing NTHi to circumvent the host's mucociliary clearance mechanisms and facilitate bacterial infection (Fink *et al.* 2002; Hendrixson & Geme 1998). The role of various adhesins, proteins and LOS involved in NTHi adherence has been reviewed by (St. Geme 2002). Cellular events, such as macropinocytosis, PAF-r and β-glucan receptor mediated invasion by NTHi *in vitro,* have also been highlighted in this review (St. Geme 2002). More recently, an *in vitro* study has demonstrated adherence of NTHi to the adhesion molecule ICAM-1 and the P5 protein was shown to up-regulate the expression of ICAM-1 on human epithelial cells (Avadhanula *et al.* 2006). The P5 protein of NTHi has been shown to bind to respiratory mucin and another adhesion molecule CEACAM-1 (Hill *et al.* 2001; Reddy *et al.* 1996). The P5 protein is a major outer membrane protein in *H. influenzae* known to share a homology with OmpA protein of *E. coli* (Munson *et al.* 1993). Another outer membrane protein in *H. influenzae* is the P2 protein, the major porin protein, which has been shown to interact with human mucin via recognition of sialic-acid containing oligosaccharides (Reddy *et*

al. 1996). As the ability of the host defence mechanisms to clear the mucous is compromised in infections such as chronic bronchitis and cystic fibrosis, the bacteria could bind to mucin and facilitate establishment of infection.

The role of OapA protein in nasopharyngeal colonisation was demonstrated in a rat model (Weiser *et al.* 1995) and adherence to human conjunctival epithelial cells *in vitro* (Prasadarao *et al.* 1999). Another highly conserved surface protein, Protein D which is also known as Lipoprotein D or GlpQ was thought to have a high affinity to human immunoglobulin D (IgD) (Raun *et al.* 1990), but, further studies have demonstrated that only encapsulated strains of *H. influenzae* and not NTHi had IgD binding capacity and is not related to protein D (Sasaki & Munson 1993). It has a role in facilitating entry of NTHi onto certain monocytic cell lines by obtaining free choline with the help of glycerophosphodiester phosphodiesteriase, encoded by the *glpQ* gene (Ahren *et al.* 2001). *H. influenzae* is known to mimic host cell components and can invade tissues by incorporating free choline into its lipooligosaccharide as phosphorylcholine. However in the absence of free choline, *glpQ* was shown to be required for promoting adherence of *H. influenzae* and obtaining choline from epithelial cells. Hence, protein D enables *H. influenzae* to obtain choline and facilitate choline transfer onto the bacterial cell surface (Fan *et al.* 2001). The other functions of Protein D include ciliary impairment and damage to ciliated cells, however, its exact mechanism is unclear (Janson *et al.* 1999).

1.2.3.2 Lipooligosaccharide (LOS)

There is considerable evidence implicating the role of LOS as a major virulent factor for *H. influenzae*. Its involvement in colonisation and invasion of mucosal surfaces is well known (DeMaria *et al.* 1997). Structurally, it is known to be similar to the lipopolysaccharide of enteric gram-negative bacteria, with the lipid A linked to a heterogenous sugar polymer by 3-deoxy-D-manno-octulosonic acid (Gibson *et al.* 1993). NTHi LOS contains many surface antigens which are known to undergo phase variations and mimic the host's glycosphingolipids, thereby, having an adaptive strategy

to adhere to and invade host cells (Harvey *et al.* 2001). Phosphorylcholine (ChoP), one of the phase-variable components of LOS is known to mediate NTHi adherence on bronchial epithelial surfaces (Swords *et al.* 2000). The role of ChoP in enhancing nasopharyngeal colonisation and cause NTHi OM has been shown in the experimental chinchilla model (Tong *et al.* 2000b).

1.2.4 Moraxella catarrhalis

Microbiology of *Moraxella catarrhalis*

M. catarrhalis is gram-negative diplococci which occasionally occurs in chains and is non-motile. It is a normal respiratory commensal but is known to cause infections such as sinusitis, OM in children, pneumonia and acute exacerbation of chronic bronchitis in adults. It was first isolated from a middle ear exudate in 1927 and was considered to be non-pathogenic. Its pathophysiology became evident in the past 2-3 decades in immunocompromised host causing septicaemia, conjunctivitis, genitourinary and respiratory tract infections (Hart 1927; MacNeely *et al.* 1977).

Pathogenesis of *Moraxella catarrhalis*

The pathogenic ability of *M. catarrhalis* is still unclear, however, several LOS and outer membrane proteins (OMPs) are known to exhibit some degree of pathogenicity (Faden *et al.* 1992). Certain surface antigens and cell wall structures such as outer membrane proteins, pili (fimbriae) and LOS have been identified and understood to contribute to its virulence (Campagnari *et al.* 1994; Hu *et al.* 2000). Although much research has been done to understand the virulence factors involved in its pathogenesis, there is less information than for NTHi and *S. pneumoniae* about the precise mechanisms involved.

1.2.4.1 Various proteins in pathogenesis of *M. catarrhalis*

The various OMPs which have been extensively studied include: CopB, OMP CD, ubiquitous surface proteins (Usp A1 and Usp A2), OMP E and iron regulated proteins

like CopB, lactoferrin binding proteins (Lbp A and B) and transferrin binding proteins (Tbp A and B) (Aebi *et al.* 1998; Bonnah *et al.* 1998; Klingman & Murphy 1994; Yang *et al.* 1997). More recently, the focus has been on the identification of potential surface-exposed protein antigens having functionality as adhesins, and their role in colonisation and pathogenesis; and the development of potential vaccine candidates to treat OM caused by *M. catarrhalis*. Various OMPs, such as Usp A1 and A2, Hag, LOS, OMP CD, and, Usp A2H, have been studied and shown to exhibit adhesive properties (Bernstein & Reddy 2000; Lafontaine *et al.* 2000; Pearson *et al.* 2002). The different OMPs along with some of their known functions are summarised in **Table 1.3.**

Table 1.3: Various outer membrane proteins of *M. catarrhalis* and their functions

Outer membrane proteins (OMPs)	Role or functions
Ubiquitous surface protein A1	adhesins
Ubiquitous surface protein 2H	
Hag protein	
McaP protein	
MID	
CopB protein	acquisition of iron
Lactoferrin binding protein A	
Lactoferrin binding protein B	
Transferrin binding protein A	
Transferrin binding protein B	
OMP B1	
Ubiquitous surface protein A2	serum resistance
OMP E and OMP CD	porin, binds to middle ear mucin, nutrient acquisition and adherence to mucosal surfaces

1.2.4.1.1 Adhesins involved in pathogenesis of *M. catarrhalis*

Usp A is a highly conserved surface protein consisting of two proteins Usp A1 and Usp A2, of which Usp A1 is known to function as an adhesin (Aebi *et al.* 1998; Lafontaine *et al.* 2000; McMichael *et al.* 1998), and Usp A2 provides resistance to complement mediated killing (Attia *et al.* 2005). The UspA1 protein has been associated with bacterial binding to epithelial cells and fibronectin protein [reviewed by (McMichael 2000)]. The binding of different bacteria to epithelial cells with the help of fibronectin binding proteins, such as MSCRAMMs, is known to mediate bacterial adherence and host cell invasion [reviewed by (Joh *et al.* 1999)]. Fibronectin binding proteins have also been shown to facilitate adherence of some bacteria to injured airways (Mongodin *et al.* 2002), as observed in COPD patients, which could contribute to the reoccurrence of *M. catarrhalis* infection in COPD patients. More recently, *in vitro* studies have found a correlation between fibronectin binding and the expression of Usp A1 and A2 and showed fibronectin to be a receptor for Usp A (Tan *et al.* 2005). Another cell adhesion molecule, CEACAM, comprised of several glycoproteins of which CEACAM 1 is highly expressed on human tissues and respiratory epithelia (Hammarstrom 1999). Recently, Usp A1 was shown to bind to CEACAM, an adhesion molecule targeted by other mucosal pathogens such as NTHi and *N. meningitidis* (Hill & Virji 2003; Virji *et al.* 2000). In addition of being an adhesin, *in vitro* tissue culture studies using transposon mutagenesis have shown that the Usp A1 protein plays an important role in biofilm formation (Pearson *et al.* 2006).

McaP protein is a recently identified adhesin known to possess esterase and phospholipase B enzymatic activity (Timpe *et al.* 2003). Phospholipase is known to destroy lung surfactant, increase vascular permeability, promote colonisation of tissues, stimulate inflammatory responses and generate signal transducers (Dorrell *et al.* 1999; Holm *et al.* 1991). Another protein, OMP CD was shown to bind to middle ear mucin (Reddy *et al.* 1997). The possibility of being a porin (Murphy *et al.* 1993) and being able to bind to mucin suggests its role in nutrient acquisition and adherence onto mucosal surfaces (Holm *et al.* 2004).

The *M. catarrhalis* IgD binding MID protein has been well characterised (Mollenkvist *et al.* 2003). It also has adhesive properties on alveolar epithelial cells, suggesting its role as an adhesin (Forsgren *et al.* 2003). The role of IgD in an immune response to respiratory infections has been hypothesised because of its high concentration found in the middle ear and nasopharynx secretions (Sorensen & Larsen 1988). MID has two functional domains, one that functions as an adhesion, promoting attachment to epithelial cells, and the second is the IgD-binding domain (Riesbeck & Nordstrom 2006). Recently, Hag protein of *M. catarrhalis* was shown to be involved with haemagglutination, binding to human IgD (Pearson *et al.* 2002) and as an adhesin to human lung epithelial cells (Holm *et al.* 2003), and to human middle ear cells (Bullard *et al.* 2005).

1.2.4.1.2 Proteins involved in serum resistance

The involvement of several OMPs like Usp A2, CopB, OMP E, OMP CD and LOS epitopes in serum resistance have been well documented (Aebi *et al.* 1998; Helminen *et al.* 1993(b); Holm *et al.* 2004; Murphy *et al.* 2000; Zaleski *et al.* 2000). Many strains of *M. catarrhalis* are known to resist complement-mediated killing by normal human serum, making serum resistence as one of the virulence factors involved in its pathogenesis (Hol *et al.* 1993). Amongst the different OMPs known to be involved, Usp A2 was recently shown to be directly involved in serum resistance (Attia *et al.* 2005). However the mechanism, by which *M. catarrhalis* confers serum resistance, is yet to be determined. Another OMP shown to be involved with serum resistance is OMP CD. Along with its ability to adhere, certain studies have documented its direct involvement in serum resistance, and protecting the bacteria from complement mediated killing (Holm *et al.* 2004).

1.2.4.1.3 Proteins involved in nutrient acquisition

OMP CD, a highly conserved surface exposed protein (Murphy *et al.* 1993; Murphy & Loeb 1989), is known to share a homology with the OprF protein in Pseudomonas

species, which functions as a porin. This homology has resulted in speculation that OMP CD could function as a porin (Murphy *et al.* 1993). More recently, an OMP from *M. catarrhalis* designated as M35 has been characterised and DNA modelling, translated sequences and functional assay have confirmed M35 as a porin. It shares homology with other known porins such as OMP K36 from *K. pneumoniae* and PorB from *N. meningitidis* (Easton 2008). In addition, the role of M35 in nutrient acquisition has confirmed that this is a substrate-specific porin (manuscript under review).

Iron is an essential element required for the growth and metabolism of most bacteria. Successful pathogenic bacteria must possess one or more efficient iron scavenging systems, which are capable of competing with or exploiting the iron transport and storage mechanisms of the host. Complex molecules such as transferrin, lactoferrin and haemoglobin are known to sequester iron in the human host (Campagnari *et al.* 1994; Luke & Campagnari 1999; Schryvers & Stojiljkovic 1999). The expression of these OMPs by *M. catarrhalis* enables it to bind to and utilise both, lactoferrin and transferrin (Schryvers & Lee 1989). The receptors of lactoferrin and transferrin are shown to be functionally and genetically related and designated as lactoferrin binding protein (Lbp) and transferrin binding protein (Tbp) respectively (Gray-Owen & Schryvers 1996). *In vitro* studies have shown that under iron restricted environment, *M. catarrhalis* has the ability to enhance the expression of lactoferrin binding protein (Du *et al.* 1998). A new OMP MhuA, which is surface-exposed and highly conserved has been characterised and shown to utilise haemoglobin as a sole iron source for its growth (Furano *et al.* 2005). Haem is the most abundant source of iron and many bacteria utilise both haem and haemoglobin for their growth (Genco & Dixon 2001; Otto *et al.* 1992). During the inflammatory process, both haem and haemoglobin are found on the mucosal surfaces as a source of nutrients for pathogens (Schryvers & Stojiljkovic 1999). The advantage to *M. catarrhalis* in utilising these in the middle ear mucosa during AOM is unknown, although a recent, study demonstrating the ability of *M. catarrhalis* to utilise haemoglobin as the sole iron source have added a new insight towards its colonisation and pathogenesis (Furano *et al.* 2005). Cop B or OMP B2 is another major surface

exposed protein, homologous to FrpB protein of Neisseria species (Aebi *et al.* 1996; Helminen *et al.* 1993a), involved in the acquisition of iron from human transferrin and lactoferrin (Aebi *et al.* 1996; Campagnari *et al.* 1994).

1.2.4.2 Lipooligosaccharides (LOS)

LOS is a major surface antigen and known to contribute to the virulence of *M. catarrhalis* (Fomsgaard *et al.* 1991). Although not essential for its survival, the LOS of *M. catarrhalis* was critical as a virulence characteristic (Peng *et al.* 2005). It consists of an oligosaccharide linked to lipid A, which is responsible for endotoxin activity. Based on the antigenic differences in their LOS moieties, *M. catarrhalis* has been divided into 3 serotypes (Masoud *et al.* 1994; Vaneechoutte *et al.* 1990). LOS consists of a carbohydrate structure and the oligosaccharide terminates into a Galα1-4Galβ1-4Glc. *M. catarrhalis* LOS reacts with a monoclonal antibody specific for the P^k (Galα1-4Galβ1-4Glc) epitope and resists complement mediated killing (Mandrell & Apicella 1993; Zaleski *et al.* 2000). Recently, an *in vitro* study has reported selective up-regulation of an adhesion molecule, ICAM-1, on human monocytes when exposed to purified *M. catarrhalis* LOS (Xie & Gu 2008). In addition, it was also reported that the LOS-activated monocytes up-regulated ICAM-1 expression and stimulated naïve monocytes to produce TNF-α. This up-regulation was also shown to be mediated by pathways such as c-Jun N-terminal kinase, NF-κβ p65 along with Toll-like receptor-4. An *in vitro* study using rat alveolar epithelial cells has shown that pulmonary ICAM-1 mediated leucocyte activation leads to enhanced lung injury (Beck-Schimmer *et al.* 2002). Stimulation of TNF-α induces neutrophil infiltration and causes increased microvascular permeability during OME in an experimental rat model (Lee *et al.* 2001).

1.2.4.3 Pili and its proteins

Pili are homologous or heterologous polymers composed of helically arranged subunits assembled and expressed on the surface of gram-negative bacteria, as reviewed in (Fernandez & Berenguer 2000). Type IV pili are responsible for infectivity and disease

manifestations and known to play a role in colonisation of host tissues, twitching motility, biofilm formation, stability and transfer of genetic material (Mattick 2002; O'Toole & Kolter 1998; Shi & Sun 2002). *M. catarrhalis* expresses type IV pili that has a role in facilitating adherence to epithelial cells (Marrs & Weir 1990; Rikitomi *et al.* 1991), and was regulated by available iron (Luke *et al.* 2004).

Overall, the various OMPs along with other components of cell wall of *M. catarrhalis* have been shown to mediate or assist in bacterial adherence and enable colonisation. Moreover, the involvement of certain OMPs in serum resistance have highlighted the importance of virulence factors in a bid by the bacterium to protect itself from host's immune system and initiate an infection. In addition, the bacterial interaction with various cell adhesion molecules suggests a significant level of redundancy that ensures *M. catarrhalis* is able to effectively colonise and survive.

1.2.5 Alloiococcus otitidis

Microbiology of *Alloiococcus otitidis*

A. otitidis is a slow growing gram-positive aerobic coccus and fastidious in nature. It was first isolated from the middle ear effusion of patients with OME (Faden & Dryja 1989). *A. otitidis* was detected in approximately 40% of the children with OME and along with the other dominant bacteria involved with OM (Beswick *et al.* 1999; Hendolin *et al.* 1999; Hendolin *et al.* 1997). More recently, it was detected along with *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in approximately 25% of middle ear effusion samples from children using multiplex PCR technique (Leskinen *et al.* 2004). Its role in the pathogenicity of OM is still unclear. However, there have been studies demonstrating its ability to stimulate $CD8^+$ cytotoxic suppressor T lymphocytes and production of IL-12 and IL-8 *in vitro* (Himi *et al.* 2000; Kita *et al.* 2000), indicating its role as a potential pathogen. More recently, the high frequency of *A. otitidis* colonisation in the nasopharynx of OM prone children has implicated its role in causing OM as commonly as other bacteria such as *S. pneumoniae*, NTHi and *M. catarrhalis* (Harimaya

et al. 2006). Given the polymicrobial nature of OM, future studies involving *A. otitidis* with the previously known predominant pathogens of OM would provide an insight to the role of this bacterium in the complex nature of bacterial interactions in OM.

1.2.6 Presence of virus during OM

Whilst OM has been considered a predominantly bacterial infection, the involvement of certain respiratory viruses in the etiology and pathogenesis of AOM is well recognised (Chonmaitree & Heikkinen 1997; Ruuskanen *et al.* 1991). The different types of respiratory viruses involved in OM include RSV, parainfluenza viruses, influenza A and B, rhinovirus, adenovirus, enterovirus, herpes simplex virus, cytomegalovirus and coronavirus (Klein *et al.* 1998; Pitkaranta *et al.* 1998). Viruses have been detected in approximately 5-48% of children suffering from AOM (Arola *et al.* 1990; Bulut *et al.* 2007; Chonmaitree *et al.* 1986; Chonmaitree *et al.* 1992; Henderson *et al.* 1982; Klein 1994; Nokso-Koivisto *et al.* 2006). In the late 1990s, with the development of highly sensitive molecular amplification-based techniques further increased the detection rate to approximately 60-90% (Pitkaranta *et al.* 1998). The inclusion of healthy controls did not change significantly the detection rates of respiratory viruses, due to the high specificity of these assays. However, the presence of RSV was found to be almost twice as frequent in patients having AOM (Uhari *et al.* 1995). This was in accordance with several other studies in which RSV was found to be the most commonly associated virus with AOM followed by influenza A virus, adenovirus and parainfluenza virus (Henderson *et al.* 1982; Ruuskanen *et al.* 1989).

The progress of disease in middle ear infection associated with a respiratory viral infection is very important, as different viruses are known to predispose host to bacterial OM differently (Giebink *et al.* 1980; Suzuki & Bakaletz 1994). A respiratory viral infection is known to aid bacterial colonisation, adherence and translocation through epithelial barrier and make way for secondary bacterial diseases. The pathogenic mechanisms involved (physical damage to respiratory epithelium, mucociliary dysfunction and ET dysfunction) and the interaction of respiratory cells and bacteria

preceding a viral infection has been previously reviewed (Hament *et al.* 1999). The ability of respiratory viruses to cause ET dysfunction has been demonstrated in both animal models and in humans (Bluestone 1996; Sanyal *et al.* 1980). As reviewed by (Heikkinen 2001), the contributions of viruses to the pathogenesis of AOM includes: ET dysfunction through the release of pro-inflammatory cytokines; enhancing bacterial colonisation; and modulating the host's immune function thereby increasing the host's susceptibility to bacterial infections.

1.2.7 Biofilms and otitis media

A bacterial biofilm is a complex association and a well structured community of bacterial cells enveloped in a matrix adherent to an inert or a living surface (Costerton *et al.* 1999). In the past few decades, the constitution of microcolonies in the extracellular matrix of the biofilm, architecture of the matrix and its adherence onto different surfaces have been well characterised in review papers (Costerton *et al.* 1987; Costerton *et al.* 1995; Dongari-Bagtzoglou 2008). Moreover in the last decade, the role of biofilms has been widely recognised in infections such as endocarditis, dental caries, and pneumonia in cystic fibrosis patients (Costerton *et al.* 1999; Dongari-Bagtzoglou 2008; Donlan 2001; Singh *et al.* 2000).

The bacteria forming biofilms are known to have a different phenotype in comparison with other free-living planktonic bacteria. In addition, they are also resistant to antimicrobial treatment and phagocytosis within the biofilm matrix (Mah & O'Toole 2001). The multiple microenvironments developed by different bacteria within the biofilm are known to vary in their nutrient availability and pH level, which enables them to form a symbiotic living community (Vroom *et al.* 1999). This symbiotic living is often known to confer antibiotic resistance to other bacteria in the biofilm. *In vitro* studies showed protection of *S. pneumoniae* by the β-lactamase produced by *M. catarrhalis*, when grown together as a continuous-culture biofilm system. This synergistic behaviour in a biofilm could explain antibiotic treatment failures in OM (Budhani & Struthers 1998).

The microcolonies in the bacterial biofilm are known to attach to each other by a process of bacterial coaggregation. Coaggregation was first observed in human dental plaques and is defined as the process of adhesion between genetically distinct bacterial species (Gibbons & Nyagaard 1979). The formation of a biofilm is a continuous process, beginning with the adhesion and multiplication of primary colonisers which forms the microcolonies. The formation of microcolonies leads to a change in the environment, which is suitable for other bacteria to adhere to and further multiply. Thus, with the involvement of microcolonies and different species of bacteria, the biofilm continues to develop into a multispecies microbial community (Busscher & Der Mei 1995). The diagrammatic representation of development of biofilm is shown in the **figure 1.1**.

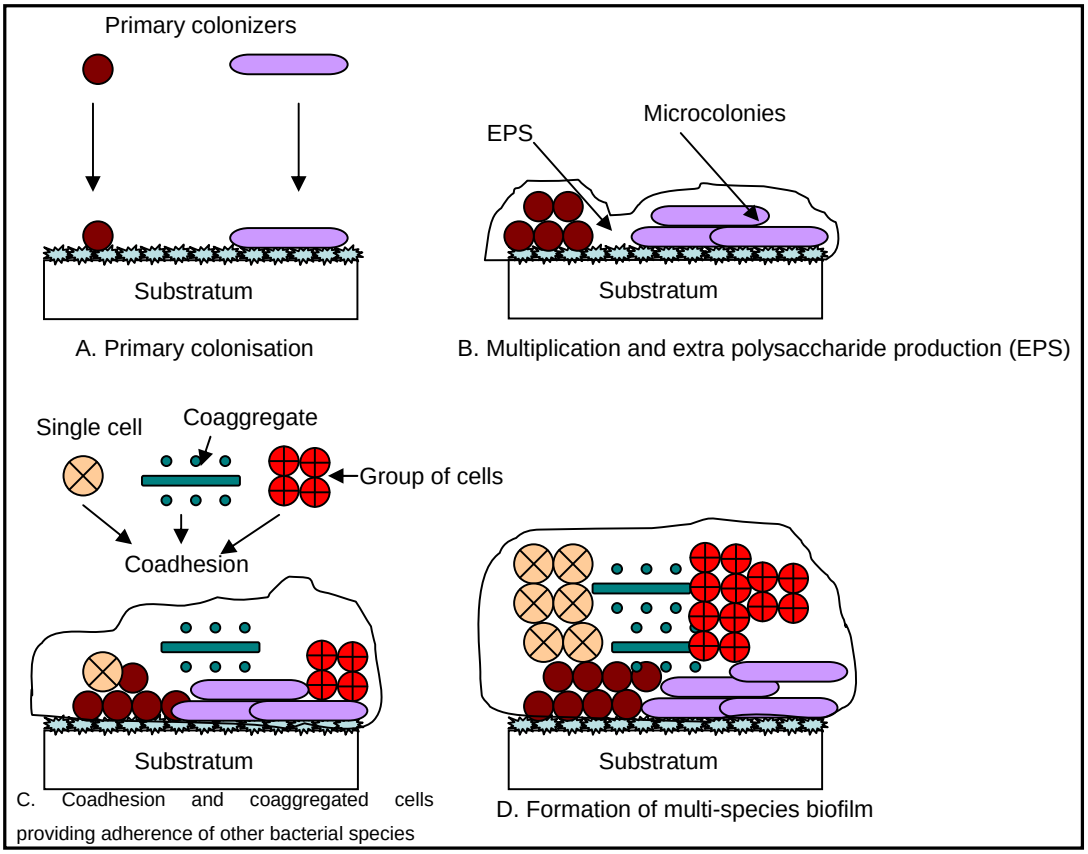


Figure 1.1: Stages involved in multi-species biofilm. A] Primary colonisation on a substratum enriched with nutrients, polysaccharides and proteins. B] Multiplication and formation of microcolonies and production of EPS. C] Coadhesion, coaggregated cells form groups of cells constituting other bacterial species. D] Fully matured multi-species biofilm [adapted from (Rickard *et al.* 2003)].

The formation of a disease-associated bacterial biofilm by *P. aeruginosa*, a primary pathogen involved in CSOM, was first documented in a nonhuman primate model. This study has provided a good understanding of the formation of a biofilm using scanning electron microscopy (Dohar *et al.* 2005). In addition to *P. aeruginosa*, NTHi is also known to form biofilms both *in vitro* and *in vivo* (Ehrlich *et al.* 2002; Murphy & Kirkham 2002). NTHi causes OME, which is characterised by the presence of middle ear effusion in absence of any symptoms. The use of amplification-based techniques has demonstrated the presence of bacterial mRNA, indicating the presence of viable and

metabolically active bacteria which are not detected by conventional culture methods (Rayner *et al.* 1998). Clinical bacterial isolates from the middle ear fluid of children have shown substantial variability in their ability to form biofilms. The involvement of major OMPs such as P2, P5 and P6 along with the expression of LOS and peroxiredoxin-glutaredoxin (PGdx) in the formation of biofilms has been recently documented (Murphy & Kirkham 2002; Murphy *et al.* 2005a). LOS produced by NTHi is known to combine with sialic acid from the host, which confers resistance to opsonisation and enhances binding to host cell receptors. NTHi has 3 sialyltransferases, SiaA, Lic3A and LsgB, which facilitate the placement of sialic acid residues on the LOS (Jones *et al.* 2002), and sialylated LOS has been shown to promote biofilm formation both *in vivo* and *in vitro* (Jurcisek *et al.* 2005; Swords *et al.* 2004). Recently, presence and identification of double stranded DNA and type IV pilin protein of NTHi within the biofilm suggests the importance of their involvement in providing structural stability to the biofilms *in vivo* (Jurcisek & Bakaletz 2007). A recent proteomic analysis of the extracellular matrix constituting the biofilm has revealed around 265 different proteins responsible for the formation of the biofilm by NTHi (Gallaher *et al.* 2006).

Apart from NTHi, bacteria such as *S. pneumoniae* and *M. catarrhalis* have also been shown to form biofilm on cellulose filter support (Budhani & Struthers 1998). The role of previously known adhesins of *M. catarrhalis*, Usp A1 and Hag protein in biofilm formation was demonstrated using *in vitro* tissue culture studies and knockout mutants of *M. catarrhalis* (Pearson *et al.* 2006). The Pearson study (2006) demonstrated contrasting roles of these proteins with the expression of Usp A1 responsible for biofilm formation, whereas, expression of Hag protein inhibited the biofilm formation. Recently, real-time measurement of exopolysaccharide and *S. pneumoniae* involved in *in situ* biofilm formation was demonstrated in a continuous culture system (Donlan *et al.* 2004). The real-time measurement could be further used for investigating the mechanisms involved in biofilm formation. More recently, the stages involved in the biofilm formation by different strains of *S. pneumoniae* was shown to be similar across all strains, however, the architecture of the biofilm was shown to be strain specific

(Allegrucci *et al.* 2006). In addition, the proteins involved in pneumococcal adherence, virulence and antibiotic resistance were significantly increased during biofilm formation. Thus, there is a growing understanding of the biofilm development, its involvement in pathogenesis, and the complexities within the biofilm.

1.3 POLYMICROBIAL DISEASE

1.3.1 Definition and introduction

A polymicrobial disease is often recognised as a condition in which two or more organisms act synergistically or in succession to produce a complex disease. It is also accepted with different terminologies such as: complex infections, complicated infections, dual or mixed infections, secondary infections, co-infections, and concurrent and polymicrobial infections [reviewed by (Brogden 2002). The polymicrobial nature of many infectious diseases were first documented in the 1920s and various factors or conditions such as: stress, alterations in the mucosal surfaces, induction of pro-inflammatory cytokines, microbial virulence and impaired immune functions involved in predisposing animals and humans to polymicrobial diseases, have been identified [reviewed by (Bakaletz 2004)]. Although recognised for many years, research into the nature of polymicrobial disease has become more focused over the past decade (Chonmaitree 2000; Hament *et al.* 1999; McCullers 2006; Tuomanen 1999).

1.3.2 Otitis media-A polymicrobial disease

OM is known to exist in several clinical forms ranging from acute to chronic situations and is well recognised to be caused by more than one bacterial agent. The bacterial species associated with OM are nasopharyngeal commensals (Infante-Rivard & Fernandez 1993). However, the ability of these commensals to become pathogens causing invasive disease is less understood. The detection of different bacteria in the middle ear effusions are generally performed using conventional culture techniques and genetic amplification-based techniques have improved the sensitivity for detecting viable bacterial presence in the middle ear fluid during OM (Bluestone & Klein 1995;

Hendolin *et al.* 1997; Matar *et al.* 1998; Ruohola *et al.* 2006). In addition, several respiratory viruses are involved in AOM, thereby making it a polymicrobial disease of both bacteria and viruses (Ruohola *et al.* 2006).

1.3.3 Isolation of bacteria during OM

In OM, the bacterial colonisation begins in the nasopharynx, which is a well recognised natural reservoir for many bacterial species. The relationship between nasopharyngeal colonisation and middle ear infection became more evident following the similarity of bacterial strains found in these areas (Loos *et al.* 1989). Late in the 1950s, prior to the discovery of antibiotics, the most commonly isolated bacteria from the middle ear fluids were beta-hemolytic Streptococci and Staphylococcal species. However during the past four decades, bacteria such as NTHi, *S. pneumoniae* and *M. catarrhalis* have been the dominant pathogens isolated (Bluestone & Klein 1995). The reasons for this change in the bacteriology of OM are uncertain.

Over the past two decades, the diagnosis of OM has been made using culture methods, antigen detection and amplification-based techniques. The detection rate of bacteria such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* was reported to be approximately 20-40% with conventional culture methods. This rate was further increased to approximately 40-60% when used in conjunction with antigen detection methods (Luotonen *et al.* 1981). The relationship between nasopharyngeal colonisation and middle ear infection in children has shown a strong relationship to the likelihood of developing OM. Carriage rates observed for *M. catarrhalis*, *S. pneumoniae* and *H. influenzae* were approximately 26%, 24% and 9%, respectively (Faden *et al.* 1997), whereas the recent study by Cohen *et al* reported 52%, 58% and 47% (Cohen *et al.* 2006). In the Australian context, especially in certain high-risk population, the prevalence of bacteria in OM has highlighted some interesting observations with regards to bacterial colonisation of the nasopharynx and early onset of OM; described clinical features and recommended treatment options; and the burden of bacterial carriage (Leach *et al.* 1994; Morris *et al.* 2007; Watson *et al.* 2006). These studies have shown

that nasopharyngeal colonisation by these bacteria was less than 20 days of age in Australia Aboriginal children (Leach *et al.* 1994), and the carriage rates were as high as 49% for *S. pneumoniae*, 50% for *M. catarrhalis* and 41% for NTHi (Watson *et al.* 2006).

Amplification-based methods such as PCR, are able to detect bacterial DNA in the middle ear effusions and increase the sensitivity of the bacterial detection in comparison with culture methods. Multiplex PCR to the commonly isolated bacteria from middle ear effusions (Hendolin *et al.* 1997) has increased detection rates to approximately 95% (Hendolin *et al.* 2000; Matar *et al.* 1998). *A. otitidis* has been frequently isolated from the middle ear effusions in children using PCR-based assays (Hendolin *et al.* 1999), and more recently, *A. otitidis* was detected in approximately 64% of the 25 middle ear effusion isolates from children aged 8 months to 10 years (Harimaya *et al.* 2006).

1.3.4 Viral-bacterial interaction during OM

The presence of respiratory viruses along with bacteria from the middle ear fluids and nasal aspirates of children with AOM implicated viral-bacterial interactions in the pathogenesis of AOM (Chonmaitree *et al.* 1986). The viral-bacterial interaction in OM was first established using an experimental chinchilla model (Giebink *et al.* 1980). Once established, this model was further adapted to investigate the incidence of bacterial OM (*S. pneumoniae or H. influenzae*) following adenovirus or influenza virus infection, respectively. Thus the role of synergism between bacteria and virus was demonstrated in the chinchilla model where the incidence of OM observed was higher in animals having combined bacterial and viral infection when compared to either bacterial or viral infection alone (Giebink *et al.* 1980; Suzuki & Bakaletz 1994). This synergism was also observed in children where both, bacteria and respiratory viruses were isolated from the middle ear effusions (Chonmaitree *et al.* 1992; Heikkinen *et al.* 1999). Furthermore, different viruses have been observed to have a preferential ability to provide a synergistic effect with some bacteria in causing OM. Influenza A virus and adenovirus has been known to enhance pneumococcal and NTHi OM, respectively (Giebink *et al.*

1980; Suzuki & Bakaletz 1994). This bacterial-viral synergy has also been observed in children. Using culture methods, *S. pneumoniae* was recovered more significantly in the middle ear effusion containing influenza virus when compared to RSV or parainfluenza virus (Heikkinen *et al.* 1999). A recent review has highlighted virus-induced mechanisms such as production of inflammatory mediators in the middle ear and delayed clearance of bacteria due to ET dysfunction (Heikkinen & Chonmaitree 2003). There is a general acceptance that viruses contribute to the pathogenesis associated with bacterial OM.

1.3.5 Bacterial adhesins in mediating adherence

The interaction between microbes and hosts begin at the mucosal surfaces. Depending on the bacterial species and the microenvironment where they reside, the host-microbial relationship can be very specific (Niederman 1990). Under normal circumstances, the lower respiratory tract is a sterile environment and bacterial colonisation is prevented by the host's defence mechanisms such as mucociliary clearance, production of pro-inflammatory mediators and antimicrobial peptides such as defensins [reviewed by (Bals & Heimstra 2004; Van Alphen 1996)]. However, in a immune compromised host, a damaged epithelium along with other inflammatory processes and the presence of pathogens, has been known to provide an ideal niche for the establishment of infection [reviewed by (Van Alphen 1996)].

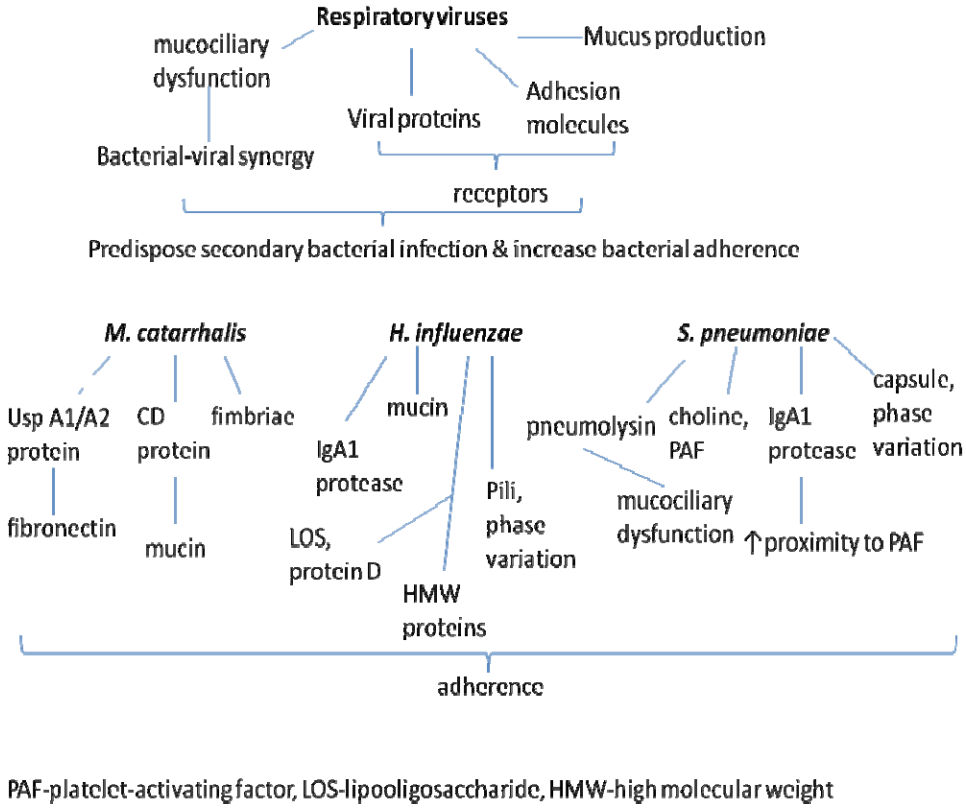
Bacterial adherence to any mucosal surfaces has been well recognised and accepted as part of the establishment of infection (Niederman 1990). Adherence is usually initiated as a result of the interaction between various adhesins found on the bacterial surface and receptors on the host's epithelial surface. Different epithelial surfaces have different receptors, and these can be targeted by different bacteria (Niederman 1990). During LRTIs and/or viral infection clinical conditions, such as: loss of cilia, mucociliary dysfunction, and increased mucous production, has been known to alter the mucosal surfaces and enhance bacterial adherence and colonisation (Niederman 1990; Wilson *et al.* 1987). The ability of respiratory viruses to predispose secondary bacterial infections

and potential mechanisms involved in bacterial adherence during viral infection has been previously reviewed by (Hament *et al.* 1999). In addition, certain adhesion molecules, such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), have been shown to be up-regulated following inflammation and virus infection (Roebuck & Finnegan 1999). The exposure of these adhesion molecules as a result of viral infection could serve as potential receptors for the infecting bacteria and facilitate enhanced bacterial adherence.

The virulence factors from *S. pneumoniae*, NTHi and *M. catarrhalis* involved in mediating adherence to various epithelial surfaces have been previously described in this thesis. The OMPs such as Usp A1, McaP, OMP CD, MID and Hag protein are well recognised adhesins of *M. catarrhalis* (Aebi *et al.* 1998; Holm *et al.* 2003; Tan *et al.* 2005; Timpe *et al.* 2003). In addition, the ability of Usp A1 to bind to certain adhesion molecules such as fibronectin binding proteins (Tan *et al.* 2005) and CEACAM (Hill & Virji 2003) highlights the importance of these surface adhesins in the pathogenesis of *M. catarrhalis* leading to lower respiratory tract colonisation and infections such as COPD exacerbations, bronchitis etc. Moreover, OMP CD has also been shown to bind to middle ear mucin (Reddy *et al.* 1997), thereby suggesting its role in causing OM. In NTHi, the LOS and certain high molecular weight proteins (HMW 1 and HMW 2), Hia protein, Hap protein have been shown to mediate bacterial adherence to respiratory epithelia and colonisation *in vivo* (St. Geme *et al.* 1993; Swords *et al.* 2001; Tong *et al.* 2000b). In addition, the majority of *H. influenzae* strains can evade the host defence actions of IgA by production of an extracellular endopeptidase IgA1 protease (Kilian *et al.* 1996). The cleavage of IgA by this protease neutralises its biological function (Poulson *et al.* 1992). The ability of NTHi to bind to the sialic acid-containing oligosaccharides of mucin in the nasopharynx has implicated the involvement of OMPs like P2 and P5 in promoting nasopharyngeal colonisation (Reddy *et al.* 1996). The CBPs, neuraminidase, pneumolysin and capsule of the pneumococcus have been shown to play an important role in promoting colonisation *in vivo* and adherence to respiratory epithelia [reviewed by (AlonsoDeVelasco *et al.* 1995; Jedrzejas 2001)].

The requirement of different virulence factors for promoting bacterial adherence and colonisation raises questions about any differences for their role in a polymicrobial environment. **Figure. 1.2** illustrates how different virulence factors from bacteria and a viral infection could promote adherence to respiratory epithelia. In a polymicrobial environment different bacteria might compete for the available receptors on the host's mucosal surface. As an example, most of the mucosal pathogens are known to express phosphorylcholine (ChoP) (Gillespie *et al.* 1996) and this moiety has also been shown to facilitate bacterial attachment to platelet-activating factor receptors (PAF-r) (Cundell *et al.* 1995; Swords *et al.* 2000). This could suggest that in a polymicrobial environment, different bacteria could compete with each other for the same available receptors in order to promote adherence and colonisation.

Figure 1.2: Factors affecting bacterial adherence in a polymicrobial environment



PAF-platelet-activating factor, LOS-lipooligosaccharide, HMW-high molecular weight

1.3.6 Inflammatory responses

The host's defence mechanisms, including the innate immune system, not only regulate the integrity of the respiratory tract by providing initial protection against pathogens, but also stimulate the adaptive immune response. The immune response of the airway epithelium to any infection or antigen, is to initiate an inflammatory reaction with the release of certain chemokines and cytokines, leading to recruitment of white blood cells and facilitation of the adaptive immune response. Cytokines that regulate innate immunity are produced by macrophages, dendritic cells, T-cells, NK cells and other leukocytes. These include TNF-α, IL-1, IL-6, IL-10, IL-12, IL-15 and IL-18 along with some chemokines like IL-8, MIP-1 and RANTES. The cytokines that regulate adaptive immunity are produced by T-cells upon specific antigen recognition and include IL-2, IL-4, IL-5, IFN-γ, TGF-β and IL-13. The other functional category of cytokines includes stimulation of growth and differentiation of immature leukocytes and are produced by bone marrow cells e.g.; colony stimulating factors, IL-3 and IL-7 [reviewed in (Kindt *et al.* 2006)]. The source of cytokine producing cells, properties of specific cytokines and their mode of action are listed in **Table 1.4.**

The innate immune system recognises the pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). This enables the immune system to recognise motifs used by pathogens but not host cells, leading to cytokine production (Girardin *et al.* 2002). Other functional characteristics of PRRs include: opsonisation of various microbes, uptake of pathogens by the process of phagocytosis and triggering secretion of antimicrobial peptides [reviewed in (Medzhitov & Janeway 2000)]. It has also been demonstrated that certain Toll proteins can detect certain bacterial species, for example; TLR4 is known to recognise the LPS in gram-negative organisms [reviewed in (Medzhitov & Janeway 2000)], whereas TLR2 recognises the lipoteichoic acid and peptidoglycan of gram-positive organisms (Yoshimura *et al.* 1999). In addition to TLRs, certain cytoplasmic proteins such as nucleotide-binding oligomerisation domain (NOD) proteins have been recently identified as recognising components of both gram-positive and gram-negative

bacterial peptidoglycans (Girardin *et al.* 2003b; Girardin *et al.* 2003a), and induce an inflammatory response (Opitz *et al.* 2004; Travassos *et al.* 2005). In contrast, the ability of bacteria to avoid immune recognition by PRRs, such as the TLRs, and invade epithelial cells is a well known strategy of invading pathogens (Akira *et al.* 2006; Inohara *et al.* 2005; Philpott & Girardin 2004).

The LPS in gram-negative bacteria consists of polysaccharide side chains (O-antigen), core saccharides consisting of N-acetylglucosamine, glucose, galactose, heptose, phosphate and ethanolamine, and, lipid A. The lipid A is known to be the main component of LPS involved in inflammation, and induces release of IL-1, IL-6, IL-8 and TNF-α from mononuclear blood cells [reviewed by (Nau & Eiffert 2002)]. The teichoic acid and lipoteichoic acid found in gram-positive bacteria are also involved in inducing an inflammatory response through the release of cytokines such as IL-1, IL-6, IL-8, IL-12 from monocytes (Bhakdi *et al.* 1991; Mattsson *et al.* 1993). The other component of gram-positive bacteria involved with induction of an inflammatory response is the peptidoglycan. *In vitro* studies have found that macrophages and monocytes release TNF-α, IL-1β and IL-6 in response to gram-positive cell wall fragments and peptidoglycan (Bhakdi *et al.* 1991; Heumann *et al.* 1994).

The contributions of respiratory viruses in the pathogenesis of AOM includes: ET dysfunction through the release of pro-inflammatory cytokines; enhancing bacterial colonisation; and modulating the host's immune function [reviewed by (Heikkinen 2001)]. In addition, a concurrent bacterial and viral infection has also been known to enhance inflammation, increase mucosal damage and affect immunological responses leading to delayed bacterial clearance from the middle ear and cause persistent middle ear effusions in young children (Chung *et al.* 1993; Monobe *et al.* 2003). Viruses on their own are also known to induce production of cytokines and mediators causing further inflammation of the nasopharynx and cause ET dysfunction [reviewed by (Chonmaitree & Heikkinen 1997)]. The concentration of pro-inflammatory cytokines, such as IL-2, IL-6, IL-8 and TNF-α, and inflammatory mediators was further increased

in the middle ear fluids following co-infection with bacteria and virus in comparison with bacterial or viral infection alone (Chonmaitree *et al.* 1994). Following local inflammatory responses, viral infections are known to interfere with the penetration of antibiotics into the middle ear, leading to treatment failure (Canafax *et al.* 1998; Chonmaitree *et al.* 1990).

During LRTIs, microbial interaction with the host is known to begin at the respiratory mucosal epithelium following inhalation of various pathogens. The respiratory mucosal epithelial cells are known to protect the host from these invading pathogens by producing pro-inflammatory mediators and antimicrobial peptides [reviewed by (Bals & Heimstra 2004)]. The epithelial cytokine response to bacterial infection was first demonstrated in patients having urinary tract colonisation by *Escherichia coli* (Hedges *et al.* 1991). Various studies have subsequently demonstrated the release of cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, on exposure of the respiratory epithelium to different human respiratory viruses (Arnold *et al.* 1994; Terajima *et al.* 1997; Yoon *et al.* 2007). Recently, synergistic inflammatory responses as a result of polymicrobial colonisation on epithelial surfaces have demonstrated the ability of commensal flora to alter the level of cytokine production and amplify the pro-inflammatory responses (Ratner *et al.* 2005). The involvement of the innate immune response has also been demonstrated in a experimental animal models (Lysenko *et al.* 2005). The Lysenko study showed neutrophil and complement mediated killing of *S. pneumoniae* when co-infected with a typeable strain of *H. influenzae*. This was further shown to be facilitated by nucleotide-binding oligomerisation domain-1 (Nod1), which recognises the peptidoglycan of *H. influenzae* (Lysenko *et al.* 2007; Lysenko *et al.* 2005). This observation speculates the involvement of innate immune responses in clearance of one species from the mucosal surfaces, and the existence of microbial competition involved in mucosal colonisation.

Table 1.4: Different cytokines along with their generic properties

Interleukin	Producing cells	Target cells and properties
IL-1	Monocytes, macrophages, dendritic cells	Works synergistically with TNF to mediate acute inflammatory response, acute phase response, activates macrophage
TNF	Monocytes, macrophages, dendritic cells, T_H 1 cells	Mediates acute inflammation, proliferation of T & B cells, cytotoxicity, up-regulates adhesion molecules, induction of inflammatory cytokines and acute phase response
IL-6	T cells, macrophages, monocytes	Differentiation of T & B cells, acute phase response, anti-inflammatory effects, inhibition of IL-1 and TNF synthesis
IL-10	T_H 1, T_H 2 cells, B cells, mast cells, monocytes	Inhibits IFN-γ and IL-2 production, inhibits MHC class II on monocytes, decreases inflammatory cytokine production, inhibits activated macrophage and dendritic cells
IFN-γ	T cells and NK cells	Stimulates antigen presentation, cytokine production, phagocytosis, TNF-α production, inhibits viral replication, increases nitric oxide production

[Source: Cytokines and chemokines (Borish & Steinke 2003)].

1.4 Animal models of OM

1.4.1 General overview

The infections caused by bacteria and/or viruses involve basic mechanisms of pathogenesis and predisposing factors, however, each disease has its own unique characteristics of pathogenesis. Polymicrobial diseases which are complex in nature require certain *in vitro* methodologies such as cell culture systems and animal models to provide a better understanding of the different mechanisms of pathogenesis [reviewed by (Bakaletz 2004)]. However, *in vitro* methods have certain disadvantages when compared with animal models. These include absence of a defined and organised organ system, no specific genetic background and no other cell type interaction to characterise the immune responses (Bakaletz 2002). There are certain requirements for an animal model through which complex human infections could be better understood. Firstly, the infection should preferentially be induced through a natural route, for example, nasopharyngeal infection in order to study respiratory tract infections. Secondly, the animal should be manipulated minimally to facilitate induction of any human infection. Finally, the potential animal should be widely available and well characterised in terms of genetic, microbiologic and immunologic determinants (Hermansson *et al.* 1988).

1.4.2 Animal models for OM

Over the last two decades, animals such as chinchillas, rats, mice, and, guinea pigs have been widely used to investigate and understand the pathogenesis involved (Giebink *et al.* 1980; Krekorian *et al.* 1991; Piltcher *et al.* 2002; Russell & Giles 1998). Moreover, while investigating the pathogenesis of OM, the route of infection in animal model has to resemble as closely as possible to the natural course of infection which is observed in humans. This has been successfully established in the chinchilla model in which nasopharyngeal induction of bacteria ascends to the ET and results in the middle ear infection (Giebink *et al.* 1979). Many animal models have focussed on OM caused by single microbial infection, however there has been no animal model standardised for mixed bacterial infections (Soriano *et al.* 2000).

The mechanism of the pathogenesis of OM involving influenza A virus and *S. pneumoniae* was first developed in an experimental chinchilla model (Giebink *et al.* 1980). This animal model has been fundamental in investigating viral-bacterial co-infection in OM. Once established, the chinchilla model was further adapted to investigate the incidence of OM with NTHi challenge preceding adenovirus infection and study the kinetics involved (Miyamoto & Bakaletz 1997; Suzuki & Bakaletz 1994). Although being an excellent nasopharyngeal colonisation model, certain combinations of bacteria and viruses were unable to induce OM, indicating the preferential ability of different viruses to have a synergistic effect with specific bacteria in causing OM (Bakaletz *et al.* 1995; Tong *et al.* 2000a). In the late 1990s, nasopharyngeal infection by NTHi in the chinchilla mimicking the natural route of infection in humans was successfully implemented to screen various potential vaccine antigens, NTHi adhesins and demonstrate the protection against antibiotic resistant NTHi with heat-killed NTHi through parenteral immunisation studies (Yang *et al.* 1998).

Amongst the different animal models used for studying polymicrobial diseases, the rat model has been well-defined in terms of immunological parameters, pharmacokinetics and gene sequences (Albiin *et al.* 1986; Hermansson *et al.* 1989). Other advantages to its use include: anatomical similarities with humans of the middle ear and ET, histological features like cell type and ciliary clearance tracts to that of humans (Albiin 1984; Albiin *et al.* 1986). However, the limitations of using a murine model included the high frequency of natural infection of the middle ear with bacteria, small size of middle ear in mice and surgical approach to block ET (Hal *et al.* 1982). Over the last four decades, several mouse strains such as BALB/c, Swiss-Webster and C57BL/6 have been widely used to investigate and elucidate the hearing defects and development of ear infections (Steel 1995), amongst which, BALB/c mice showed susceptibility to the common causative bacteria of AOM (Melhus & Ryan 2003). In addition, the inflammatory responses caused by various cytokines, mediators and ET obstruction have also been investigated (Johnson *et al.* 1994b; Johnson *et al.* 1994a).

Recently, a non-invasive rat model has been developed which promises potential for investigating immunological aspects of OM such as vaccination studies (Tonnaer *et al.* 2003). This model is based on the introduction of high positive nasopharyngeal pressure resulting in the transfer of the bacteria to the middle ear cavity. However, the limitations include haemorrhages in the tympanic membrane if the pressure is not maintained to 50kPa (Sato *et al.* 1997), generation of increased initial pressure before the animal regains consciousness and failure of reliable induction of OM.

Despite certain drawbacks in the various animal models used in OM, they have provided a better understanding of OM and led to the discovery and development of potential vaccine antigens, therapeutic antibiotic and treatment regimes. In addition, the use of mice as a preferred laboratory animal has significantly increased recently due to success in establishing appropriate OM models, advances in analytical procedures and the availability of genetically modified animals (Wasserman *et al.* 2007).

1.4.3 Concluding remarks

Current understanding of the complex mechanisms and bacterial interactions involved in OM is limited because no animal model has been established to investigate the polybacterial nature of OM. Previous studies have identified different virulence factors and their role in the pathogenesis of OM, developed potential vaccine antigens, investigated bacterial-viral synergy leading to greater incidence of OM. In addition, the clinical representation of OM, particularly the rate of bacterial colonisation, relationship between nasopharyngeal colonisation and incidence of OM, and the increased risk of recurrent OM in children with high nasopharyngeal carriage rates have highlighted the burden and significance of this disease. However, the involvement of multiple microbes in the nasopharynx, questions relating to bacterial load and order of bacterial infection, and their affect on the incidence and severity of OM, along with the knowledge of the real-time kinetics of disease progression could provide a better understanding of the significance of the polymicrobial environment in this disease and advance the

knowledge required for developing new strategies for preventing and managing infections.

1.5 AIMS AND OBJECTIVES

The mechanisms of host-bacterial relationships, microbial interactions, and the resulting inflammatory response in polybacterial OM have many questions that have not been investigated. A better understanding of these mechanisms would provide greater insight into the significance of microbial interactions and microbe-host dynamics in a polymicrobial environment, and advance in the development of improved therapeutic approaches for OM and respiratory tract infections. An appropriate experimental animal model investigating the predominant bacteria involved with OM will not only offer a more realistic representation of the disease state observed in children, but also advance our understanding of the complexities involved in bacterial OM. The research in this thesis has established a polybacterial otitis media infection animal model with the bacteria *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*, and co-infection with a respiratory virus for comparison of this trigger in predisposing bacterial infections (Chapter 2). The general objectives of this study were to further explore the effects of polymicrobial infections on the incidence and severity of OM.

The specific aims of this study were to:

1. Investigate the effect of the complex bacterial composition of the nasopharynx and its role in influencing the incidence and severity of OM *in vivo*;
2. Determine whether increased bacterial load affects the progression of bacterial colonisation from the nasopharynx to the lower respiratory tract *in vivo*;
3. Determine whether the acquisition of a new bacterium in the nasopharynx affects the incidence of OM in the presence of a pre-existing coloniser within the nasopharynx *in vivo*;
4. Investigate the dynamics of polybacterial colonisation patterns, localisation of the bacteria within the nasopharynx and middle ear, and the effect of sequential

bacterial infection on the incidence of OM, through real-time monitoring of disease progression *in vivo*; and

5. Investigate the microbe-host relationship involved and the inflammatory response generated *in vitro*, by determining the effect of polybacterial infection on bacterial adherence and inflammatory cytokine release.

CHAPTER 2

The incidence of *Streptococcus pneumoniae* otitis media is affected by the polymicrobial environment particularly *Moraxella catarrhalis* in a mouse nasal colonisation model

2.1 ABSTRACT

Otitis media (OM) is a highly prevalent paediatric disease commonly caused by *Streptococcus pneumoniae,* non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* with respiratory viruses recognised as major triggers that also affect bacterial adherence and colonisation. Various animal models of this disease have generally ignored the polymicrobial nature of the infection. This study investigated combinations of the above bacteria in the presence and absence of a respiratory virus (Sendai virus) in a mouse nasal colonisation model. The model has shown that the respiratory virus significantly contributed to bacterial OM for all combinations ($p<0.001$). We found that *S. pneumoniae* consistently dominated as the causative bacterium of OM and that when co-infected with *S. pneumoniae, M. catarrhalis* and not NTHi significantly affected pneumococcal OM ($p<0.001$) by increasing the OM incidence rate, infection bacterial load and duration of infection. Nitric oxide levels in the middle ear, as an indicator of inflammation, peaked at day 3 in single bacterium groups, but at day 1 in mixed bacterial groups and was produced in all bacteria inoculated groups even in the absence of viable bacterial recovery from the middle ear. The study has also found that phagocytic cells were recruited rapidly following nasal inoculation but that over time their numbers did not correlate with the individual bacterial loads. The study has shown that NO levels had a peak response that related to time and bacterial composition post-inoculation rather than with bacterial load and that the co-presence of different bacteria along with a respiratory viral infection significantly affected the incidence rate, duration of infection and bacterial load (severity) of OM.

2.2 INTRODUCTION

Otitis media (OM) is a common childhood infection and manifests itself as an inflammation of the middle ear along with the presence or absence of effusion due to bacterial or viral infection (Bakaletz 2002; Cripps & Otczyk 2006; Darrow *et al.* 2003). The incidence of acute OM is highest in the second half of the first year of life, except in certain OM-prone groups, and appears to have a second lower peak between the ages of

four and five years (Teele *et al.* 1989). Approximately, 30% of infants will have at least one episode of AOM within the first year (Klein *et al.* 1990). By three years of age, more than 80% of children have had at least one episode of AOM, and by seven years of age, almost 40% of children have had six or more episodes of AOM (Teele *et al.* 1989). The multifactorial nature of OM and the various risk factors involved have been well documented (Froom *et al.* 2001; Lubianca *et al.* 2006). Although not directly associated with the pathogenesis of OM, the risk factors influence the disease through mechanisms such as Eustachian tube (ET) dysfunction, aiding bacterial adherence and impairing mucociliary clearance (Froom *et al.* 2001; Gitiban *et al.* 2005; Lubianca *et al.* 2006; St. Sauver *et al.* 2000). A respiratory viral infection is well recognised as a trigger for secondary bacterial upper respiratory tract infections by enhancing bacterial adherence, colonisation and translocation through the epithelial barrier (Hament *et al.* 1999). The ability of various respiratory viruses to trigger bacterial OM also differs. The polymicrobial etiology of OM has been well established from the concurrent presence and isolation of respiratory virus and bacteria within the nasopharynx and from the middle ear. Amongst the nasopharyngeal commensal bacteria, *S. pneumoniae,* NTHi and *M. catarrhalis* are known to be the predominant bacteria responsible for bacterial OM (Bakaletz 2002; Heikkinen *et al.* 1999; Klein 2000) with percentage incidence rates reported in the same order (Faden 2001). The introduction of the pneumococcal conjugate vaccine has not had a major impact on the overall incidence rate of OM (Palmu *et al.* 2008) and the USA which had one of the highest coverage rates for serotypes in this vaccine is now reporting significant evidence of serotype replacement (Pichichero & Casey 2007).

Certain ethnic groups such as the Inuits, Native Americans and Australian Aborigines have higher rates of chronic OM (Cripps *et al.* 2005). The high incidence of OM has been of serious concern in Australian Aboriginal children. In this group, the median age for nasopharyngeal colonisation with any bacteria involved in OM is less than 20 days of age when compared to about 270 days in non-Aboriginal children (Leach *et al.* 1994). In particular *M. catarrhalis* is one of the early colonisers in these infants (Leach *et al.*

1994). Early colonisation and higher carriage rates in the first 3 months of age have also been identified as risk factors associated with children who are OM-prone (Faden 2001; Faden *et al.* 1997). It is well accepted that the microbial environment in the nasopharynx, whether in a commensal or disease state is an important contributing factor in the occurrence of OM. Carriage rates of *S. pneumoniae, M. catarrhalis* and NTHi in Aboriginal children have been reported as 49%, 50% and 41%, respectively, compared with 25%, 25% and 11% in non-Aboriginal children (Watson *et al.* 2006). By as early as 2 months of age *S. pneumoniae* and *M. catarrhalis* had been isolated from 37% and 36% of Aboriginal children and only from 11% and 12% of non-Aboriginal children, respectively. In addition to the well established positive association between virus (rhinovirus and adenovirus) and bacteria, analysis of colonisation patterns has shown that there are significant positive associations between pairs of bacteria, in particular, *M. catarrhalis* with either *S. pneumoniae* or *H. influenzae* (Jacoby *et al.* 2007).

In acute and chronic inflammatory diseases many cellular processes are activated and compounds produced as part of the host's response. As part of this response, cells such as macrophages, neutrophils and vascular endothelial cells produce nitric oxide (NO), a short-lived inorganic free-radical known to be involved in cellular signaling and inflammation (John *et al.* 2001; Li, W. *et al.* 2000; Ryan & Bennett 2001). NO is produced by the NADPH-dependent enzyme nitric oxide synthase (NOS) which is present in 3 distinct isoforms namely; endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). Both eNOS and nNOS are constitutively expressed whereas iNOS is mostly expressed by cells such as macrophages and neutrophils upon stimulation with cytokines and other microbial products. The importance of NO in the development of mucoid middle ear effusion first became evident in the mid 1990s. In OM, inflammatory mediators induce leukocytes and macrophages to release pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-10, IFN-γ (Cripps & Kyd 2007; Juhn *et al.* 2004). Associated with this pro-inflammatory response is the expression of iNOS (Jeon *et al.* 2006). Thus, detection of NO is an indicator of inflammation even in the absence of

detectable microbes and is a contributing inflammatory mediator in the pathology of OM. By the nature of its location, the middle ear cavity should normally be in a quiescent state since the only exposure of the mucosal surface to foreign agents is via ascension through the ET.

Over the last two decades, various animal models have provided a better understanding of OM and its pathology and have included the use of rats, mice, chinchillas, gerbils, guinea pigs, monkeys and ferrets. These studies have led to the discovery and development of potential vaccine antigens, therapeutic antibiotics and treatment regimes. Only recently has the use of mice increased significantly due to the success in establishing appropriate OM models, advances in analytical procedures and the development of new genetically modified mouse strains (Wasserman *et al.* 2007). The significance of understanding the complexity of the synergistic role between the different bacteria and viruses, ET dysfunction and virulence factors responsible for causing the disease is a high priority in preventing OM (Bakaletz 2002; Bakaletz 2004; Lim *et al.* 2002; TongFisher *et al.* 2000; Winther *et al.* 2007).

To date, most studies have considered individual bacterial-viral relationships, whereas OM is associated with a complex polymicrobial state. This study aimed to establish an animal model to test the hypothesis that the presence of multiple microbes in the nasopharynx differentially affects the incidence of OM associated with *S. pneumoniae,* NTHi and *M. catarrhalis*. In particular, this study examined how the complex bacterial composition of the nasopharynx differentially affected the rates of occurrence, incidence and severity of OM. In addition to the detection of bacteria in the middle ear, NO was measured as an indicator of middle ear inflammation. One of the challenges with these animal models has been the choice of virus. It is well documented that not all human respiratory viruses have the same level of association with OM in children and many of these viruses do not cause similar disease etiology in animal models. Sendai virus is a well accepted murine counterpart of human type-1 parainfluenza virus and a naturally pneumotropic murine pathogen, not reported to cause human diseases (Bousse *et al.*

2006; Faisca *et al.* 2005). Human parainflueza and Sendai virus belong to the family *Paramyxoviridae* and genus *Respirovirus,* sharing substantial amino acid sequence identities (Bousse *et al.* 2006). Human parainfuenza virus is known to cause croup, bronchiolitis and viral pneumonia in children and accounts for approximately 12% of the acute lower respiratory tract infections in hospitalised adults (Vilchez *et al.* 2003). Sendai virus causes acute respiratory infections in its natural hosts; mice, rats, guinea pigs and hamsters. Because of this similarity, Sendai virus has been used as a prototype model for studying human respiratory infections in experimental animals (Takao *et al.* 1997) and was selected as the viral trigger in this study.

This study found that the presence of multiple colonising bacteria in the nasopharynx significantly increased the rate of incidence and severity (bacterial load) of OM. *S. pneumoniae* was the dominant organism recovered in the middle ear and the incidence of OM was significantly enhanced in mice pre-infected with a respiratory virus. The bacterial and not the viral infection induced an inflammatory response in the middle ear as measured by phagocytic cell recruitment and nitric oxide production. The timing of the peak NO response was affected by the polymicrobial infection. Phagocytes were recruited into the middle ear within the first three days of infection, but were not isolated at the day 7 and 14 timepoints when the bacterial load peaked. A significant finding was that the presence of *M. catarrhalis* as a co-coloniser of the nasopharynx significantly exacerbated OM caused by *S. pneumoniae*. This is the first experimental evidence that demonstrates that the presence of *M. catarrhalis* in a polymicrobial environment can affect the disease state that appears to be caused by other bacteria.

2.3 MATERIALS AND METHODS

2.3.1 Bacteria and virus preparation

The rodent respiratory Sendai virus (10^4 $TCID_{50}$) has been previously described (Moore *et al.* 2001) and was recovered from frozen stocks. *M. catarrhalis* K65 (kindly provided by Dr Barbara Chang) was a clinical isolate from an adult sputum recovered at Sir Charles Gardiner Hospital, Perth, Australia. NTHi 289 was a clinical isolate from the

sputum of an adult patient with chronic bronchitis. Both were grown on chocolate Brain-Heart Infusion (BHI) agar (Oxoid Ltd., Hampshire, England) supplemented with 5% partially lysed defibrinated horse blood. *S. pneumoniae* serotype 14, obtained from GlaxoSmithkline, Belgium was grown on Blood agar (Oxoid Ltd., Hampshire, England) supplemented with 5% defibrinated horse blood (Oxoid Australia Pty. Ltd.). All bacteria were grown overnight at 37ºC in a humidified 5% CO_2 incubator, harvested, suspended in sterile phosphate buffered saline (PBS) and washed three times by centrifugation. The bacterial concentration was estimated by calculation from a regression curve based on the optical density at 405nm. *M. catarrhalis* and NTHi were adjusted to $2x10^{10}$ CFU/ml and *S. pneumoniae* to $1x10^{10}$ CFU/ml, with the concentration subsequently confirmed by overnight culture and purity by Gram staining.

2.3.2 Selection of mouse strain

The incidence of bacterial OM with and without pre-viral infection was compared in BALB/c, C57BL6, CBA/CaH, C3H(He) and DBA/2J mice (Animal Resources Centre, Perth, Australia). The mice were sedated by intraperitoneal injection of 0.25 ml ketamine plus xylazine (5mg/ml ketamine hydrochloride and 2mg/ml xylazine hydrochloride) in PBS. Seventy two hours prior to the bacterial infection (day minus 3), half the mice received 10 µl volumes of 10^4 $TCID_{50}$ Sendai virus to each nare. At day 0 (72 hr after Sendai virus infection), the mice were sedated as above and all mice received a bolus of viable bacteria; *M. catarrhalis* K65, NTHi 289 (at 10^7 CFU each) or *S. pneumoniae* serotype 14 (at 10^6 CFU), intranasally as above. After 24 hours of infection, the animals were euthanised by an intraperitoneal injection of 0.2 ml pentobarbital sodium (60 mg/ml). Both middle ear cavities of each mouse were lavaged by multiple recoveries of 50 µl volumes of PBS with a total lavage volume of 150 µl collected. The presence and quantity of bacteria in the middle ear was determined by titration of the middle ear lavage (MEL) on to appropriate media BALB/c mice were selected on the basis of high numbers of animals with culture positive MEL **(Table 2.1)**.

2.3.3 Experimental groups

Specific pathogen free (SPF) BALB/c mice (Animal Resources Centre, Perth, Australia), male, aged 6-8 weeks old were used in this study. A total of 278 mice were used and on an average 70 mice were euthanised at each time point; day 1, 3, 7 and 14. In addition to this, 10 mice were included in the non-infected group and 20 mice were infected with Sendai virus only.

For each of the following bacterial groups half the mice received Sendai virus 3 days before nasal bacterial inoculation (see below). The experimental groups used were:

A] Single bacteria: a) *M. catarrhalis*; b) NTHi; or *c*) *S. pneumoniae* alone.

B] Double bacterial combination: a) *M. catarrhalis* + NTHi; b) *M. catarrhalis* + *S. pneumoniae*; or c) NTHi + *S. pneumoniae*.

C] Triple bacterial combination: *M. catarrhalis* + NTHi + *S. pneumoniae*.

2.3.4 Induction of infection

Following intranasal infection with Sendai virus (as mentioned above), the virus-infected mice were housed in an infection containment facility to prevent any transmission of virus to the non-infected mice. Three days after virus infection (day 0), all mice were infected with 10µl (5µl per nare) of the relevant bacteria or bacterial combination, intranasally. For double and triple bacterial combinations, equal ratios of appropriately adjusted bacterial concentrations were used. The mice were euthanised at day 1, 3, 7 and 14 timepoints following the bacterial inoculation. Blood was collected by heart puncture with a 26G needle and the serum separated by centrifugation and stored at -20^{0}C for future antibody analyses. MEL was collected as above. Nasal lavages were performed by injecting and recovering 50 µl of sterile PBS into each nare with a 26G needle attached to a 1ml syringe. Bacteria in MEL and nasal lavages from each mouse were titrated by serial 10-fold dilutions onto the relevant media. Colony identity was confirmed by gram-staining and morphology. An aliquot of MEL was cytospun onto a glass slide, stained with Diff-Quik® staining kit (Dade Behring Inc., USA) and differential cell counts performed as described previously (Kyd *et al.* 2000). The remaining MEL was centrifuged at 1500 x g for 10 minutes and the supernatant stored at

-80^{0}C. The cell pellet was re-suspended in PBS and methylene blue for enumeration of the total number of white blood cells recovered. The total cell count was calculated after making the necessary correction for prior removal of aliquots.

2.3.5 Nitric oxide assay

The total concentration of nitric oxide in MEL was measured using a Total Nitric Oxide assay kit (Endogen, Pierce, Illinois, USA). The assay was carried out in 96 well microtitre plates (Nunc, Thermo Fisher Scientific Inc., Demark). Prior to the assay, all samples were deproteinised using 0.15M zinc sulphate and 0.15M barium hydroxide in order to prevent any interference with the proteins. Following deproteinisation all samples were diluted 1:2 and used in the assay. The assay was performed according to the manufacturer's instruction and the concentration determined by calculation from the nitrate standards and the linear standard curve.

2.3.6 Statistical analysis

The significance of bacterial recovery in MEL and nasal lavage between groups infected with different bacterial combinations and with or without the pre-viral infection was analysed using a two-way ANOVA with a Bonferroni post hoc comparison. The significance of NO production at different timepoints was performed using a one-way ANOVA with Kruskal-Wallis and Dunns post tests.

2.4 RESULTS

2.4.1 Establishment of the animal model

Initially, several strains of mice were investigated for their capacity to naturally acquire a bacterial OM with and without pre-viral infection **(Table 2.1)**. Mice (sedated) received a bolus of live bacteria intranasally either alone or 3 days post inoculation with Sendai virus. BALB/c mice were selected as the most suitable strain to investigate OM for all three pathogens in this study because they were the strain most consistently infected by all three bacteria. A pre-viral infection resulted in the highest incidence rate of bacterial

OM. It should be noted that for future studies focusing only on *M. catarrhalis*, DBA/2J may warrant consideration as a suitable strain of mice.

Table 2.1: Incidence of bacterial otitis media from different strains of mice

	Number of animals with culture positive middle ear lavage					
Mouse strain[a]	*S. pneumoniae*		*NTHi*		*M. catarrhalis*	
	-virus	+virus	-virus	+virus	-virus	+virus
BALB/c	1/4	4/4	2/4	3/4	0/4	3/4
C57BL6	3/4	4/5	0/4	2/4	0/4	1/5
CBA/CaH	0/5	4/5	0/5	1/5	-	-
C3H(He)	0/5	0/5	1/5	0/5	0/5	0/5
DBA/2J	1/4	2/4	3/5	4/5	4/5	0/4

[a] Different strains of mice were intranasally inoculated with a live bolus of bacteria either alone or 3 days post inoculation with Sendai virus. Values indicate the number of mice/group that were bacteria culture positive.

- denotes that *M. catarrhalis* infection was not performed on CBA/CaH

2.4.2 Overall incidence of bacterial OM

In BALB/c mice, the incidence of bacterial OM was based on the recovery of bacteria by culture methods. Bacterial recovery was determined from (MEL) collected at 1, 3, 7 and 14 days post intranasal inoculation. Eighty four (30.10%) of the total of 278 mice developed OM as defined by recovery of viable bacteria from the middle ear. From the 84 MEL culture positive animals, 89.3% cultured *S. pneumoniae*, 9.5% NTHi and 1.2% *M. catarrhalis*. These were recovered either as the only bacterial agent or as part of any given bacterial combination. The recovery of bacteria in the nasal lavage from the same mice showed that, similarly to the middle ear, *S. pneumoniae* was overall the dominant species, followed by NTHi and then *M. catarrhalis*. This profile is consistent with the reported dominance and distribution of these bacteria for OM in children and validated

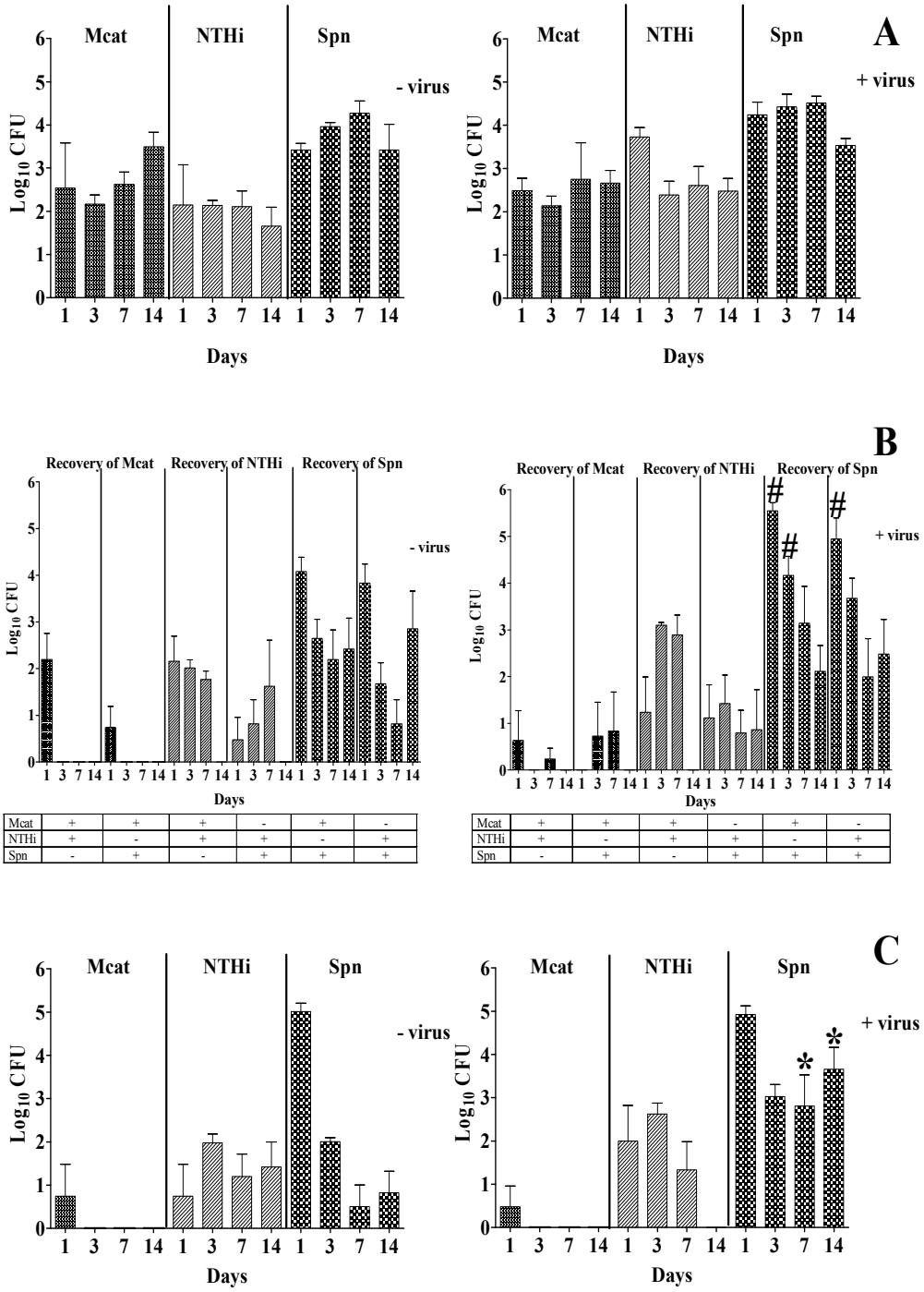
the relevance of the model with a good correlation for disease incidence rate and bacterial etiology.

2.4.3 Recovery of bacteria in nasal lavage

The recovery of any given bacteria inoculated singly or in combination in non- or pre-virally infected groups of mice is shown in **Figure. 2.1A-2.1C**. In single bacterial infections, each bacterium was successful in colonising the nasopharynx over the 14 day infection period. Bacterial recovery remained consistent over this period. A pre-viral infection did not have any significant effect on bacteria recovered in the nasal lavage **(Figure. 2.1A)**. There were a number of significant observations in the double bacterial combination groups **(Figure. 2.1B)**. In the virus infected group, the recovery of *S. pneumoniae* in *S. pneumoniae* + *M. catarrhalis* was almost 10^6 and 10^4 fold higher than the recovery of *M. catarrhalis* in the same group at 24 hr and 3 days post infection ($p<0.001$). Similarly, the recovery of *S. pneumoniae* in *S. pneumoniae* + NTHi was almost 10^4 fold higher than the recovery of NTHi in the same group at 24 hr post-infection ($p<0.001$). The recovery of *S. pneumoniae* in *S. pneumoniae* + *M. catarrhalis* (+virus) was significantly higher compared to its recovery in *S. pneumoniae* + NTHi (-virus) ($p<0.001$). The recovery of *S. pneumoniae* was enhanced in the nasopharynx at 24 hr post-infection when co-infected with either *M. catarrhalis* or NTHi compared to a single infection ($p<0.001$). Furthermore, a pre-viral infection and time post-inoculation (duration) were found to be important factors in the recovery of bacteria in the nasopharynx ($p<0.001$). In the triple bacterial infection groups, *S. pneumoniae* were the only bacterium to be recovered throughout the infection period in both virus and non-virus infected groups, although some NTHi were recovered **(Figure. 2.1C)**. The pre-viral infection enhanced nasopharyngeal colonisation with *S. pneumoniae* especially at days 7 and 14 post-infection ($p<0.01$). In this polybacterial environment, *M. catarrhalis* was never recovered by culture post a 24 hr time point and although NTHi was recovered during most of the infection period, the presence of virus did not affect recovery of either of these from the nose. Overall the recovery of bacteria in the triple polymicrobial infection was dominated by *S. pneumoniae*>NTHi>*M. catarrhalis*.

Figure. 2.1: Nasal bacterial recovery in Sendai virus-infected and non-virus infected BALB/c mice inoculated with different bacterial combinations. Bars represent the mean ± SEM of the bacteria recovered from nasal lavage (n=5 at each time point) on day 1, 3, 7 and 14 post-bacterial infection with A] Single bacterium (*M. catarrhalis*; NTHi or *S. pneumoniae*), B] Double bacterial combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; NTHi + *S. pneumoniae*), and C] Triple bacterial combination (*M. catarrhalis* + NTHi + *S. pneumoniae*). Mice were pre-infected with (right panel) or without (left panel) Sendai virus. # $p<0.001$, recovery of *S. pneumoniae* vs *M. catarrhalis* in the virus infected (*M. catarrhalis* + *S. pneumoniae*) group; recovery of *S. pneumoniae* vs NTHi in the virus infected (NTHi + *S. pneumoniae*) group. *$p<0.01$, recovery of *S. pneumoniae* in virus infected and non-virus infected groups.

Nasal bacterial recovery in Sendai virus-infected and non-virus infected BALB/c mice inoculated with different bacterial combinations



2.4.4 Recovery of bacteria in MEL

The recovery of bacteria from the middle ear was different to the nasopharynx for the different bacteria **(Figure. 2.2A-2.2C)**. In the single bacterial groups, *S. pneumoniae* was the dominant bacteria recovered at all timepoints in the infection period, followed by NTHi and *M. catarrhalis*. *S. pneumoniae* recovery at days 3 and 7 post-infection was enhanced by the pre-viral infection ($p<0.01$) **(Figure. 2.2A)**. When two different bacteria were co-inoculated, the presence of *M. catarrhalis* in *S. pneumoniae* + *M. catarrhalis* (+ virus) resulted in a significant 10^4-fold increase in the recovery of *S. pneumoniae* by 24 hr post-infection ($p<0.001$). The recovery of *S. pneumoniae* in *S. pneumoniae* + NTHi was almost 10^3 fold higher than the recovery of NTHi in the same group ($p<0.001$) **(Figure. 2.2B)**. As per the nasopharyngeal colonisation data, the choice of bacterium and the presence of virus were significant factors in the recovery of *S. pneumoniae* in double bacterial combination groups in the MEL. At day 3 post-infection, the recovery of *S. pneumoniae* in *S. pneumoniae* + *M. catarrhalis* (+ virus) was significantly higher when compared to its recovery in *S. pneumoniae* + NTHi (- virus) ($p<0.001$). In contrast to *S. pneumoniae*, in double bacteria co-infections, the recovery of NTHi was very low and *M. catarrhalis* could not be recovered throughout the infection period. Even though it was not recovered in the MEL, the presence of *M. catarrhalis* in *S. pneumoniae* + *M. catarrhalis* resulted in a significantly greater incidence of OM, a more sustained duration of infection and increased bacterial load (severity) for pneumococcal OM **(Figure. 2.3)**. A pre-infection with respiratory virus and the time post-inoculation (duration) were both important in the recovery of bacteria ($p<0.001$ and $p<0.01$, respectively). In the triple polymicrobial infection, the co-presence of both *M. catarrhalis* and NTHi in pre-virally infected mice did not result in a significant increase in the recovery of *S. pneumoniae* **(Figure. 2.2C)**. Similar to the double bacterial combination group, the recovery of NTHi was not significant and *M. catarrhalis* was not recovered at all throughout the infection period. Overall, the recovery of bacteria was dominated by *S. pneumoniae*>NTHi>*M. catarrhalis*.

Figure. 2.2: Middle ear bacterial recovery in Sendai virus-infected and non-virus infected BALB/c mice inoculated with different bacterial combinations. Bars represent the mean ± SEM of the bacteria were recovered from middle ear lavage (n=5 at each time point) on day 1, 3, 7 and 14 post-bacterial infection with A] Single bacterium (*M. catarrhalis*; NTHi or *S. pneumoniae*), B] Double bacterial combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; NTHi + *S. pneumoniae*), and C] Triple bacterial combination (*M. catarrhalis* + NTHi + *S. pneumoniae*). Mice were pre-infected with (right panel) or without (left panel) Sendai virus. * $p<0.01$-recovery of *S. pneumoniae* in virus infected vs non-virus infected group. # $p<0.001$-recovery of *S. pneumoniae* vs *M. catarrhalis* in the virus infected (*M. catarrhalis* + *S. pneumoniae*) group; recovery of *S. pneumoniae* vs NTHi in the virus infected (NTHi + *S. pneumoniae*) group.

Middle ear bacterial recovery in Sendai virus-infected and non-virus infected BALB/c mice inoculated with different bacterial combinations

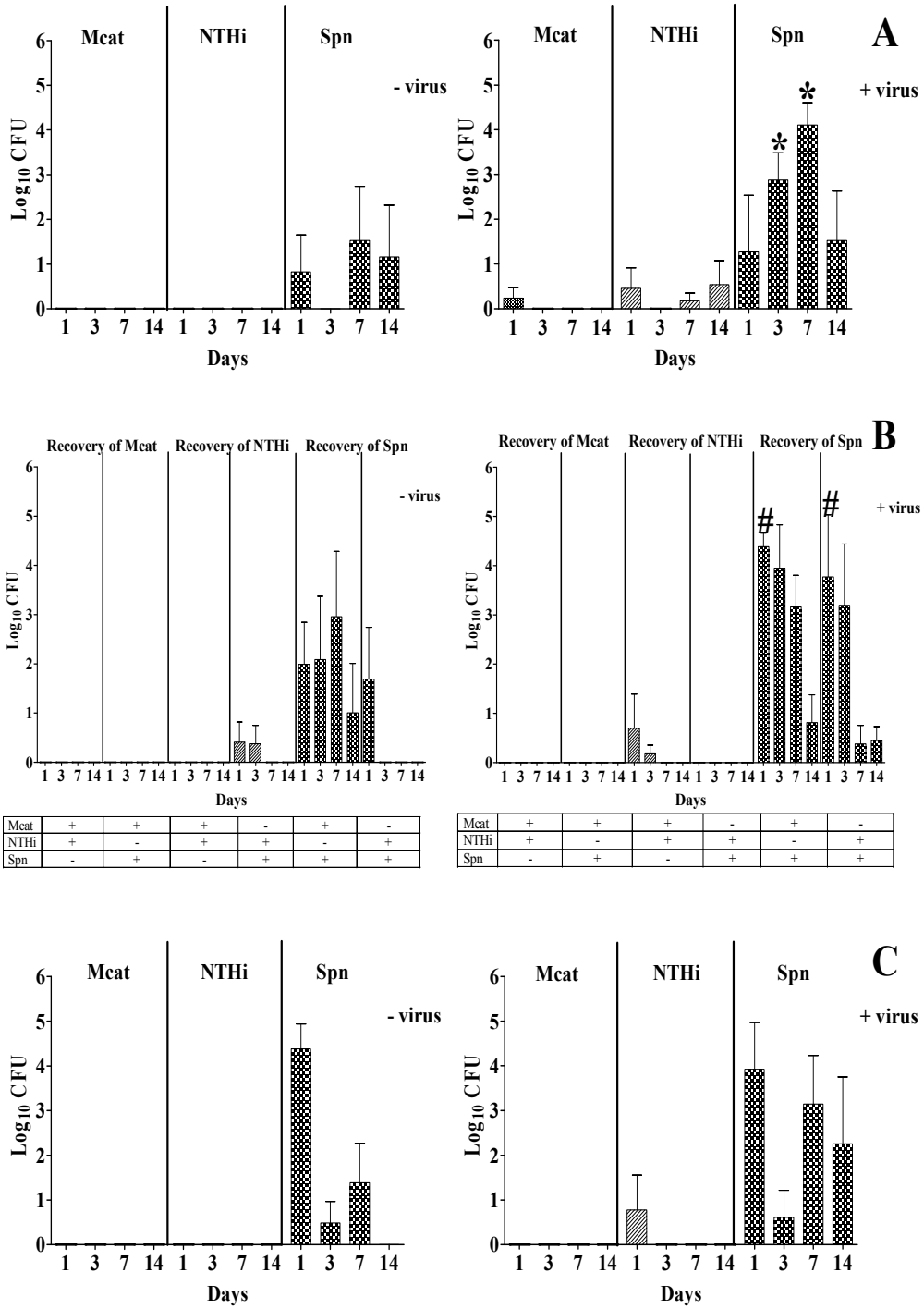
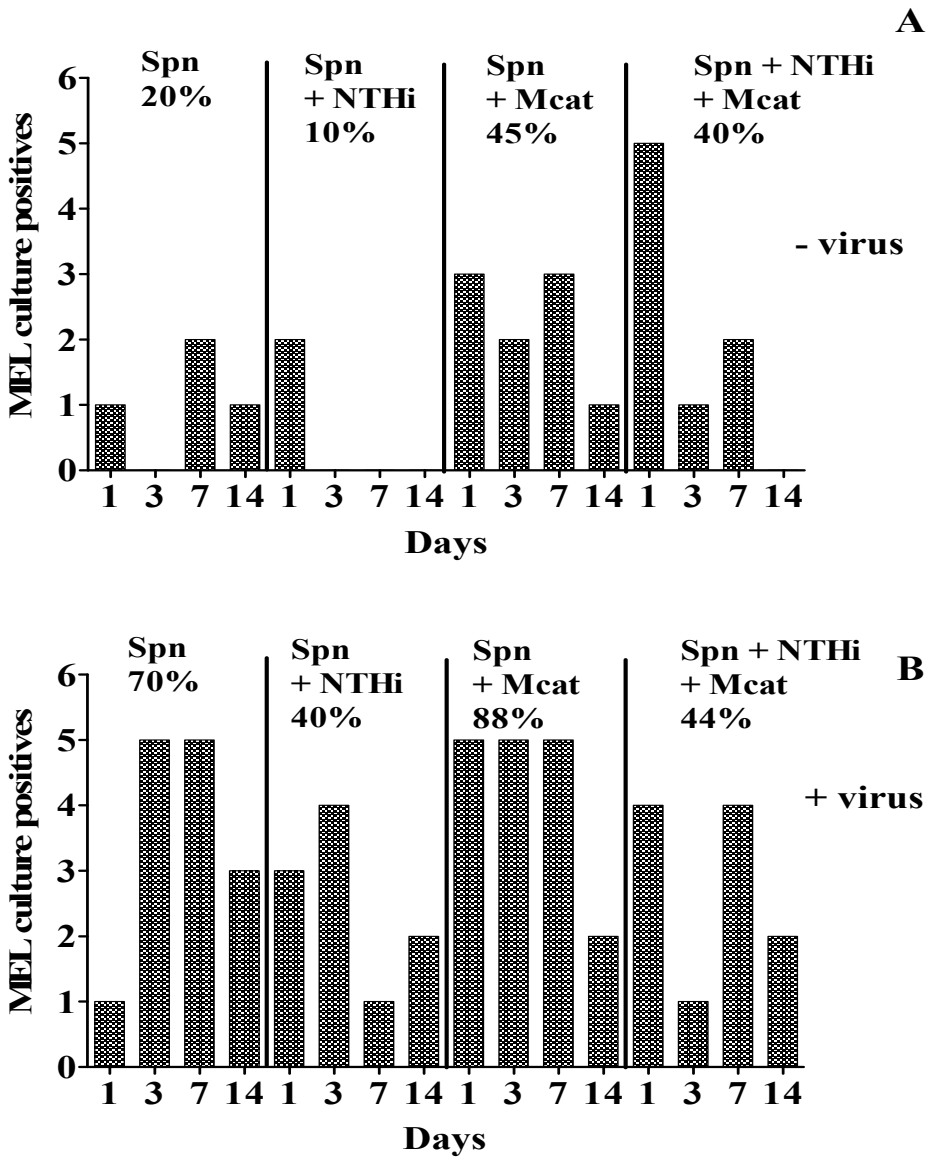


Figure. 2.3: Incidence of *S. pneumoniae* OM. Number of mice with culturable *S. pneumoniae* in the middle ear following nasal co-inoculation with either NTHI or *M. catarrhalis* or NTHI + *M. catarrhalis* in the presence and absence of the pre-viral infection (n=5 mice at each time point with a total of 20 mice per infection group).

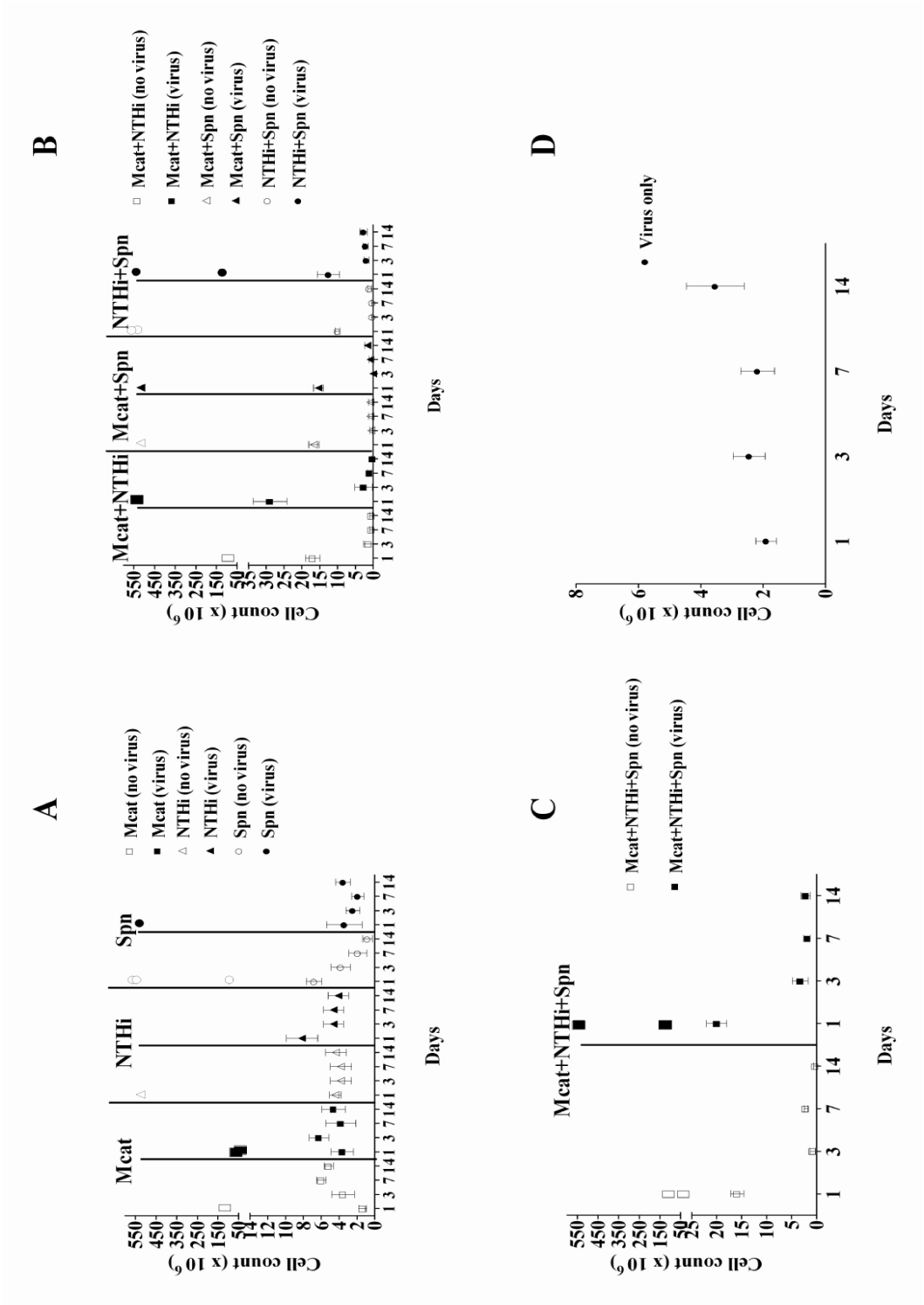


2.4.5 White blood cell count in MEL

Intranasal inoculation of bacteria, whether single, double or triple infections and regardless of viral infection status induced the recruitment of white blood cells into the middle ear at day 1 post-infection **(Figure. 2.4A-2.4C)**. The numbers of white blood cells recruited to the middle ear was reduced at latter timepoints despite the sustained bacterial burden in some groups. White blood cell recruitment was better induced by bacterial infection than by viral infection **(Figure. 2.4D)**. Differential cell counts showed that polymorphonuclear neutrophils (PMN) were the predominant cell type in all groups followed by macrophages.

Figure. 2.4: White cell blood counts in the middle ear. The total viable white blood cell counts in the middle ear lavage were enumerated using methylene blue on day 1, 3, 7 and 14 post-intra nasal inoculation with A] Single bacterium (*M. catarrhalis*; NTHi or *S. pneumoniae*); B] Double bacterial combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; NTHi + *S. pneumoniae*); C] Triple bacterial combination (*M. catarrhalis* + NTHi + *S. pneumoniae*), in the presence or absence of a pre-viral infection; and D] Sendai virus alone.

White cell blood counts in the middle ear



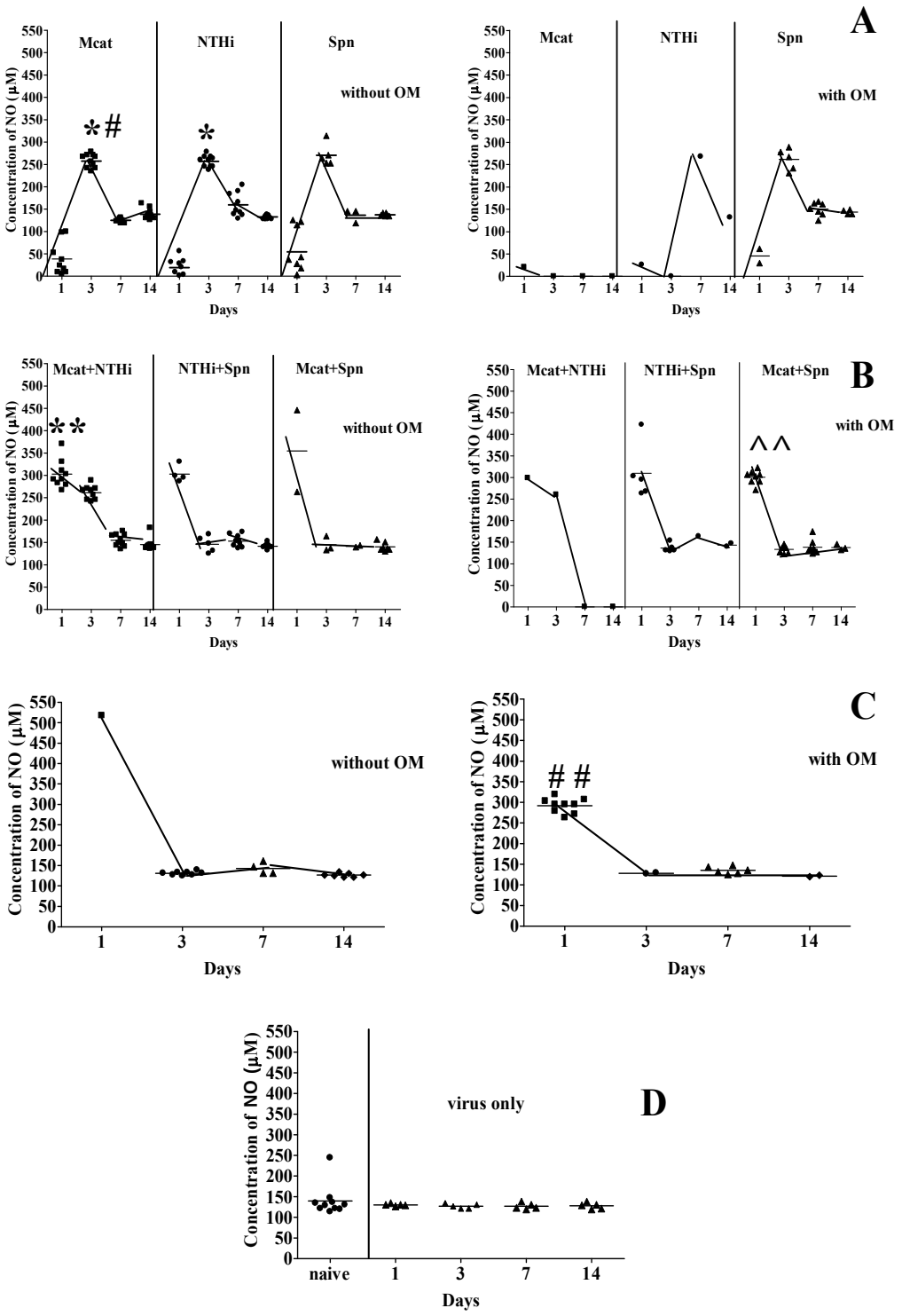
2.4.6 Levels of nitric oxide in MEL

The effect of different single and polymicrobial infections on levels of nitric oxide (NO) (as an indicator of the inflammatory response) detected in culture positive and culture negative MEL is shown in **Figure. 2.5A-2.5C**. NO was measured even in the absence of recoverable bacteria from the middle ear. The nitric oxide response peaked earlier (day 1) when mice were infected with multiple bacterial species as compared with a single species (day 3). In culture negative MEL, the level of NO peaked on day 3 post-infection with single bacterium species only, as shown in the comparison between *M. catarrhalis* and NTHi single infections and the triple species infection ($p<0.05$) **(Figure. 2.5A, 2.5C)**. The levels of NO at day 3 post-infection with *M. catarrhalis* were greater than *M. catarrhalis* + *S. pneumoniae* group ($p<0.001$). In contrast to the NO levels which peaked on day 3 post-infection with single bacterium, the levels of NO peaked on day 1 post-infection in double and triple bacterial inoculation groups **(Figure. 2.5B-2.5C)**. The NO response at day 1 was significantly higher in the *M. catarrhalis* + NTHi group than in the NTHi group with culture negative MEL ($p<0.05$). Similarly, the NO response was also higher in the *M. catarrhalis* + *S. pneumoniae* group compared with the *M. catarrhalis* group with culture positive MEL ($p<0.05$). The NO levels in culture positive MEL at day 1 were higher from mice co-infected with all three bacteria than from mice infected with only *M. catarrhalis* or NTHi ($p<0.001$). Comparisons with culture positive groups of *M. catarrhalis* and NTHi were not possible due to the low numbers of animals that were culture positive at the timepoints **(Figure. 2.5A)**. The NO levels in Sendai virus-infected only and naïve control animals ranged from 125μM - 150μM which was much lower than the NO responses observed in bacterial infected groups **(Figure. 2.5D)**.

Figure. 2.5: Nitric oxide levels in the middle ear. The inflammatory response in both culture positive (with OM) and culture negative (without OM) MEL was measured using total nitric oxide on day 1, 3, 7 and 14 post-intra nasal inoculation with A] Single bacterium (*M. catarrhalis*; NTHi or *S. pneumoniae*), B] Double bacterial combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; NTHi + *S. pneumoniae*), C]

Triple bacterial combination (*M. catarrhalis* + NTHi + *S. pneumoniae*), along with ± Sendai virus and, D] Sendai virus only. n=5 at each time point and n=10 in an uninfected group. * $p<0.05$-levels of NO in *M. catarrhalis* and NTHi vs triple bacterial group (culture negative MEL); # $p<0.001$- levels of NO in *M. catarrhalis* vs *M. catarrhalis* + *S. pneumoniae* (culture negative MEL). ** $p<0.05$- levels of NO in *M. catarrhalis* + NTHi vs NTHi (culture negative MEL); ^^$p<0.05$- levels of NO in *M. catarrhalis* + *S. pneumoniae* vs *M. catarrhalis* (culture positive MEL). ## $p<0.001$- levels of NO in triple bacterial group vs *M. catarrhalis* and NTHi in single bacterium group (culture positive MEL).

Nitric oxide levels in the middle ear



2.5 DISCUSSION

Polymicrobial infections usually include two or more organisms which act synergistically to produce a complex disease (Bakaletz 2004). The polymicrobial etiology of OM has been demonstrated by the presence of bacteria and respiratory virus in the middle ear of children. In the development of animal models of OM, the choice of virus has often been challenging. Human respiratory viruses have differing abilities to predispose for bacterial OM and can cause different disease etiology in animal models. For example, influenza A virus will trigger *S. pneumoniae* infection and adenovirus is known to influence NTHi OM in animal models (Bakaletz 1995). Therefore in this study, a well established murine respiratory virus (Sendai virus) was included as a viral trigger for bacterial OM. To date, a number of animal models have considered the contribution of individual bacterial-viral interactions in causing OM. In contrast, this study reports the development of a polymicrobial OM infection model to investigate the effects of co-infection of the three predominant bacteria isolated in clinical cases of OM. This was studied in the context of a predisposing viral infection on nasopharyngeal colonisation and the ability of the bacteria to cause a middle ear infection. This study also determined if a polymicrobial infection induces an inflammatory response in the middle ear even in the absence of culturable bacteria. The data provides evidence that following intranasal inoculation of different bacteria either singly or in combination, recovery of *S. pneumoniae* in both the nasopharynx and the middle ear was greater than NTHi and *M. catarrhalis* and was enhanced by co-infection with *M. catarrhalis*. In addition, the recovery of bacteria in the nasopharynx and middle ear was enhanced by a pre-infection with the respiratory virus. Similarly, the duration of bacterial recovery over the period of the infection was increased by the viral infection. There is evidence that a polymicrobial bacterial infection alters the timing of the inflammatory cascade in the middle ear; the peak NO response observed at day 3 post infection for single bacterial infections was later when compared to day 1 for polymicrobial bacterial infections. The inflammatory response was observed in middle ears even in the absence of recoverable bacteria. This suggests that culturing of bacteria alone from middle ear aspirates is not sufficient to assess OM status.

The association between rates of nasopharyngeal colonisation by bacteria such as *S. pneumoniae*, NTHi and *M. catarrhalis* and the incidence of OM is well cited in the literature. This association is most apparent in Australian Aboriginal communities where infants are colonised with these bacteria within a few days of birth (Leach *et al.* 1994). In Aboriginal infants, the carriage rates for *S. pneumoniae* and *M. catarrhalis* ranges from 50%-70% and 40%-60% for *H. influenzae* and they are known to carry multiple pathogens which increases the risk for OM (Garcia-Rodriguez & Martinez 2002; Leach *et al.* 1994; Watson *et al.* 2006). Similar high and consistent rates of nasopharyngeal colonisation were observed in the murine OM model, particularly after single bacterial inoculation. A significant finding of this study was that the presence of different bacteria affects not only the rates and burden of nasopharyngeal colonisation but can enhance the incidence and severity of OM. In our model, the overall incidence rate of OM as defined by recovery of viable bacteria from the middle ear was 30.1%, a level of infection that compares with the overall incidence rate of 30% for infants in their first year (Klein *et al.* 1990). Importantly, this rate of incidence was dependent on a predisposing respiratory viral infection, and on the nature of the bacteria in the polymicrobial infection. For example, this study shows that the recovery of *S. pneumoniae* in both the nasopharynx and the middle ear was significantly higher when co-infected with *M. catarrhalis* in pre-virally infected mice compared to when co-infected with NTHi.

The interaction and competition between a type b *H. influenzae* and *S. pneumoniae* during nasopharyngeal colonisation was reported to show a reduction in recovery and killing of *S. pneumoniae* (Lysenko *et al.* 2005). A subsequent report suggested that activation of the cytoplasmic signaling molecule, Nod-1 (nucleotide-binding oligomerisation domain-1) contributed to the killing of *S. pneumoniae* by *H. influenzae* (Lysenko *et al.* 2007). Our findings differ to these, most likely due to differences between type b and nontypeable *H. influenzae in vivo* and the use of immunodeficient mice compared to wild type. However, both studies demonstrate the importance of the composition of the microbial environment on both the host and microbe responses. The significance of co-occurrence of *S. pneumoniae* with other bacterial and viral pathogens

in the upper respiratory tract has also recently been established using statistical covariance modelling (Jacoby *et al.* 2007). The authors found a positive association between *S. pneumoniae* and *M. catarrhalis* that was higher than between *S. pneumoniae* and NTHi, thereby suggesting that the ability of *S. pneumoniae* to colonise is greater in the presence of *M. catarrhalis* than NTHi. The mechanisms by which the concurrent presence of bacteria is able to affect colonisation and infection and in particular, the mechanism(s) by which *M. catarrhalis* affects *S. pneumoniae* are yet to be elucidated. An understanding of how bacteria interact such that they increase the potential to cause disease is important to the design of better prophylactic and therapeutic strategies.

In the present study, the recovery of bacteria from the middle ear of mice infected with different combinations of bacteria was based on being able to culture them on agar. We found that the overall recovery of bacteria was dominated by *S. pneumoniae* followed by NTHi and *M. catarrhalis*. The enhanced recovery of *S. pneumoniae* in both the nasopharynx and the middle ear was significantly higher when co-infected with *M. catarrhalis* than with NTHi, in pre-virally infected mice. The data is consistent with the notion of respiratory viruses having a role in predisposing bacterial infection by facilitating nasopharyngeal colonisation of bacteria (Bakaletz *et al.* 1998; Gates 1999). It is possible that the poor recoveries of NTHI and *M. catarrhalis* are associated with culture as a method of measurement. It is known that standard culture techniques have limited capacity for the recovery of bacteria from the middle ear, for example are often non-culturable in aspirates from CSOM. Moreover, the formation of biofilms by *H. influenzae* and *S. pneumoniae* in the middle ear mucosa (Hall-Stoodley *et al.* 2006) may impede detection by culture techniques. The differences in the nasopharyngeal colonisation patterns in the polymicrobial infections composed of two or more bacteria suggest either changes to the colonisation architecture (such as biofilm formation) or competition dynamics. It is also possible that aspirates may not recover bacteria if they are inaccessible to the wash for example if they reside intracellularly. NTHi is known to readily invade respiratory epithelial cells as part of its defence mechanism (St. Geme & Falkow 1990). Whether or not the low yield of NTHi and *M. catarrhalis* in this study is

due to the formation of biofilms or some other host (e.g. neutralisation and killing) or microbial factor (e.g. intracellular residence, competition, killing) is the subject of ongoing work in our laboratory.

During OM, leukocytes and macrophages release pro-inflammatory cytokines including tumor necrosis factor and interleukin 1β (Maniscalco *et al.* 2007), which can induce iNOS via transcriptional factors such as nuclear factor κβ leading to the production of NO. Following the expression of iNOS, large amounts of NO are produced and known to persist as long as the enzyme is present in the cell or tissue. Bacterial components such as lipopolysaccharide are inducers of high levels of nitric oxide and other reactive oxygen species (ROS) in the middle ear (Jeon *et al.* 2006). Both nitric oxide and ROS interact to produce reactive nitrogen species such as N_2O_3 and peroxynitrite causing mucociliary dysfunction in the middle ear, which could feasibly promote bacterial infection. A concentration of NO as low as 100 parts per billion is considered sufficient for some sensitive bacteria (Maniscalco *et al.* 2007). It is believed that small amounts of NO are continuously produced by the middle ear mucosa which may assist in maintaining the sterile condition of the middle ear cavity (Morineau *et al.* 2001). Thus, the detection of NO is an indicator of inflammation and a contributing inflammatory mediator in the pathogenesis of OM. A recent study showed a high concentration of NO in the middle ear effusion of patients with mucoid OM and demonstrated the need to measure total NO (John *et al.* 2001). Nitric oxide contributes to the host response by increasing vascular permeability and neutrophil migration, as supported by the rapid and high white blood cell counts in this study. The recruitment of white blood cells to the site of an infection is another indicator of the inflammatory response. This study used both NO and white blood cell recruitment as measures of inflammation and may be a better indication of OM status.

The earlier peak in NO response observed in the middle ear following polymicrobial (double or triple combination) compared to single infection is indicative of complex bacteria-bacteria and bacteria-host interactions. Synergistic pro-inflammatory responses

following co-stimulation with a type b *H. influenzae* and *S. pneumoniae* have been observed *in vivo* (Ratner *et al.* 2005). According to Ratner *et al*. (Ratner *et al.* 2005), increases in the production of pro-inflammatory cytokines like macrophage inflammatory protein-2 (*in vivo*) were significant following co-infection. Although the *in vivo* study used a type b *H. influenzae* strain, similar increases were observed for interleukin-8 from respiratory epithelial cells *in vitro* with an NTHi strain. Therefore both studies provide evidence that bacterial combinations can amplify or alter the inflammatory responses in the host. Ongoing work in our laboratory will investigate in further detail of the nature of the inflammatory response in the polymicrobial OM model, and how different combinations of bacteria alter the host responses.

The recruitment of white cells to the middle ear was found to be high and rapid (day 1 post-infection) in the bacteria and bacteria + virus infected groups when compared to the virus only infected group. This suggests that the respiratory virus was not responsible for a measurable middle ear inflammatory response. The inflammatory response (recruitment of white blood cells and NO) was observed even in the absence of recoverable bacteria from the middle ear and there was no correlation between white blood cell numbers and bacteria recovered. This supports the clinical observations of detection of inflammation in acute OM infections in the absence of detectable bacteria and in CSOM where there is persistent inflammation yet the recovery of bacteria from the effusion is very limited and often non-recoverable. The observation that the numbers of white blood cells was significantly reduced by days 7 and 14 despite the presence of bacteria at these timepoints warrants further investigation. Clearly, measures of inflammation like NO and white blood cell recruitment may be used as a measure of the incidence of OM even in the absence of recoverable bacteria.

2.6 CONCLUSION

In conclusion, this is one of the first murine models to compare the complexity of the interactions of the three major bacteria involved in OM in the presence or absence of a respiratory virus infection. The study demonstrates that the presence of different

bacteria affects nasopharyngeal colonisation and the incidence of OM. Additionally, in both the nasopharynx and middle ear, the presence of *M. catarrhalis* with *S. pneumoniae* in the nasopharynx resulted in increases in *S. pneumoniae* infection. Both the co-presence of different bacteria in addition to a respiratory viral trigger significantly affects the incidence rate, duration of and bacterial load (severity) of OM. During a respiratory viral infection, the severity of middle ear infection was most significant following the co-infection of *S. pneumoniae* and *M. catarrhalis* and/or *S. pneumoniae* and NTHi. The study has also found that the presence of multiple bacteria significantly affected the inflammatory response in the middle ear even in the absence of recovery of viable bacteria from the middle ear. This model can be used to investigate some of the pathogen and host complexities associated with susceptibility to acute and chronic OM infections in children.

CHAPTER 3

Effect of bacterial concentration in the nasal inoculum on respiratory tract infections

3.1 ABSTRACT

Bacteria such as *S. pneumoniae*, NTHi, *M. catarrhalis* are part of the natural colonising flora of the nasopharynx, but under certain conditions, such as predisposing respiratory viral infection, are also the etiological agents of both lower and upper respiratory tract infections. Recent studies have suggested that the high density nasal bacterial carriage of these bacteria is linked to the incidence of bacterial infections and persistence of respiratory tract infections, including OM. This study aimed to investigate how a change in the concentration of the nasal bacterial inoculums affects the induction of respiratory tract infections and compare how a viral co-infection contributes to such differences in lung and middle ear infections using a BALB/c mouse experimental model. The study found that there was a difference in the ability of nasal inoculation to induce lung and middle ear infection. In particular, increasing the concentration of NTHi and *M. catarrhalis* did not result in increases in the recovery of bacteria from the lungs or bronchoalveolar lavage (BAL), however, there was a significant increase in NTHi recovery from the lung tissue in association with a viral infection. Intranasal inoculation with an increased concentration of *S. pneumoniae* both with and without respiratory viral infection did affect the amount of bacteria recovered in both the lung (particularly the BAL) and middle ear, supporting the dominance of this bacterium in this model. In contrast, the recruitment of white blood cells, as an indicator of the host's inflammatory response to infection, showed that there were consistently higher numbers of these cells in both the BAL and MEL associated with the higher concentration of nasal inoculums. There were also differences in the dominance cell types with PMNs dominating in the middle ear and macrophages in the BAL. The results of this chapter demonstrated that both the bacterial load and time post-inoculation were important in the recovery of *S. pneumoniae* from bronchial washes, and not in causing invasive lung infection. In contrast, the incidence of pneumococcal OM increased with the nasal bacterial load, presence of respiratory virus, and time post-inoculation.

3.2 INTRODUCTION

Infections of the lower respiratory tract, such as tuberculosis, pneumonia, exacerbations of chronic obstructive pulmonary disease (COPD), and influenza are included as leading infectious disease health problems that result in significant mortality and morbidity in humans. Annually, approximately four million deaths are reported due to LRTIs (Moellering 2002). LRTIs are caused by both bacterial and viral etiological agents with some of the most common agents being *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, Influenza A or B virus, parainfluenza virus, rhinovirus, RSV and adenovirus (Garbino *et al.* 2004; Kais *et al.* 2006). An epidemiological study in the United States reported that the highest incidence of pneumococcal disease is in both children less than 2 years of age and adults aged 65 years and above (Robinson *et al.* 2001). Of the 1162 hospitalised children in the Gambia, approximately 60% were diagnosed with pneumococcal pneumonia [reviewed by (Greenwood 1999)]. The incidence rate of pneumococcal pneumonia in developing countries, in children less than 5 years of age, is reported to be in the 100s to 1000s per year, and higher rates of mortality exist in countries such as sub-Saharan and south-east Asia (Cashat-Cruz *et al.* 2005; Rudan *et al.* 2008). The isolation of bacteria such as NTHi, *M. catarrhalis* and *S. pneumoniae* from COPD patients is well documented (Murphy *et al.* 2005b). Moreover, the occurrence of exacerbations in COPD patients is known to be associated with the acquisition of new strain of these bacteria (Sethi *et al.* 2002). A recent study in children aged 2 months to 24 months reported that the recovery of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* in approximately 23%, 18% and 17%, respectively, in a study cohort of 1819 AOM cases (Palmu *et al.* 2004). OM is one of the complicating consequences of the events in URTI and has caused significant concern in paediatric health care. Approximately 30% of infants have at least one episode of AOM within one year of age, increasing to approximately 80% by three years of age (Klein *et al.* 1990; Teele *et al.* 1989). In this context, nasopharyngeal commensal bacteria, such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, are etiological agents responsible for both LRTI and URTIs.

The incidence rate of AOM following an URTI in children was found to be approximately 36% during the age of 6-11 months, 29% during 12-23 months and 15% during 24-35 months of age (Revai *et al.* 2007). The association of OM with the nasal bacterial load was recently investigated (Smith-Vaughan *et al.* 2006). In the Smith-Vaughan study (2006), the pneumococcal and *M. catarrhalis* nasal load was significantly higher in the Aboriginal children when compared with non-Aboriginal children. In addition, it also predicted that the probability of suppurative OM increases with the pathogen load, and demonstrated a positive correlation between nasal bacterial load (when infected alone and during co-infection) and various representations of OM. In the context of LRTIs, *S. pneumoniae* has been the causative agent for bacterial pneumonia in approximately 44% of the 154 children (Michelow *et al.* 2004). In Australia, 6.8% of children under 14 years of age were likely to contract pneumonia, of which approximately 40% of these children were likely to require hospitalisation (MacIntyre *et al.* 2003). The relationship between bacterial colonisation, changes in the bacterial load and frequency of exacerbations in COPD along with the resulting inflammation has provided a greater understanding of the disease pathogenesis (Hurst *et al.* 2006; Hurst *et al.* 2005; Patel *et al.* 2002; Sethi *et al.* 2007; Tumkaya *et al.* 2007).

The immune response to respiratory tract infections involves both cellular and humoral components of the host's immune system. However, under immune compromised situations, recruited inflammatory cells control the infection [as reviewed by (Wagner & Roth 2000)]. Macrophages and polymorphonuclear cells (PMNs) are the phagocytic cells that respond to bacterial infections in both the ear and lungs. In the lung, alveolar macrophages are an important part of the innate defence system, providing both a surveillance function to keep the airways clear and responding to infectious agents, such as bacteria, as a function of the host's innate defences. The tympanic bulla of the middle ear is normally sterile and is not known to have the same resident surveillance macrophages as the lungs. These cells facilitate up-regulation of the host's innate immune responses by producing antimicrobial molecules and being a source of pro-inflammatory cytokines, such as TNF-α. In the lungs, this response from the alveolar

macrophages is important for the recruitment of the PMNs (Dockrell *et al.* 2003). The specific relationship between macrophages and PMNs in the middle ear has not been characterised.

Many studies involving bacterial colonisation and host innate immune responses have provided a greater understanding of the pathology associated with infections, microbe-host interactions and the role of the innate immune system in specific areas of the respiratory tract. This study aimed to compare the responses in the lungs and middle ear to *S. pneumoniae*, NTHi and *M. catarrhalis* in the nasal cavity and the effect of a respiratory viral infection on these responses. Since changes in colonisation load of bacteria have been suggested to be an important risk factor for OM in children, a three-fold increase in the bacterial inoculums was investigated to determine how shifts in the colonising load affected both middle ear and lung infections.

3.3 MATERIALS AND METHODS

3.3.1 Bacteria and virus nasal inoculations

The bacterial strains and their preparation along with the mouse respiratory virus (Sendai) used in this chapter have been previously described in Chapter 2 (Section 2.3.1). In order to investigate the effect of bacterial concentration on the incidence of both lung and middle ear infections, two different bacterial concentrations were used in this study. Based on the optical density at 405 nm, the starting concentration for each bacterium was 10^{10} CFU (1x) and 3 x 10^{10} CFU (3x). Ten µl (5µl per nare) of 10^{10} CFU (1x) and 3 x 10^{10} CFU (3x) each, were intranasally inoculated to the designated experimental groups. The resulting final nasal inoculation dose for each bacterium was 10^8 CFU/mouse (1x groups) and 3 x 10^8 CFU/mouse (3x groups). Half the animals in each group were also intranasally infected with 20 µl of 10^4 $TCID_{50}$ Sendai virus (10µl/nare).

3.3.2 Experimental groups

In this study, a total of 120 BALB/c mice were used. These were divided so that 50% of all groupings received the Sendai virus infection on day minus 3 and for sacrifice at two time points; day 1 and 3 post-bacterial nasal inoculation. There was also an equal distribution into the 1x group (10^8 CFU/mouse) and 3x group (3 x 10^8 CFU/mouse) for the two bacterial concentrations. In addition, 10 mice were infected with Sendai virus alone. Three days after the Sendai virus was inoculated in half the mice, all mice were divided into the following experimental groups with half the mice in each of the following groups also infected by the virus:

1) *M. catarrhalis* (n = 20/concentration/day)
2) NTHi (n = 20/concentration/day)
3) *S. pneumoniae* (n = 20/concentration/day)

3.3.4 Induction of infection

The procedure for the induction of infection has been described previously in Chapter 2 (Section 2.3.4). At day 1 and day 3 post-nasal inoculation with the bacteria, the mice were sacrificed using 0.2ml pentobarbital sodium (60mg/ml), injected intraperitoneally. The trachea was exposed through an incision in the neck for collection of the bronchoalveolar lavage (BAL) by instillation and recovery of 0.5 ml of sterile PBS. The thoracic cavity was opened for removal of the lungs which were placed into 2 ml of sterile PBS and homogenised using a tissue homogeniser (Heidolph DIAX 600, Electro GmbH & Co., Kelheim, Germany) at 9500 rpm. The middle ear fluid was collected by straightening the ear canal to visualise the tympanic membrane and instillation and recovery of 3 x 50 µl volumes of sterile PBS injected into the middle ear through the tympanic membrane and collected through a 26G needle attached to a 1ml syringe. The nasal lavage was performed by injecting and recovering 100µl of sterile PBS through each nare with a 26G needle attached to a 1ml syringe. The lung homogenate, BAL, middle ear lavage (MEL), and nasal washes from each mouse were plated onto the relevant media by titration of 10-fold serial dilutions. The bacterial colonies were further identified using gram-staining and morphological characteristics. The number of white

blood cells collected in the MEL and BAL were enumerated, followed by differential cell counting, as previously described in Chapter 2 (Section 2.3.4).

3.3.5 Statistical analysis

Analysis for the significance of the effect of the inoculum concentration on differences in the BAL, lung, nasopharyngeal and middle ear samples was analysed with a two-way ANOVA with a Bonferroni post-hoc comparison. All samples that had no bacterial colonies detected were designated to have a value of half the lowest limit of detection to enable statistical analysis of the data set for that group.

3.4 RESULTS

The incidence of LRTIs, nasopharyngeal colonisation and OM was based on the culture of recovered bacteria from the lung homogenate, BAL, nasal washes and MEL. The effect of the nasal bacterial inoculum concentration on recovery of bacteria in different compartments of the respiratory tract is shown in **Figure. 3.1**. The limit of detection is shown by the dotted line across each figure and groups that are represented below this line were assigned a baseline value at half the limit of detection for analysis purposes **(Figure. 3.1A-3.1D)**. The recovery of bacteria from each day following infection with the two different bacterial concentrations (1x and 3x) and in the presence and absence of the Sendai virus infection is shown in each graph. The significance of differences between groups is shown by connected lines in each graph.

3.4.1 Effect of the nasal inoculum concentration on lung infection

The effect of the bacterial load delivered intranasally on the level of infection within the lungs was measured by the recovery of bacteria from both the lung homogenates and BAL and compared also to the effect of a respiratory virus on lung infection. At day 3 post-infection, the recovery of *S. pneumoniae* in the 1x concentration group was significantly greater in the virus-infected group in comparison to its recovery in both

virus and non-virus infected 3x concentration groups ($p<0.01$ and $p<0.05$, respectively; **Figure. 3.1A**). In contrast, the day 1 results did not show significant differences in the amount of *S. pneumoniae* recovered at either concentration or was associated with the viral infection. In the BAL, however, there was a significant increase in the amount of *S. pneumoniae* recovered at day 1 in association with the increase in concentration of the bacteria which was observed in both the presence and absence of the viral infection ($p<0.001$ and $p<0.01$, respectively; **Figure. 3.1B**). By day 3, there was still a significantly higher level of *S. pneumoniae* in the BAL between the 1x concentration groups with and without viral infection ($p<0.01$) and the 1x and 3x concentration groups without viral infection ($p<0.01$) **(Figure. 3.1B)**. While there was no significant difference between the 1x and 3x concentration groups and the presence of a viral infection at day 3, both these groups had high levels of *S. pneumoniae* recovered in the BAL.

Overall in the context of the recovery of *S. pneumoniae*, the concentration of the bacteria delivered to the nasal cavity and the presence of a respiratory viral infection did not result in some difference in the induction of the lung infection. In addition, higher levels of *S. pneumoniae* were recovered from the BAL, rather than the lung tissue, suggesting that during the first 3 days of lung infection the bacteria tended to remain predominantly within the bronchoalveolar spaces. However, viral infection had resulted in a significant ($p<0.001$) increase in the bacteria recovered in the 1x group at day 3 with a similar trend in the 3x groups with and without virus, reflective of the higher concentrations of *S. pneumoniae* present in the BAL.

In contrast, NTHi infection in the lung resulted in a different pattern of infection to that observed for *S. pneumoniae*. At day 1 post-nasal inoculation the presence of the respiratory viral infection was associated with a significantly higher recovery of NTHi in the 1x concentration group which was also significantly higher than the 3x concentration group ($p<0.05$; **Figure. 3.1A**). However, the recovery of NTHi in the 3x concentration group in conjunction with the viral infection was not significantly

different from either the 1x group with virus or the 3x group without virus. It does appear that the respiratory virus did make a significant difference at the lower concentration, but that when the inoculum increased, this difference was no longer apparent. The lack of any difference and the lower levels of recovery of NTHi at day 3 are consistent with previous observations that NTHi does not persist in high numbers over time in the mouse lung infection models. In the BAL, the groups that had the viral co-infection, there were equivalent levels of NTHi recovered from both the 1x and 3x concentration groups at days 1 and 3, respectively, with day 3 recoveries lower than day 1. In contrast to the observation in the lung where the 1x and 3x group recoveries of NTHi in the absence of viral infection were equivalent at day 1 **(Figure. 3.1A)**, in the BAL, the recovery of NTHi from the 1x group was significantly higher than the 3x concentration group ($p<0.05$; **Figure. 3.1B**), with a similar trend between these groups observed in the mice euthanased at day 3, although this difference did not reach statistical significance possibly due to reaching the time limits for detecting any NTHi in the lungs in this model.

3.4.2 Effect of the nasal inoculum concentration on middle ear infection and nasal colonisation

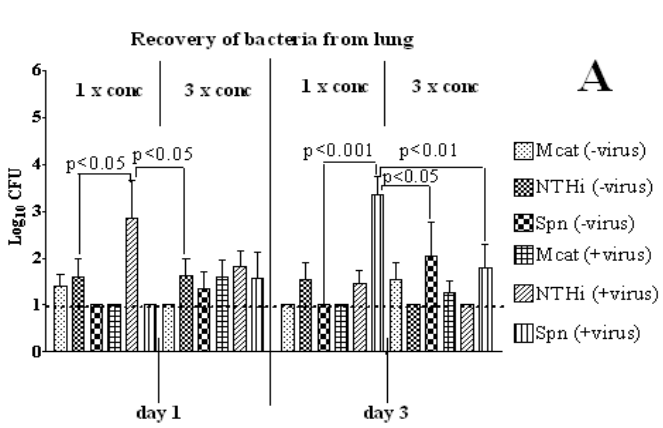
The recovery of bacteria from MEL and nasal washes following intranasal inoculation with a change in bacterial concentration from 1x to 3x the dose used in this model and the effect of concurrent respiratory viral infection on middle ear infection and nasal colonisation is shown in **Figure. 3.1C and 3.1D**, respectively. The recovery of *M. catarrhalis* and NTHi from the MEL was lower in comparison with *S. pneumoniae* at day 1 and was not detected at day 3. Thus increasing the concentration of the nasal inoculum did not have a significant effect on their recovery in this model. There were some increases in bacterial recovery at day 1 for the viral infection groups that was consistent with the observations in Chapter 2, but the three-fold increase in the nasal inoculums did not result in any significant differences between the relevant groups.

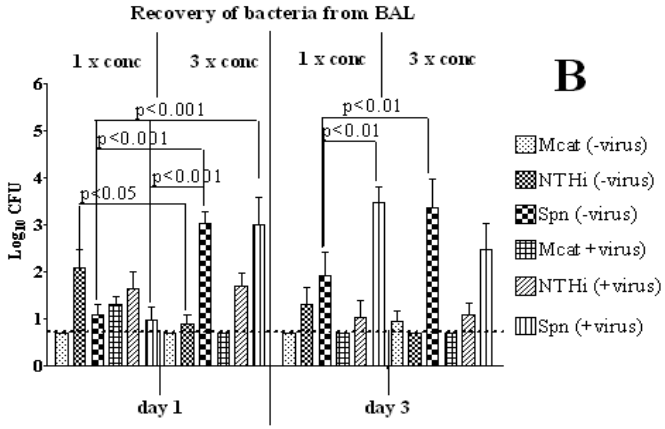
In contrast, the recovery of *S. pneumoniae* in the MEL was significantly affected by both the changes to concentration of the nasal inoculums, presence of viral infection and time post-inoculation **(Figure. 3.1C)**. In the absence of virus, there was significantly more *S. pneumoniae* recovered at day 1 ($p<0.05$) and day 3 ($p<0.001$) in the 3x concentration groups compared with that in the 1x concentration groups. Similarly, in the presence of the viral infection, the 3x concentration groups at days 1 ($p<0.01$) and 3 ($p<0.001$) had significantly higher levels of *S. pneumoniae* recovered in the MEL. The level of *S. pneumoniae* in the MEL on day 3 post-inoculation was higher in the respective groups when compared with day 1 **(Figure. 3.1C)**, indicating increasing infection loads over time in these groups. The recovery of *S. pneumoniae* in the 3x concentration group on day 3 was almost 10^5-fold higher than the 1x concentration group in the absence of a viral infection on day 1 ($p<0.001$). Overall the recovery of *S. pneumoniae* from MEL was higher when infected with higher bacterial concentration (3x), increased over time post-inoculation and as reported in Chapter 2, increased in the presence of a respiratory viral infection.

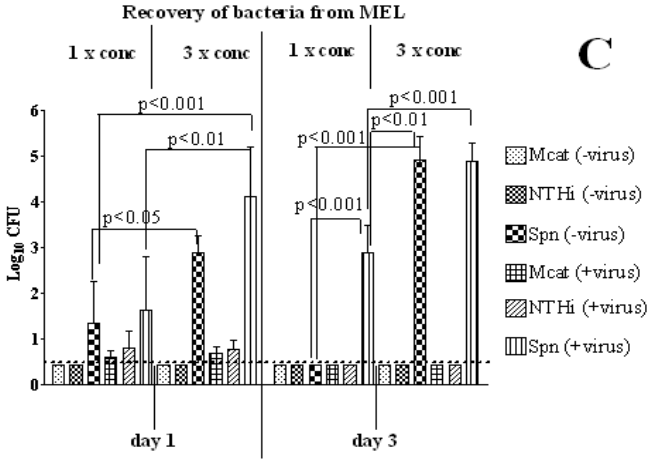
All bacteria were able to establish a consistent nasal colonisation at day 1 post-inoculation **(Figure. 3.1D)**, with levels of bacteria recovered being generally consistent between the respective groups at day 1 and 3. As found in chapter 2, the respiratory viral infection did not generally influence the bacterial levels in the nasal cavity. The exceptions in this study was a significant difference in the recovery of *S. pneumoniae* in the non-virus infected groups between the 1x and 3x concentration groups at day 1 ($p<0.05$). However, by day 3 there was no longer a significant difference between these two groups. At day 3 post-inoculation, the recovery of *M. catarrhalis* and NTHi tended to be much lower in the 3x groups than observed in the 1x groups, respectively, although neither reached statistical significance. However, for NTHi, the lower recovery at day 3 in the 3x group resulted in a significant difference with the 3x group in the presence of the viral infection ($p<0.01$) (right panel **Figure. 3.1D)**.

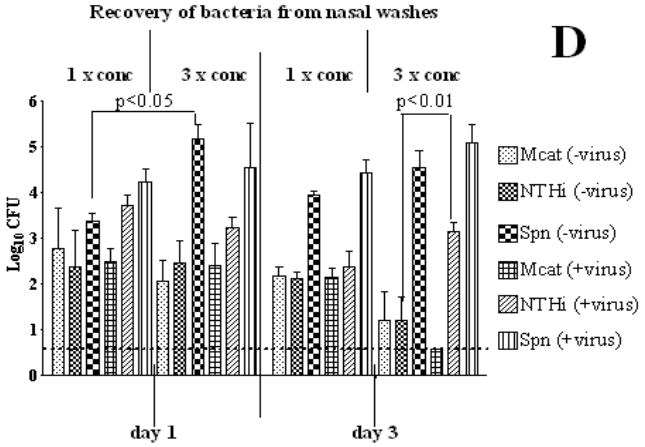
Figure. 3.1: Effect of changing the concentration of the nasal inoculum on bacterial respiratory tract infections. The bars represent the mean ± SEM of the bacteria recovered from A] lung, B] BAL, C] MEL and D] nasal washes following nasal inoculation with either 10^8 CFU/mouse (1x groups) or 3 x 10^8 CFU/mouse (3x groups) at day 1 and 3 days post-nasal inoculation of the bacteria. Co-infection with Sendai virus groups are indicated as +virus in the legend. The data is expressed as the mean ± SEM for n=5 mice per group and the statistical significance of differences between relevant groups is shown by connected lines in each group with *p*-values as indicated.

Effect of changing the concentration of the nasal inoculum on bacterial respiratory tract infections









3.4.3 Total white blood cell counts in MEL and BAL

The numbers of white blood cells enumerated from the MEL and BAL on day 1 and day 3 post-nasal inoculations can also be used to determine if the change in concentration of the nasal inoculums resulted in differences in inflammatory responses. The total numbers of white blood cells in the MEL and BAL in the 1x concentration groups were consistently lower than from the higher 3x concentration groups **(Figure. 3.2A-3.2D)**. The white blood cell numbers in the MEL at day 1 in the 3x groups for NTHi and *M. catarrhalis* were relatively high in both the non-viral and viral infection groups, even though it was only the viral infection groups where measurable levels of bacteria were recovered in the MEL. This would suggest that despite the lack of recoverable bacteria in these mice, the increase in the concentration of the nasal inoculums had affected the inflammatory responses in the middle ear. For mice with *S. pneumoniae* recovered from the middle ear, the difference in white blood cell numbers between the 1x and 3x groups (non-viral groups) at day 3 correlated with the differences in bacterial recovery in the MEL, whereas the low number of white blood cells in 1x group with viral co-infection group does not correlate with the significant bacterial recovery from the middle ear. The differential blood cell counts in the MEL revealed that polymorphonuclear neutrophils (PMNs) were the predominant cell types followed by macrophages at both days post-infection irrespective of the treatment group **(Figure. 3.3A)**.

The numbers of white blood cells enumerated from BAL were higher in the 3x groups when compared with 1x groups **(Figure. 3.2C-3.2D)**. The 1x concentration groups did not differ from the number of cells lavaged from the Sendai virus infection alone group which had very low counts (data shown with dotted line; **Figure. 3.2C-3.2D**). The exception was the NTHi 1x group with viral co-infection at day 1**(Figure. 3.2C)**. The higher number of white blood cells in the BAL for this group corresponds with the higher bacterial recovery from the lung **(Figure. 3.1A)**, but not the numbers of bacteria in the BAL **(Figure. 3.1B)**, suggesting the induction of an inflammatory response associated with bacterial invasion into the lung tissue. The white blood cell numbers in the BAL on day 3 were low in all 1x groups even though the numbers of *S. pneumoniae*

recovered in the BAL were significant and higher in the viral group **(Figure. 3.1B)**. The numbers of bacteria recovered in the 1x group with viral infection did not differ significantly from the bacterial numbers recovered in the 3x concentration groups (with and without viral infection; **Figure. 3.1B**), even though the 1x group had much lower white blood cell numbers. Different to the MEL, in all BAL collected following bacterial infection at the different bacterial concentrations, macrophages were the dominant cell type followed by PMNs **(Figure. 3.3B)**.

Figure. 3.2: Total white blood cell counts in the MEL and BAL. White blood cell numbers recovered in the MEL at day 1 A] and day 3 B], and BAL at day 1 C] and day 3 D]. The bars represent the mean ± SEM for n=5 mice/group at each time point with the indicated bacterium at either 10^8 CFU/mouse (1x groups) or 3 x 10^8 CFU/mouse (3x groups). The dotted line represents the white blood cell numbers recovered from a control groups with or without viral infection only.

Total white blood cell counts in the MEL and BAL

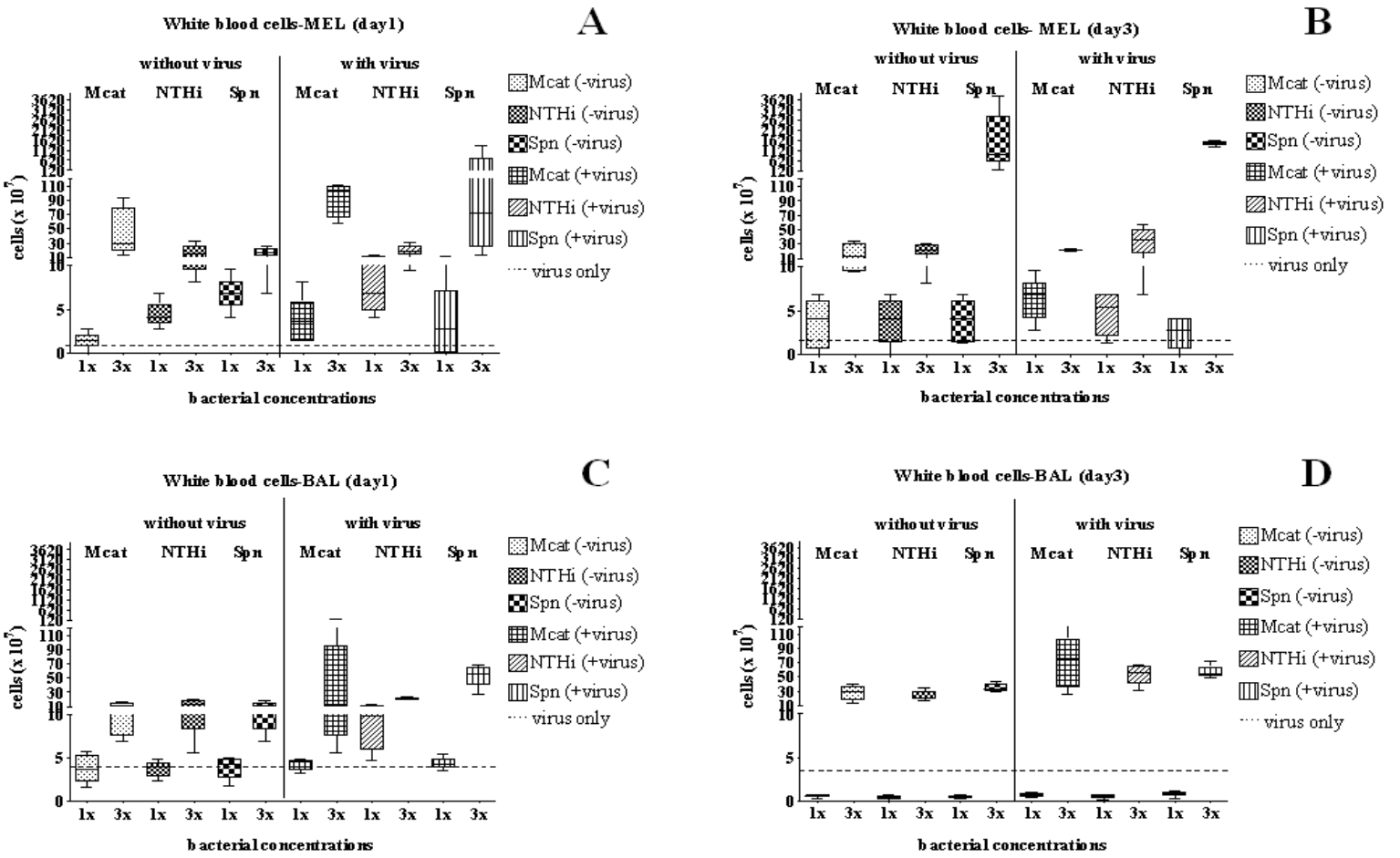
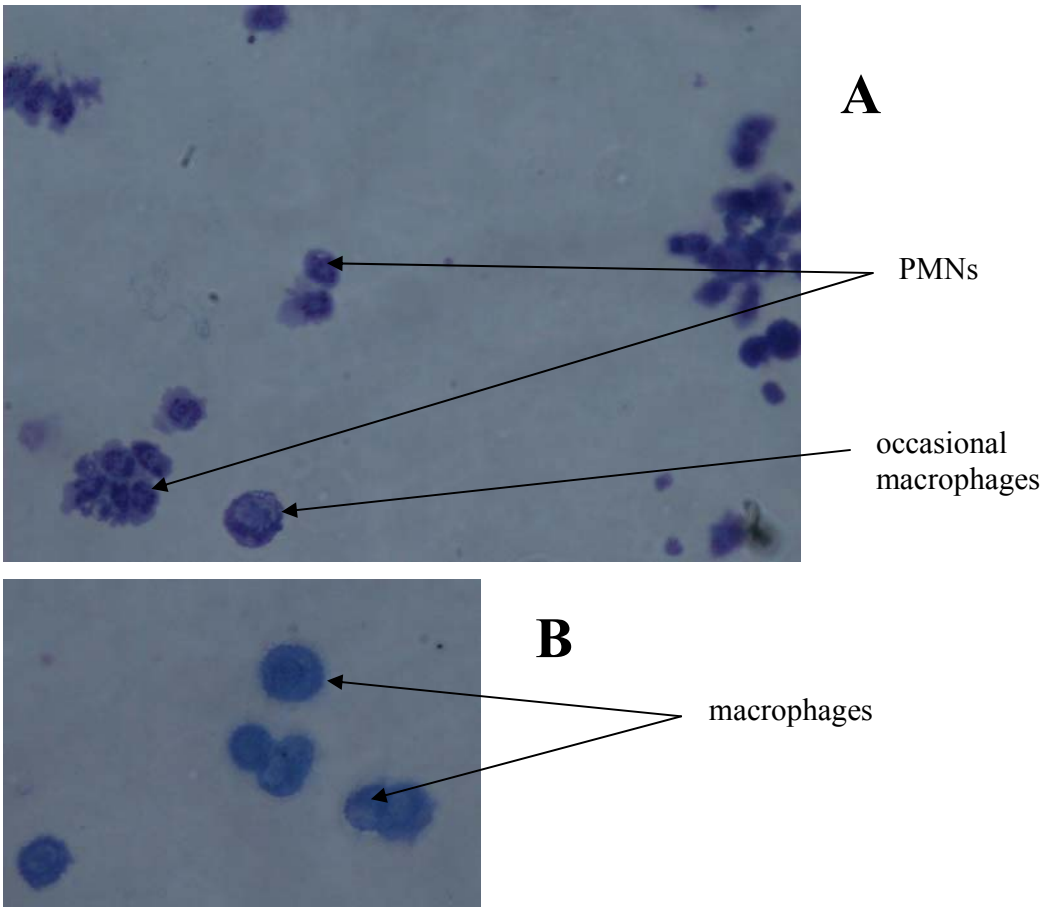


Figure. 3.3: Presence of PMNs in MEL and macrophages in BAL. The figure is from stained cytospin of A] MEL showing the presence of predominant PMNs and an occasional macrophage (as indicated by arrows), and B] BAL showing the presence of predominant macrophages (as indicated by arrows).



3.5 DISCUSSION

The nasopharyngeal commensals such as *M. catarrhalis*, NTHi and *S. pneumoniae* are commonly involved in causing both upper and lower respiratory tract infections. The previous chapter of this thesis found there was an increase in the incidence of OM that was associated with nasal colonisation and through a complex interaction between these different bacteria, was also affected by a respiratory viral infection in an investigation using an experimental BALB/c mouse model. This finding addressed the situation

associated with observing bacterial interactions at one concentration only. In context with OM, there is a positive correlation between the nasal bacterial load (when infected alone and in combinations) and the disease state in the ear. In addition, the high density bacterial carriage was predicted to be responsible for the persistence of the disease (Smith-Vaughan *et al.* 2006). Whereas in LRTI, such as exacerbations in COPD, changes in the bacterial load in the lower airways was shown to be an unlikely cause of these exacerbations (Sethi *et al.* 2007). In contrast, this study has investigated how a change in the nasal colonisation load for the different bacteria affects both upper and lower respiratory tract infections. Inhalation and nasal aspiration techniques are commonly used methods for inducing bacterial lung infections, but have been less commonly used to investigate middle ear infections.

In this study, changing the bacterial concentration resulted in some distinct differences in the induction of LRTI, in particular, those caused by *S. pneumoniae* and NTHi. In addition, the incidence of OM and the patterns of nasal colonisation also revealed a number of differences, in particular reinforcing the dominance of *S. pneumoniae* in this model of OM. Assessing the white blood cell recruitment as an indicator of an inflammatory response to infection revealed evidence of the incidence of infection, sometimes in the absence of recovery of significant concentrations of bacteria from both the lungs and middle ears. There was also a difference in the predominance of the white cell types recruited in the MEL and BAL, with PMNs and macrophages differently dominating, respectively.

There are a few theories suggesting mechanisms by which bacteria may gain access to the middle ear via the ET. The ascension of the bacteria from the nasopharynx could be possible due to aspiration of nasopharyngeal secretions through the ET upon equalising pressure between the nasopharynx and the tympanum, and through a compromised ET itself (Miyamoto & Bakaletz 1997). A recent review has suggested induction of inflammation at the opening of ET in the nasopharynx after intranasal inoculation, and inflammatory responses along with the action of respiratory viruses on respiratory

epithelium to be responsible for progression of OM. However, the mechanisms of actual spread of any microbial infection from the nasal cavity into the middle ear have not been studied extensively (Sabirov & Metzger 2008). Respiratory viruses are known to predispose the host to subsequent bacterial infections in the respiratory tract, facilitating enhanced adherence and translocation through the epithelial barrier [reviewed by (Hament *et al.* 1999)]. This mechanism would suggest that the bacteria upon colonising the nasopharynx in association with a viral infection of the respiratory epithelium, would likely trigger middle ear and lower respiratory tract infections.

In this study, intranasal inoculation of bacteria with different bacterial concentrations resulted in some distinct differences in the induction of LRTI, in particular, those caused by *S. pneumoniae* and NTHi. The recovery of *S. pneumoniae* was different in both BAL and lung tissue. The difference could be due to the response of the innate immune system in clearing the bacteria and allowing fewer bacteria to invade the lung tissue. The lower recovery of *S. pneumoniae* from the lung tissue on day 3 post-infection with the higher inoculum could be due to the combined action of the alveolar macrophages and PMNs. Alveolar macrophages are the predominant macrophages in the lung, constituting approximately 93% of the pulmonary macrophage population (Marriott & Dockrell 2007). The ability of the macrophages to clear the lower inoculum of bacteria without recruiting inflammatory cells has been observed elsewhere (Marriott & Dockrell 2007). However, when the bacterial numbers are overwhelmingly large, as seen in chronic infections, the inflammatory cells, particularly, neutrophils are recruited and together with macrophages clear the invading bacteria (Dockrell *et al.* 2003; Knapp *et al.* 2003). In addition, the influx of macrophages in response to the infection could also trigger the release of cytokines, such as TNF-α, initiating recruitment of PMNs to facilitate enhanced clearance of the bacteria.

In this study, the pre-viral infection enhanced NTHi adherence especially on day 1 when infected with the lower bacterial concentration. However at both day 1 and day 3 post-infection, NTHi was recovered from the lung tissue to a lesser level when infected at the

higher bacterial concentration both in presence and absence of the respiratory virus. This suggests that the ability of respiratory virus to predispose the host to bacterial infection might be masked by the presence of higher bacterial inoculum and does not enhance the infection by facilitating NTHi adherence. Moreover, NTHi is an exclusively human pathogen, and hence rapidly cleared from the lung and BAL within 24 post-infection by the normal innate immune responses in the mice (Clarke 2008; Murphy 1996). In addition, a previous study has reported the rapid clearance and lower recovery of NTHi in the presence of Sendai virus in rats (Clarke 2008). The Clarke study (2008) also suggested that the recruitment and activation of phagocytic cells along with increased levels of TNF-α and IL-6 proteins in these rats (involved with activations of neutrophils) might be responsible for the enhanced clearance of NTHi.

The clinical relevance of the nasal bacterial load has been previously shown to increase the risk of OM (Smith-Vaughan *et al.* 2006). The Smith-Vaughan study (2006) also suggested that the high density of nasal bacterial carriage could explain the persistence of OM in certain high-risk communities. The results of this chapter showed that by increasing the bacterial concentration, the incidence of nasal colonisation by *S. pneumoniae* significantly increased on day 1 post-inoculation. In this context with pneumococcal OM, the importance of a pre-viral infection and time post-inoculation (duration) (Chapter 2) has also been demonstrated in this chapter. In addition, the results of this chapter also showed that the incidence of pneumococcal OM was dose dependant.

Certain clinical studies have previously addressed the issue of lower airway bacterial load and its effect on exacerbations of COPD in adults (Sethi *et al.* 2002; Sethi *et al.* 2007). Although these studies have demonstrated the relative risk of an exacerbation in association with the acquisition of a new bacterial strain, the changes in the bacterial load were not found to be associated with the exacerbations in COPD. In contrast, this study demonstrated the increase in the recovery of *S. pneumoniae* from the BAL in the presence of the higher bacterial inoculum. However, this relationship could not be

observed in the lung tissue. It has been demonstrated that there is a linear relationship between bacterial load and airway inflammation in patients with chronic bronchitis, however no relationship was observed with lung infection and the bacterial load (Adam *et al.* 2000). The Adam study (2000) used inflammatory markers such as IL-8, leukotriene B4 and leukocyte elastase activity from sputum samples to assess airway inflammation. This chapter has shown a linear relationship between bacterial load and total white blood cells in both MEL and BAL, supporting the use of inflammatory responses as markers of the effect of an infection.

In this study, large numbers of macrophages in the bronchial washes were evident, followed by PMNs. The recruitment of white blood cells following increased bacterial load were higher than during infection with the lower bacterial concentration. This indicates changes to the host's innate response against increasing bacterial load in facilitating bacterial clearance (Marriott & Dockrell 2007). The route of infection could also play a role in the recruitment of white blood cells to the bronchoalveolar spaces. An intratracheal bacterial inoculation as opposed to an intranasal inoculation used in this chapter resulted in a dominant influx of macrophages during the first few hours of infection and that was rapidly exceeded by the numbers of PMNs during the course of infection (Foxwell *et al.* 1998; Kyd *et al.* 2000). In addition, the total white blood cells recruited in the bronchi and the middle ear due to Sendai virus infection alone was much lower than during bacterial infection.

Although the number of white blood cells recovered from both BAL and MEL increased in response to higher bacterial inoculation, it did not relate to the bacterial levels. This suggests the involvement of other factors other than recruitment of cells to the site of infection. The presence of the white blood cells recruited to the middle ear is an important observation that supports the clinical findings where inflammation has been detected in AOM infections in the absence of detectable bacteria and in chronic suppurative OM where there is persistent inflammation, yet, the recovery of bacteria from the effusion is very limited and often non-recoverable.

3.6 CONCLUSION

In conclusion, this study demonstrated the effect of different nasal bacterial loads on the incidence of respiratory tract infections. The bacterial load and time post-inoculation were important in the recovery of *S. pneumoniae* from bronchial washes, however was not significant in causing invasive lung infection. In contrast, the bacterial load, presence of respiratory virus, and time post-inoculation were all significant in causing pneumococcal OM. In addition, this study has shown that there is a difference in the type of white blood cell associated with clearing the bacteria from different areas of the respiratory tract.

CHAPTER 4

The effect of sequential nasal bacterial inoculation on the incidence rate of OM

4.1 ABSTRACT

The polymicrobial etiology of OM, involving the concurrent presence of various respiratory virus and bacteria has been well documented as a significant factor to the high incidence of the disease in infants and young children. It is also well known that early bacterial colonisation of the nasopharynx is associated with early onset of OM, and high nasopharyngeal carriage rates have been identified as increasing the risk of recurrent OM in young children. Previous research has also established a positive association between specific virus and bacteria, and between different bacterial combinations, providing an insight into the possibility that certain colonising pattern of bacteria also contributes to an infection. The role of acquisition of a new bacterium in the nasopharynx and its effect on the incidence of OM in presence of an already existing coloniser has not been experimentally assessed in animal models. This study has investigated whether a sequential nasal bacterial inoculation affects the incidence of OM and how a respiratory viral infection impacts on sequential colonisation and OM. In this study, half the BALB/c mice were infected with the Sendai virus prior to nasal inoculation of the bacteria. The first bacterium was nasally inoculated 2 days after the viral infection and the second bacterial species was inoculated a further 24 hours later. There were six sequential combinations comprising combinations of *S. pneumoniae*, NTHi or *M. catarrhalis* as either the first or second bacterium. The results of this study have shown that sequential nasal bacterial inoculation did not significantly alter the nasal colonisation patterns and that the respiratory viral infection had no effect, either. In contrast, in the absence of viral infection, the sequential combinations involving *S. pneumoniae* and *M. catarrhalis* resulted in a significant increase in the amount of *S. pneumoniae* recovered from the middle ear than the *S. pneumoniae* and NTHi combinations ($p<0.05$). Additionally, the viral infection increased the bacterial recovery of *S. pneumoniae* in all combinations, but there was a significant increase in the combination involving *M. catarrhalis* as the first bacterium compared with *M. catarrhalis* as the second bacterium ($p<0.05$). In conclusion, although nasopharyngeal colonisation of bacteria was unaffected by the sequential infection, pneumococcal OM

was enhanced by both the respiratory viral infection and the order of nasal colonisation with *M. catarrhalis*.

4.2 INTRODUCTION

Bacterial OM is one of the most common childhood diseases that is of significant health concern and cost to paediatric health care. The polymicrobial etiology of OM involving the concurrent presence of various respiratory viruses and bacteria within the nasopharynx and the middle ear is well known. Amongst the nasopharyngeal commensal bacteria, *S. pneumoniae,* NTHi and *M. catarrhalis* are predominantly responsible for causing bacterial OM (Heikkinen *et al.* 1999; Klein 2000). The high incidence rate of OM in infants and young children as mentioned in the previous chapters is a significant cause of visits to the doctor and contributor to health costs (Alsarraf *et al.* 1999). Studies have documented the relationship between bacterial colonisation of the nasopharynx at an early age and the onset of OM, and the correlation of this with increased risk of recurrent OM (Faden *et al.* 1997). The nasopharynx is normally colonised by bacteria such as *S. viridians*, Diphtheroids, nonhaemolytic *Streptococci*, along with *S. pneumoniae*, NTHi and *M. catarrhalis* (Garcia-Rodriguez & Martinez 2002). During upper respiratory illness and OM, bacterial colonisation with bacteria such as *S. pneumoniae*, NTHi and *M. catarrhalis* increases significantly (Faden *et al.* 1991). The nasopharyngeal secretions containing bacteria that spread between individuals and these may potentially become pathogens. Therefore, nasopharyngeal carriage is important with regards to the development of the disease and spread of the pathogens.

The mean age of first acquisition of *S. pneumoniae* and/or *H. influenzae* and/or *M. catarrhalis* is as early as 6 months of age [reviewed in (Garcia-Rodriguez & Martinez 2002)], and in certain high risk population it can be as early as 8-10 days (Leach *et al.* 1994). Some children are often colonised by different types of bacteria during the first year of life, while others are colonised with different strains of the same bacteria (Faden 2001). Although there have been studies related to the bacterial colonisation and

incidence of OM, nasopharyngeal carriage rates, there is not much information regarding sequential infection with different bacteria and the incidence of OM. In Australian Aboriginal children, the median age for nasopharyngeal colonisation with any bacteria involved in OM has been reported to be less than 20 days of age, as opposed to about 270 days in non-Aboriginal children, thereby increasing the risk of recurrent OM due to high bacterial nasopharyngeal carriage rates (Leach *et al.* 1994). The nasopharyngeal carriage rates of *S. pneumoniae, M. catarrhalis* and NTHi in Aboriginal children have been reported as 49%, 50% and 41%, respectively, compared with 25%, 25% and 11%, respectively, in non-Aboriginal children (Watson *et al.* 2006). By as early as 2 months of age *S. pneumoniae* and *M. catarrhalis* had been isolated from 37% and 36% of Aboriginal children and only from 11% and 12% of non-Aboriginal children, respectively (Watson *et al.* 2006). The impact of OM in Australian Aboriginal children has been significant with this group amongst the world's most susceptible ethnic groups. A recent study has found that of the 1300 children studied, only 10% had normal ears, 42% had OM with effusion and 15% had chronic middle ear infections (Morris *et al.* 2007). In addition to the well established positive association between virus and bacteria, analysis of colonisation patterns has shown significant positive association between pairs of bacteria, especially, *M. catarrhalis* with *S. pneumoniae* and *H. influenzae* (Jacoby *et al.* 2007). A similar association between *M. catarrhalis* and *S. pneumoniae* was also observed in the polymicrobial OM infection model that showed enhanced incidence of pneumococcal OM due to the presence of *M. catarrhalis* (Chapter 2).

Although there have been studies related to the bacterial colonisation, the incidence of OM, and nasopharyngeal carriage rates, there is little information regarding the contribution of sequential infection with different bacteria to the incidence of OM. The observations in Chapter 2 that there was a high inflammatory response in the middle ear (as observed by nitric oxide levels in the middle ear) on day 3 post-infection with single colonising bacterium, raised the question about the effect of an acquisition of a new bacterium in the nasopharynx on the incidence of OM in presence of an already existing

response to a different bacterium. This chapter describes the investigation of sequential nasal bacterial inoculation on both nasal colonisation and bacterial recovery in the middle ear 24 hr after the final nasal bacterial inoculation.

4.3 MATERIALS AND METHODS

4.3.1 Bacteria and virus nasal inoculations

The bacterial strains and their preparation along with the mouse respiratory virus (Sendai) used in this study have been previously described in Chapter 2 (Section 2.3.1). Based on the optical density at 405 nm, the starting concentration for each bacterium was 2 x10^{10} CFU/ml, for *M. catarrhalis* and NTHi, respectively, and 1 x10^{10} CFU/ml for *S. pneumoniae*. Ten µl (5 µl per nare) from this concentration was intranasally inoculated into the designated experimental cohorts under anaesthesia. The resulting infectious dose for each bacterium was 10^8 CFU/mouse. Briefly, half the animals in each group were intranasally infected with Sendai virus under anaesthesia. Two days after inducing the viral infection (day minus 1), the animals were inoculated with either 10µl (5µl per nare) of *S. pneumoniae* or NTHi or *M. catarrhalis*, intranasally under anesthesia. The following day (day 0), the *S. pneumoniae* group was further infected with 10µl (5µl per nare) of either NTHi or *M. catarrhalis*, the NTHi group with 10µl (5µl per nare) of either *S. pneumoniae* or *M. catarrhalis*; and the *M. catarrhalis* group with 10µl (5µl per nare) of either NTHi or *S. pneumoniae*, intranasally under anesthesia. Day 0 in this model has been designated as the day that corresponds to the final nasal inoculation and begins the day count for the post inoculation infection analyses.

4.3.2 Experimental groups

In this study, a total of 60 BALB/c mice were used in these experiments. Half the animals in each group were intranasally infected with Sendai virus. Two days after Sendai virus infection, all the animals were intranasally infected with the first bacterium. The following day, all animals were intranasally infected with the second bacterium. The experimental groups were:

1) *S. pneumoniae* followed by *M. catarrhalis* (n=10)
2) *S. pneumoniae* followed by NTHi (n=10)
3) *M. catarrhalis* followed by NTHi (n=10)
4) *M. catarrhalis* followed by *S. pneumoniae* (n=10)
5) NTHi followed by *S. pneumoniae* (n=10)
6) NTHi followed by *M. catarrhalis* (n=10)

4.3.3 Assessment of infection

Twenty four hours (24 hr) after the final bacterial inoculation, all animals were sacrificed by intraperitoneal injection of 0.2 ml pentobarbital sodium (60mg/ml)/mouse. The nasal washes and the MEL were performed using sterile PBS, as previously mentioned in Chapter 2 (Section 2.3.4). Bacterial culture was performed on both the MEL and nasal washes from each mouse by titrating 10-fold serial dilutions onto appropriate culture media. The bacterial colonies were further identified using gram-staining and morphological characteristics. In addition, total white blood cell counts and differential cell counts were performed on MEL using methylene blue, and Diff-Quik® staining kit (Dade Behring Inc., USA), respectively.

4.3.4 Statistical analysis

The analysis for significance of the effect of sequential infection on the incidence of nasopharyngeal colonisation and the middle ear infection was analysed using a two-way ANOVA with a Bonferroni post hoc comparison. The nonparametric significance of the effect of sequential infection on the incidence of the pneumococcal OM caused by infection was compared using the Mann Whitney U-test.

4.4 RESULTS

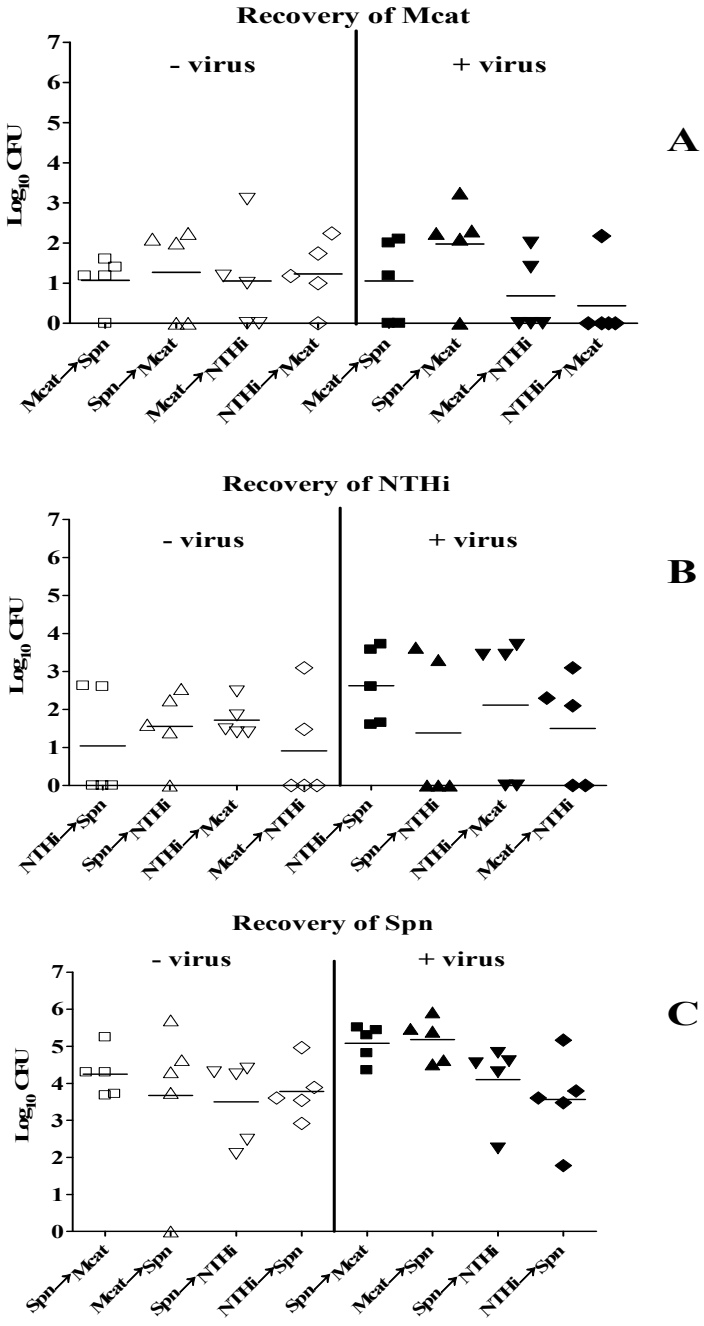
4.4.1 Bacterial recovery from the nasal washes

The recovery of bacteria from the nasal washes following intranasal sequential infection is shown in **Figure. 4.1A-4.1C**. The bacterial recovery was not significantly affected by

the sequential infection as all bacteria were recovered equivalently from the nasal washes irrespective of when the mouse received that bacterium. The recovery of *M. catarrhalis* was lower than NTHi which also tended to be lower than *S. pneumoniae* **(Figure. 4.1A-4.1C)**. There was a higher recovery of *S. pneumoniae* in all combinations and irrespective of whether *S. pneumoniae* was infected first or second **(Figure. 4.1C)**. The recovery of *S. pneumoniae* in combination with NTHi tended to be similar in both viral and non-viral groups, whereas the combination of *S. pneumoniae* with *M. catarrhalis* tended to be higher for *S. pneumoniae* in the virus-infected groups **(Figure. 4.1C)**.

Figure 4.1: Bacterial recovery in nasal lavage. The results of sequential bacterial inoculation on nasal colonisation in non-virus infected (open symbols) and virus-infected (closed symbols) mice (n=5). The bars represent mean $\log_{10}$ CFU of the recovery of A] *M. catarrhalis*, B] NTHi, and, C] *S. pneumoniae*, for the indicated groups. The arrow points from the first bacterium to the second infecting bacterium; for example: Mcat→Spn = Mcat followed by Spn.

Effect of the sequential infection on nasopharyngeal colonisation

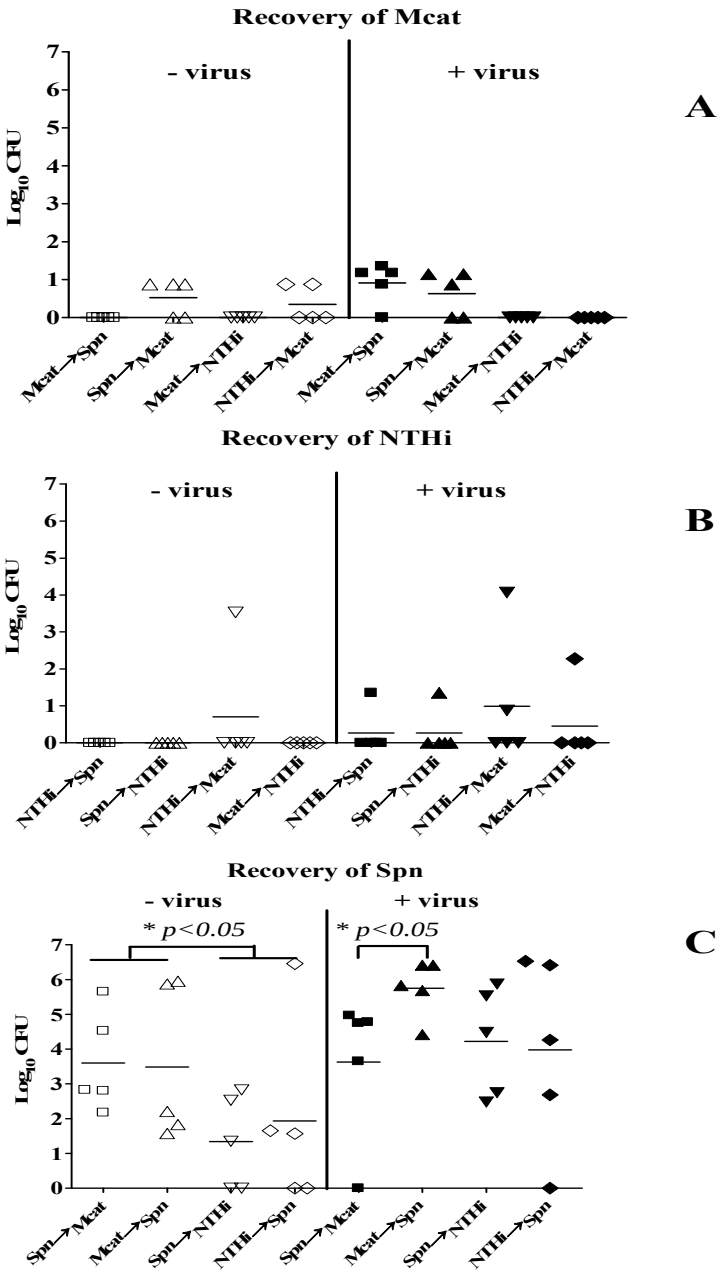


4.4.2 Bacterial recovery from the middle ear lavage

The incidence of middle ear infection based on the recovered bacteria and associated with sequential bacterial nasal inoculation is shown in **Figure. 4.2A-4.2C**. Overall, the

recovery of bacteria from the MEL was dominated by *S. pneumoniae* followed by *M. catarrhalis* then NTHi. The recovery of *M. catarrhalis* and NTHi in all combinations was very low irrespective of the order of infection **(Figure. 4.2A-4.2B)**, however, in many cases the bacteria were recovered in a co-infection with *S. pneumoniae*. The recovery of *S. pneumoniae* in the virus-infected group was significantly enhanced following nasal inoculation with *M. catarrhalis* (*p<0.05) **(Figure. 4.2C)** compared to *M. catarrhalis* as the second bacterium. In addition, the recovery of *S. pneumoniae* in the non-virus infected group in the presence of *M. catarrhalis* (in any order) was significantly greater than *S. pneumoniae* in combination with NTHi (in any order) (*p<0.05).

Figure 4.2: Bacterial recovery in the middle ear lavage. The results of sequential bacterial nasal inoculation of bacteria on inducing middle ear infection in non-virus infected (open symbols) and virus-infected (closed symbols) mice (n=5). The bars represent mean $\log_{10}$ CFU of the recovery of A] *M. catarrhalis*, B] NTHi, and, C] *S. pneumoniae*, for the indicated groups. The arrow points from the first bacterium to the second infecting bacterium; for example: Mcat→Spn = Mcat followed by Spn. *p<0.05).



4.4.3 Total white blood cell counts

The infiltration of the total white blood cells into the middle ear following sequential intranasal bacterial inoculation in non-virus and virus infected BALB/c mice is shown in **Figure. 4.3**. The total number of white blood cells in the MEL was slightly higher in the pre-viral infection group compared to the non-virus infected groups. In a number of

mice there were insufficient cells to yield an accurate enumeration of the differential count suitable for statistical purposes; however PMNs were consistently the dominant cell type **(Figure. 4.4)**.

Figure 4.3: Total white blood cell counts in MEL. The results show the effect of the sequential bacterial infection on the recruitment of total white blood cells in the MEL. The bars represent the mean ± SEM of the total white blood cells enumerated from MEL. The arrow points from the first bacterium to the second infecting bacterium; for example: Mcat→Spn = Mcat followed by Spn.

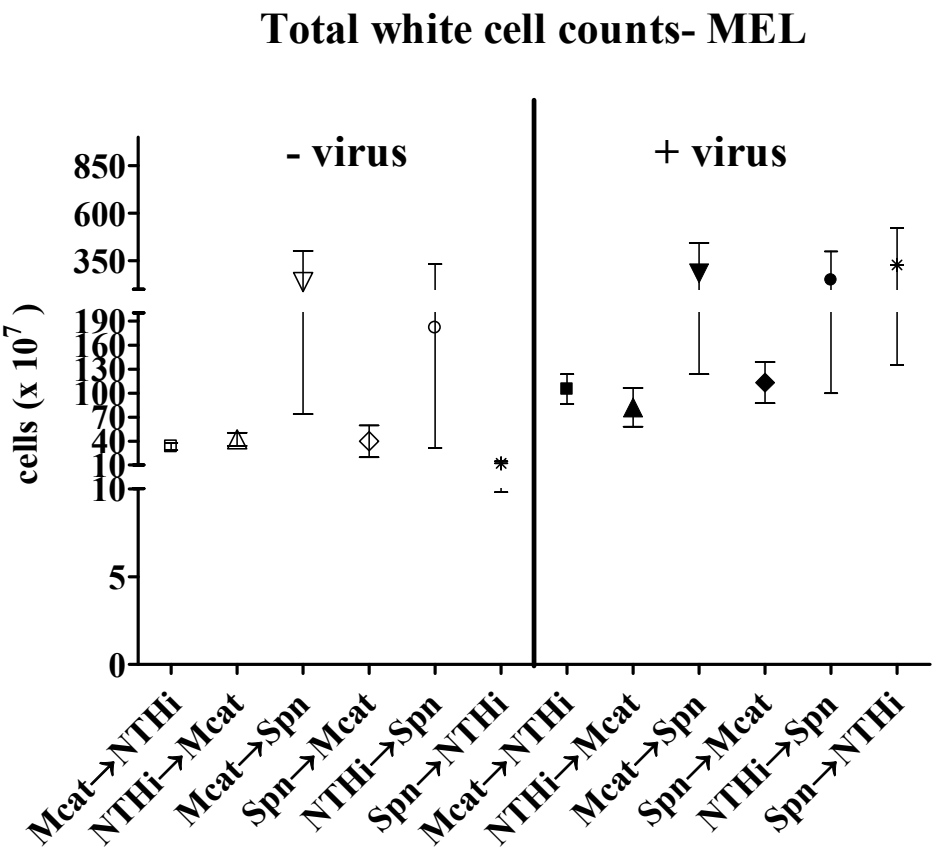
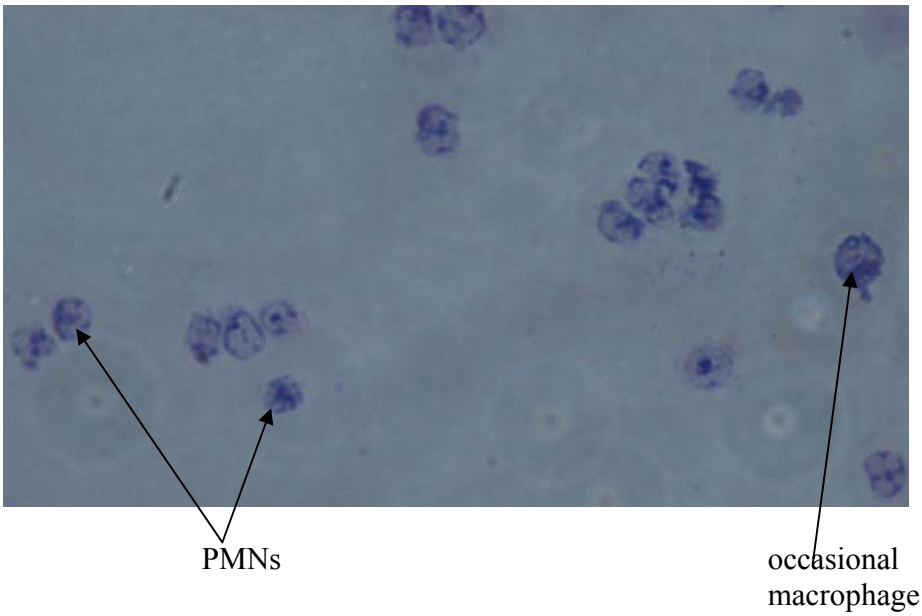


Figure 4.4: PMNs in the MEL. The figure is from a stained cytospin of MEL and shows the presence of PMNs and a macrophage (as indicated by arrows) in the MEL.



4.5 DISCUSSION

The presence of *M. catarrhalis* as a colonising agent in the nasopharynx was found to have significantly contributed to enhance pneumococcal OM (Chapter 2). In addition, a viral infection resulted in increased bacterial (*S. pneumoniae*) numbers and correlated with a greater incidence rate of pneumococcal OM supporting the hypothesis that both bacterial and viral interactions contribute to OM (Chapters 2 and 3). These significant findings led to further exploration of the significance of the order of bacterial colonisation on the incidence rate of OM. The effect of the presence of one bacterial species on colonisation by a second bacterium has not been addressed in this context. Moreover, once established in the nasopharynx, how the carriage of the initial strain is affected by the acquisition of a second bacterial strain has also been not been studied experimentally to confirm the role in developing OM. Colonisation is a dynamic process with regular acquisition and loss of many bacterial strains.

The modeling of clinical data has revealed significant associations between bacteria and viruses, and bacterial interactions with each other and the host (Jacoby *et al.* 2007). The data in the Jacoby study (2007) supports the previous findings indicating viral infection can predispose an individual to bacterial carriage. In addition, it also demonstrated negative associations between bacteria such as *S. aureus* and both *M. catarrhalis* and *H. influenzae*. This could suggest the possibility of bacterial competition in the URT during colonisation. This negative association has also been reported in a clinical study, especially between *S. aureus* and different *S. pneumoniae* serotypes that are covered by the pneumococcal conjugate vaccine (Regev-Yochay *et al.* 2004) This study implied that this could indicate a potential for replacement by bacteria that negatively compete in addition to the identified pneumococcal serotype replacement observed post-immunisation with the conjugate vaccine (Veenhoven *et al.* 2003).

The association between different bacteria along with serotype and species replacement shown clinically has also been demonstrated using experimental *in vivo* animal models. The presence of different serotypes of the same bacterial species and their effect on the nasopharyngeal carriage was demonstrated in mouse studies (Lipsitch *et al.* 2000). In the Lipsitch study (2000), intranasal carriage of one pneumococcal strain was shown to inhibit the acquisition of a second strain in a dose dependent manner. In addition, acquisition of a second strain did not affect the colonisation of the resident strain. The mechanism of *in vivo* inhibition between the colonising strains of *S. pneumoniae* and the new acquisition was postulated to be a biological mechanism and might provide further understanding of the serotype replacements observed clinically in children aged 1-7 years (Mbelle *et al.* 1999; Obaro *et al.* 1996; Veenhoven *et al.* 2003). There has been an increase in the non-vaccine pneumococcal serotypes in children following the introduction of the pneumococcal conjugate vaccine (Pichichero & Casey 2007) and all these observations speculate upon the involvement and existence of complex bacterial interactions in the nasopharynx and bacterial carriage. This can be extended to speculation on the relationships in these dynamic interactions that could lead to the initiation of an infection.

In this study, the effect of the order of nasal inoculation of the different bacteria on the incidence of OM was investigated. Although the recovery of bacteria from the nasal washes was dominated by *S. pneumoniae* followed by NTHi and *M. catarrhalis*, the order of nasal inoculation did not significantly affect the quantity of bacteria recovered in the nasal lavage. There were some interesting trends that did not reach statistical significance, such as the higher quantity of *S. pneumoniae* in combination with *M. catarrhalis* and viral infection. This coincided with a higher recovery of *S. pneumoniae* in the MEL in the group where *M. catarrhalis* preceded *S. pneumoniae*, although higher *S. pneumoniae* numbers were recovered in all viral infection combinations. This study differed to the Lipsitch study (2000) which used different serotypes of the same bacterial species and demonstrated the inhibitory effect of the resident strain on the acquisition of a secondary challenge strain, and showed that colonisation of the resident strain was unaffected by the acquisition of the secondary strain (Lipsitch *et al.* 2000). The Lipsitch study (2000) demonstrated the ability of different pneumococcal strains to compete and establish nasopharyngeal colonisation. In contrast, this study has shown that competitive nasal colonisation between these three bacterial species does not appear to have any negative associations when sequentially inoculated, at least within one day of being inoculated with the second bacterium. The quantity of bacteria recovered did not differ from those recovered at 24 hours following concurrent nasal inoculation with the bacterial combinations (Chapter 2).

The incidence rate of pneumococcal OM was higher in the *M. catarrhalis* combinations as opposed to the NTHi groups. This difference did not occur when in association with the viral infection, except there was a significant difference between the reciprocal orders of *S. pneumoniae* and *M. catarrhalis*. These findings are consistent with the high nasopharyngeal carriage rates of *S. pneumoniae* and *M. catarrhalis* in Australian Aboriginal children less than 2 months of age (Watson *et al.* 2006), thereby, this specific combination may be a major contributor to the increased risk of recurrent OM. Moreover the ability of *M. catarrhalis* and to a lesser extent NTHi, to enhance pneumococcal OM shown in this chapter also supports the previously known positive

association between these bacteria in children less than 2 years of age (Jacoby *et al.* 2007). This association was also observed in the co-infection studies in Chapter 2.

Many studies have demonstrated that a respiratory virus predisposes the host to a secondary bacterial infection and in animal models these are generally only investigated for infection with a single bacterium and virus (Bakaletz 1995; Suzuki & Bakaletz 1994). The co-presence of different bacteria in addition to a respiratory viral trigger significantly affected the incidence rate and severity of OM (Chapter 2). However, in this study, the recovery of bacteria following sequential inoculation was almost similar in both, virus-infected and non-virus infected groups. A possible explanation for this conflicting observation between the studies could be due to different mechanisms of colonisation by these bacteria when establishing themselves in the nasal cavity.

The recruitment of white blood cells following an infection is a well known host response that is indicative of the activation of the cascade of inflammatory processes. The middle ear inflammation, as seen by the recruitment of the total white blood cells in this study, indicates an inflammatory response to each of the combinations. The middle ear is normally a sterile site, so the presence of white blood cells in the lavage is indicative of the presence of an infectious agent having reached the middle ear cavity. While there was a distribution of the number of white blood cells in some of the groups, these differences were not significant and were virus independent. The recruitment of white blood cells did not directly or inversely correlate with the amount of bacteria recovered in the MEL, although in both the non-virus and virus-infected groups the combinations of *M. catarrhalis* followed by *S. pneumoniae* and NTHi followed by *S. pneumoniae* tended to have higher numbers of white blood cells. Synergistic pro-inflammatory responses following co-stimulation with a type b *H. influenzae* and *S. pneumoniae* have been observed *in vivo* (Ratner *et al.* 2005). According to the Ratner study (2005), increases in the production of pro-inflammatory cytokines like macrophage inflammatory protein-2 (*in vivo*) were significant following co-infection. Although the *in vivo* study by Ratner *et al.* used a type b *H. influenzae* strain, similar

increases were observed for interleukin-8 from respiratory epithelial cells *in vitro* with an NTHi strain. Therefore, both *in vivo* and *in vitro* studies provided evidence that bacterial combinations can amplify or alter the inflammatory responses in the host.

4.6 CONCLUSION

This study investigated the effects of sequential intranasal bacterial inoculation on the incidence rate of OM. In conclusion, this study showed increased pneumococcal OM due to the presence of a pre-coloniser (*M. catarrhalis*) in the nasal cavity. In addition, it also showed that nasal colonisation was unaffected by sequential bacterial infection or a respiratory viral infection.

CHAPTER 5

Real-time monitoring of disease progression: a novel approach to assess the incidence rate of otitis media in a polymicrobial environment

5.1 ABSTRACT

The high incidence of bacterial OM in infants and young children along with the early onset of nasopharyngeal bacterial colonisation is well known. Moreover, early nasopharyngeal colonisation with multiple strains of bacteria has been associated with increased risk of chronic and recurrent OM. Assessment of the incidence of OM in the previous studies relied on assessment of lavage samples, middle ear washes and invasive techniques. Although these studies have provided an understanding of the pathogenesis and impact of OM, the bacteriology, especially with concurrent different bacterial species residing within nasal and middle ear tissues, could be underestimated. More recently, bioluminescent imaging technology has provided promising results in cellular and molecular research, especially with regards to whole-body imaging, monitoring of drug efficacy, bacterial biofilm and gene expression *in vivo*. This technology has been adapted recently in the field of middle ear research to monitor disease progression using a single bacterium. In this study, the dynamics of polybacterial nasopharyngeal colonisation patterns and localisation of bacteria within the nasopharynx and the middle ear were investigated using bioluminescent NTHi and *S. pneumoniae* in BALB/c mice pre-inoculated with adenovirus. In addition, this study also investigated whether sequential acquisition of a new bacterium affected colonization of this bacterium in the nasopharynx and induction of OM. This study has found that there are differences in the colonising patterns of bacteria within the nasopharynx and it appears that *S. pneumoniae* and NTHi localise in different niches within the nasal cavity when present singly and in a polybacterial situation. Furthermore, it also showed that the extent and intensity of *S. pneumoniae* colonisation of the nasopharynx was greater when in combination with NTHi and *M. catarrhalis*.

5.2 INTRODUCTION

Over the last two decades, the use of various animal models in the understanding of OM has been previously mentioned in certain reviews (Bakaletz 2004; Lim *et al.* 2002). Most of these studies have considered individual bacteria or bacterial-viral relationships

and in general ignored the complexity of the polymicrobial nature of nasopharyngeal colonisation on OM. Moreover, these animal studies, including those in this laboratory, rely on interpretation of the incidence of OM and other infections by analysis of lavage samples, middle ear washes and nasal washes involving invasive techniques. There is now sufficient evidence, including results presented in the previous chapters, that the use of bacteriology by culture could be an underestimation as a measure of OM. This especially does not enable analysis of the complexities in colonisation and infection dynamics associated with the co-presence of different bacteria residing in the nasal and the middle ear tissues.

Bioluminescence is a process in which a visible light is emitted in living organisms such as bacteria, fish, fireflies and algae with the help of an enzyme, luciferase. The luciferase in the luminescent system (*lux*) along with the genes involved have been isolated from bacteria such as *Vibrio*, *Photobacterium* genera and *Xenorhadbus* genera (Meighen 1993). This phenomenon has been adapted for bioluminescent imaging technology and its applications have shown promising potential in the field of cellular and molecular research. It has been successfully applied in *in vivo* whole-body imaging of pathogens, tumors and gene expression patterns in living animals (Contag 2002). The monitoring of disease processes in living animals along with drug efficacy testing was first demonstrated in a mouse model using *Salmonella entrica* serovar Typhimurium (Contag *et al.* 1995). This was further adapted and proved to be a valuable tool in monitoring bacterial biofilm, effectiveness of antibiotics in a chronic biofilm infection and lung infection models using bioluminescent *S. aureus*, *P. aeruginosa* and *S. pneumoniae* (Francis *et al.* 2001; Kadurugamuwa *et al.* 2003). More recently, continuous monitoring of infection and the disease processes within the nasopharynx, ET and the middle ear with bioluminescent NTHi in an experimental OM animal model has added a new dimension to middle ear research (Novotny *et al.* 2005).

This thesis has already reported the enhancement in the incidence of pneumococcal OM associated with the presence of *M. catarrhalis* in a polymicrobial environment in the

nasopharynx (Chapter 2). The subsequent chapters reported studies that investigated how bacterial concentration and the order of bacterial acquisition in the nasal cavity affect the incidence of pneumococcal OM (Chapters 3 and 4 respectively). The significance of these findings led to questions associated with the dynamics of polymicrobial nasopharyngeal colonisation patterns and localisation of bacteria within the nasopharynx and the middle ear. In addition this study has aimed to determine how the presence of a new bacterium affected the pre-existing nasal coloniser and induction of OM. In Chapter 4, sequential bacterial infection and its effect on the incidence of OM were investigated using conventional culture methods of lavage samples. Although this study and other studies in this thesis have provided an insight to the complex bacterial interactions involved in OM, the results of assessment of the inflammatory responses suggests that bacterial culture may have underestimated the extent of bacterial OM. Therefore, this study aimed to investigate nasopharyngeal colonisation patterns associated with the different bacteria when nasally inoculated alone or in combination. In addition, this study aimed to investigate the effect of the acquisition of a new bacterium in the nasopharynx on the incidence rate of OM when there was a pre-existing coloniser within the nasopharynx. To investigate these questions real time monitoring of the progression of colonisation along the nasopharynx and infection into the middle ear of luminescent bacteria was used. This approach allowed visualisation of the distribution of the bacteria in a manner that was not possible using conventional culture methods.

5.3 MATERIALS AND METHODS

5.3.1 Bacteria and virus preparation

The bacterial strains included in this experiment were *M. catarrhalis* 1857, (paediatric middle ear effusion clinical isolate from The Children's Hospital, Columbus, Ohio, USA), NTHi 86-028NP/pKMLN-1 (expressing luciferase, engineered by Ms Laura Novotny and Dr Kevin Mason, The Nationwide Children's Hospital, Columbus, Ohio, USA) and NTHi 86-028NP (paediatric clinical isolate from The Children's Hospital, Columbus, Ohio, USA) and *S. pneumoniae* serotype 19F, expressing luciferase (kindly

provided by Dr Jonathan McCullers, St. Jude Children's Research Hospital, Memphis, Tennessee, USA). *M. catarrhalis* and both strains of NTHi were grown on BBL™ Chocolate II Agar (Becton Dickinson). The luciferase expressing *S. pneumoniae* was grown on Trypticase™ Soy Agar supplemented with 5% sheep blood (Becton Dickinson) and containing 50 μg/ml of kanamycin. The bacteria were incubated overnight at 37^0 C with 5% CO_2 and the bacterial concentration adjusted following harvesting by centrifugation and resuspending in sterile saline (0.9%). Adenovirus serotype 1, (paediatric clinical isolate from The Children's Hospital, Columbus, Ohio, USA) was used to pre-infect the BALB/c mice in this study. The preparation of adenovirus has been previously described (Bakaletz *et al.* 1993; Suzuki & Bakaletz 1994).

5.3.2 Bacterial and viral inocula concentrations used

Based on the optical density at 405 nm, the starting concentrations of bacteria used to infect the BALB/c mice, intranasally under anaesthesia, using a mixture of ketamine 100mg/kg and xylazine 10mg/kg in sterile water, were: *S. pneumoniae* serotype 19F (10^9 CFU/ml), NTHi 86-028NP/pKMLN-1(10^{10} CFU/ml), NTHi 86-028NP (10^{10} CFU/ml), and *M. catarrhalis* 1857 (10^{10} CFU/ml). The luminescence for *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1 was confirmed with a luminometer, and the bacterial inoculum was also confirmed for luminescence pre- and post-inoculation. In addition, the total viable cell count for each bacterium was confirmed by a colony count on appropriate culture media. Following the bacterial preparation, the designated cohorts were intranasally inoculated with *S. pneumoniae* 19F (10^6 CFU/nare/mouse), NTHi 86-028NP/pKMLN-1 (10^7 CFU/nare/mouse), NTHi 86-028NP (10^7 CFU/nare/mouse) or *M. catarrhalis* 1857 (10^7 CFU/nare/mouse). For double and triple bacterial combinations, equal volumes of each bacterial suspension were mixed and used for the intranasal inoculation. The starting concentration of adenovirus serotype 1 was 6 x 10^6 $TCID_{50}$ in minimum essential medium (BioWhittaker, Walkersville, USA). The resulting infectious dose of adenovirus serotype 1 was 10^5 $TCID_{50}$ per animal with the inoculum being divided equally between the nares.

5.3.3 Experimental groups

Specific pathogen free male BALB/c mice aged 6-8 weeks were used in this study. A total of 40 mice including the controls (virus-only infected group) were used. All the animals were intranasally pre-inoculated with adenovirus serotype 1 (10^5 $TCID_{50}$, 10 µl per nare/mouse), under anaesthesia using ketamine/xylazine. Cohorts 1-5 were used to understand the nasal cavity colonisation patterns and assess the subsequent incidence and duration of occurrence of OM associated with ascension of the luminescent NTHi 86-028NP/pKMLN-1 or *S. pneumoniae* 19F to the middle ear when nasally inoculated alone or in combination. Cohorts 6 and 7 were used to investigate the effect of sequential bacterial infection on the incidence of OM. In addition, a virus-only inoculated group was used as a control group.

Control group

Virus-only inoculated group (n=5): This group was used as a control and inoculated with adenovirus alone.

Single bacterium and concurrent bacterial combination groups

All the animals were intranasally inoculated with adenovirus on day minus 3 (10^5 $TCID_{50}$, 10 µl per nare/mouse), under anaesthesia. Three days after the viral infection, all animals were intranasally inoculated with the designated bacteria or bacterial combinations, under anaesthesia. The experimental cohorts were:

1) Cohort 1-luminescent *S. pneumoniae* serotype 19F (n=5)
2) Cohort 2- luminescent NTHi 86-028NP/pKMLN-1(n=5)
3) Cohort 3- luminescent NTHi 86-028NP/pKMLN-1 + *M. catarrhalis* 1857 (n=5)
4) Cohort 4- luminescent *S. pneumoniae* serotype 19F + NTHi 86-028NP (n=5)
5) Cohort 5- luminescent *S. pneumoniae* serotype 19F + NTHi 86-028NP + *M. catarrhalis* 1857 (n=5)

Sequential bacterial inoculation groups

Cohorts 6 and 7 were used to investigate how sequential nasal colonisation events might affect both the colonisation niche of the newly acquired bacterium and induction of OM. All the animals in these cohorts were intranasally pre-inoculated with adenovirus serotype 1 (10^5 $TCID_{50}$, 10 µl per nare/mouse), under anaesthesia. Three days after the viral inoculation, all animals were intranasally inoculated with the first bacterium. Three days after the bacterial infection, the animals were then intranasally infected with the second bacterium. The experimental cohorts were:

Cohort 6- *M. catarrhalis* 1857 followed 3 days later by luminescent *S. pneumoniae* serotype 19F (n=5)

Cohort 7- *M. catarrhalis* 1857 followed 3 days later by luminescent NTHi 86-028NP/pKMLN-1 (n=5)

5.3.4 Induction of infection

These experiments were performed in the Children's Research Institute at the Nationwide Children's Hospital, Columbus, Ohio, USA, and all work was approved by their animal ethics committee. Three days after the viral infection, mice in cohorts 1-5 were intranasally inoculated with 10 µl (5 µl per nare) of the appropriate bacteria or bacterial combination and this was designated as day 0 for subsequent analyses. The localisation of luminescent *S. pneumoniae* 19F or NTHi 86-028NP/pKMLN-1 was assessed daily following inoculation of the bacteria (day 0) from day 1 till day 7 by biophotonic imaging of anaesthetised mice using the Xenogen *in vivo* imaging system. On day 7 post-infection, 3 mice from each cohort were sacrificed using Pentobarbitol (200 µl intraperitoneal injection), imaged, and bacteriology was performed on nasal washes, bullae washes, and tissue homogenates such as nasopharyngeal tissues, left and right middle ear mucosal epithelium.

To investigate the effect of sequential nasal bacterial inoculation on the incidence of OM, adenovirus-infected BALB/c mice in cohorts 6 and 7 were inoculated with 10 µl (5 µl per nare) of *M. catarrhalis* 1857. Three days after this initial *M. catarrhalis* infection,

cohorts 6 and 7 received a secondary challenge of 10 µl (5 µl per nare) of *S. pneumoniae* 19F or NTHi 86-028NP/pKMLN-1, respectively. Twenty-four hours after this secondary intranasal bacterial inoculation, cohorts 6 and 7 were imaged to record the luminescence expressed by *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1. The localisation of luminescent *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1 was assessed daily thereafter until 96 hours. Following 96 hour post-inoculation with *S. pneumoniae* 19F or NTHi 86-028NP/pKMLN-1, 3 mice from each cohort were sacrificed using Pentobarbitol (200 µl intraperitoneal injection), imaged, and bacteriology was performed on the samples mentioned above.

The samples and the tissues (before homogenisation) from all cohorts were imaged and then serially diluted for determining the colony count. The tissues were weighed before homogenisation **(Appendix B.8)** and the recovery of bacteria was expressed as CFU/mg of tissue. The skulls from all the animals were split opened and the nasal cavity and bullae were imaged. The bacteriology was not performed on the remaining 2 mice in each cohort, which were used for imaging and have been kept for future histology studies.

All the mice from the negative control (virus-only inoculated) group were also imaged.

5.4 RESULTS

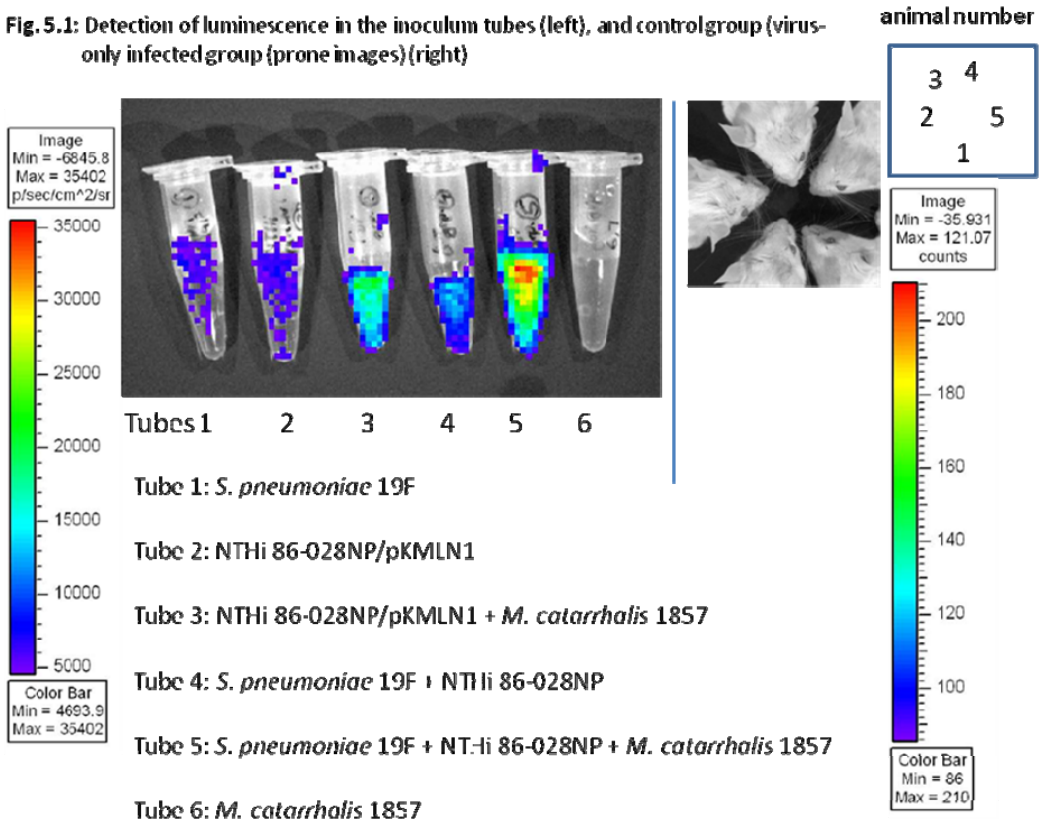
The results of this study are based on measurement of the luminescence from NTHi 86-028NP/pKMLN-1 and *S. pneumoniae* 19F. The bacterial luminescence in the inocula used for the different groups (imaged before infecting the mice) and images of the virus-only inoculated group is shown in **Figure. 5.1**. The colonisation patterns of *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1 when infected alone and in combinations with either NTHi 86-028NP (non-luminescent) and/or *M. catarrhalis* 1857 are visualised with the relative luminescent signal intensities, and are shown in **Figure. 5.2-5.9**. The effect of sequential bacterial inoculation on the colonising patterns of luminescent *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1 in BALB/c mice

pre-infected with *M. catarrhalis* are shown in **Figure. 5.10-5.12**. In the top-right corner of the figures there is a legend showing the orientation of the individual animal positions while imaging and the colour bars indicate the scale for the relative luminescent signal intensities. The left lateral, right lateral and the images of various lavage and homogenates are shown in the Appendix section of the thesis **(Appendix B.1-B.7)**.

5.4.1 Measurement of luminescence in the inoculums

The inoculum vials were imaged just before infecting the animals and their relative luminescence along with image of the virus-only infected group is also shown in **Figure. 5.1**. The level of luminescence was consistent with results for this concentration of bacteria. Vials 3-5 appeared to have luminescence at a higher intensity even though the specific luminescent bacterium in these vials was at the same concentration as in tubes 1 or 2. The difference is that these bacteria are mixed with other non-luminescent bacteria, as indicated. It is not known whether this influences expression of the *luciferase* gene. No background luminescence was detected in the virus-only control mouse cohort over the duration of the experiment.

Figure 5.1: Detection of luminescence in the inoculum tubes and assessment of the virus-only infected group. Each tube is numbered and the contents are described in the legend. The mice were the virus only group and the legend in the top-right corner of the figure shows the orientation of the individual mice keep constant throughout the experiment for imaging assessment. The colour bars indicate the relative luminescent signal intensities.



5.4.2 Colonising patterns of bacteria when infected alone or in combination

5.4.2.1 Colonising patterns of *S. pneumoniae*

The detection of luminescent *S. pneumoniae* within the pharynx when inoculated alone is shown in **Figure. 5.2**. Luminescent *S. pneumoniae* were visible within the anterior nares and pharynx in 2/5 mice at day 1 post-infection. Over a period of time, luminescence was detected in other mice with the pattern of detection changing, for

example, mouse 3 had distinctive luminescence patterns on days 2-4, whereas mouse 5 had a different pattern detected on days 1 and 5 only. Although the luminescence signal was low within the pharynx **(Figure. 5.2)**, stronger signals were seen in the nasal cavities **(Figure. 5.3A)**. This could suggest that *S. pneumoniae* was able to colonise within the nasal tissues and localise within certain niches in the nasal cavity. A low level of luminescence was also detected in the left bulla of animal 3. The level of luminescence in the nasal cavity upon excision was significantly greater once exposed than could be detected by whole body imaging. All mice displayed some degree of luminescence, with animals 3 and 5 showing the most intense readings.

Figure 5.2: Cohort 1- Detection of luminescent *S. pneumoniae*. This figure shows the luminescent *S. pneumoniae* 19F cells within the pharynx when inoculated alone. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. The day represents the time point post nasal inoculation with the luminescent bacterium (day 0).

Fig. 5.2: Cohort 1-Detection of luminescent *S. pneumoniae* 19F over 7 day period (prone images)

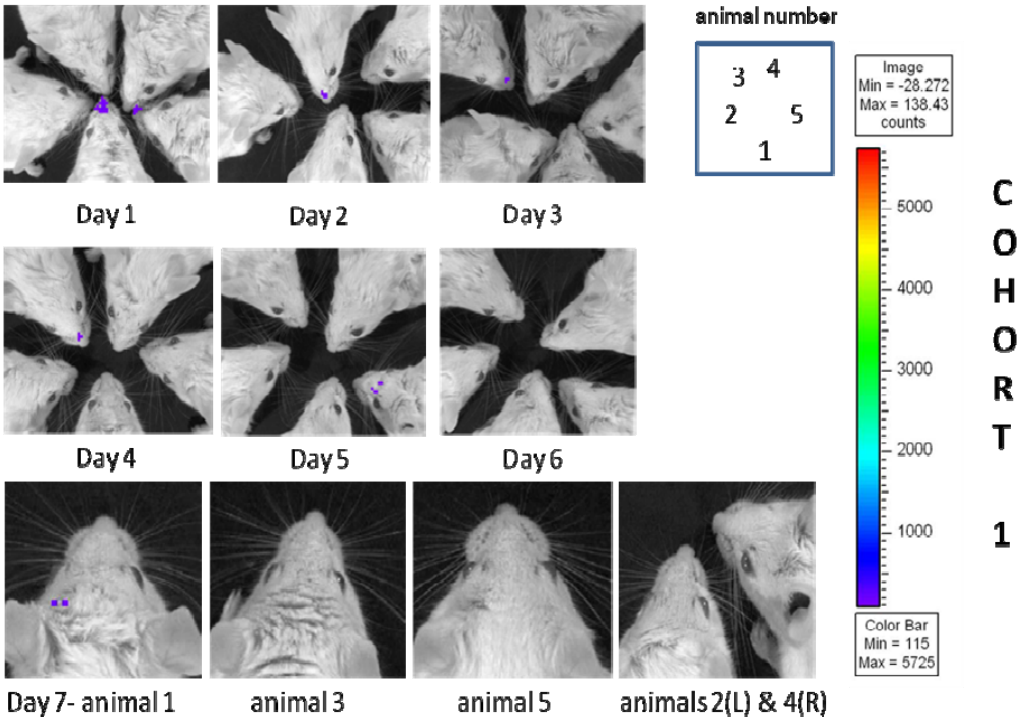
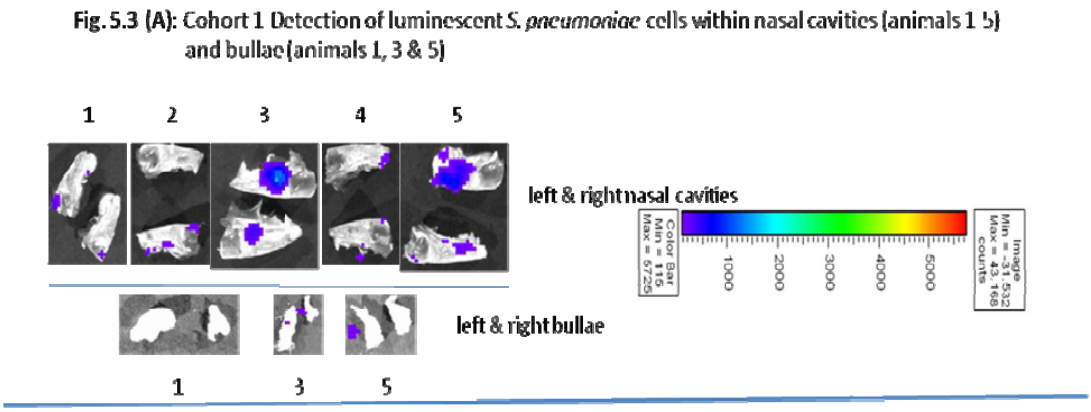
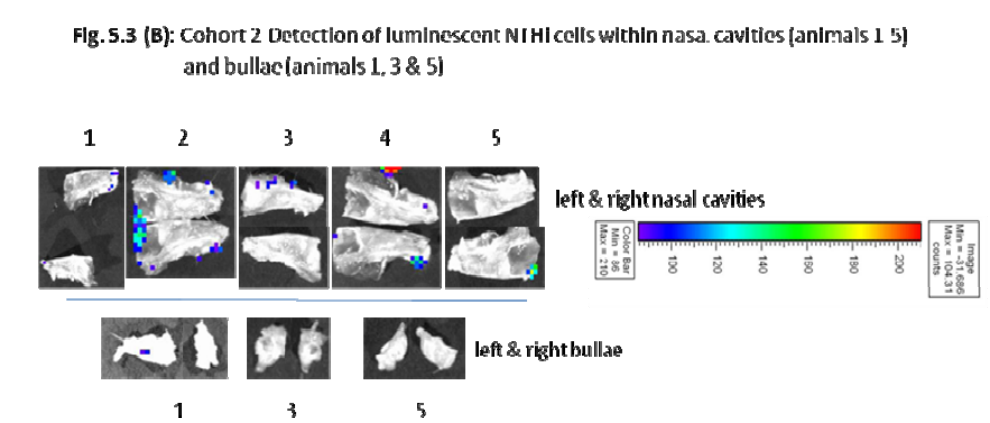


Figure 5.3: Cohorts 1 & 2- Detection of luminescent *S. pneumoniae* (A) and NTHi (B) in the nasal cavities and bullae. This figure shows the location of luminescent *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1, respectively, within the nasal cavities (mice 1-5) and bullae (mice 1, 3 and 5), as indicated, when inoculated into mice alone. The colour bars indicate the relative luminescent signal intensities.





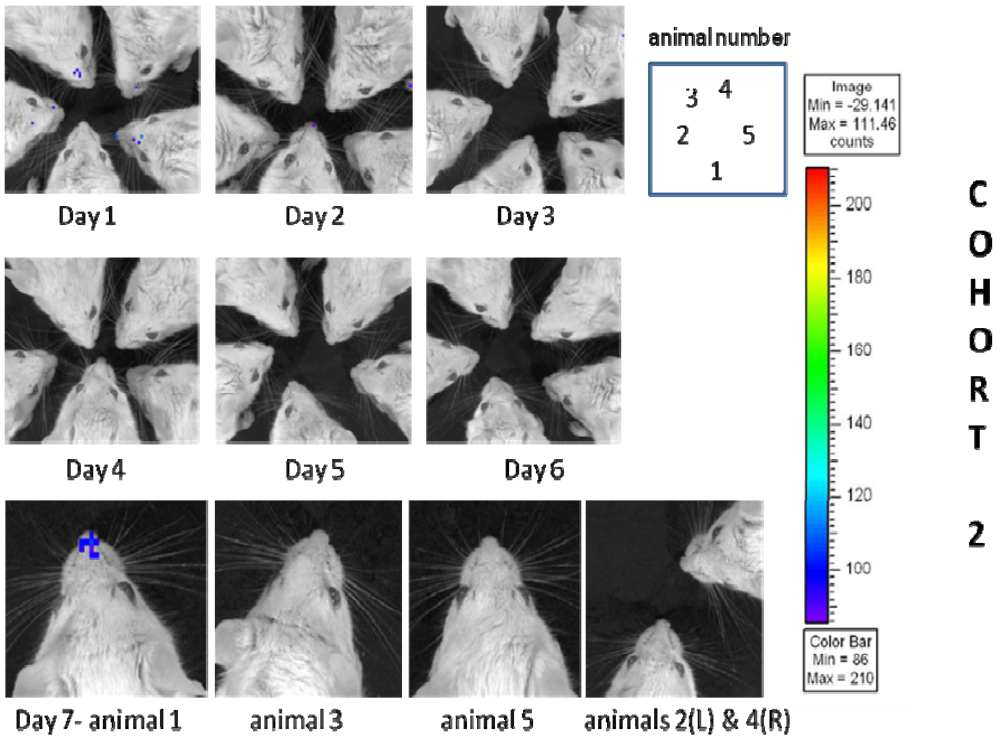
5.4.2.2 Colonising patterns of NTHi

The detection of luminescent NTHi 86-028NP/pKMLN-1cells within the pharynx when inoculated alone is shown in **Figure. 5.4**. At day 1 post-bacterial inoculation, low levels of luminescent NTHi cells were visible within the anterior nares and pharynx in 4/5 animals. No luminescence was detected in the subsequent days and only 1/5 animals showed strong luminescent signal within the anterior nares on day 7 post-infection. Upon termination, luminescent NTHi were detected in all mice in their nasal cavity and

these were located on the distal and proximal ends of the nasopharynx **[Figure. 5.3B]**. A low signal of luminescence was also seen in the left bulla of animal 1.

Figure 5.4: Cohort 2- Detection of luminescent NTHi 86-028NP/pKMLN-1. This figure shows luminescent NTHi cells within the pharynx when inoculated alone. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. The day represents the time point post nasal inoculation with the luminescent bacterium (day 0).

Fig. 5.4: Cohort 2-Detection of luminescent NTHi 86-028NP/pKMLN-1 over 7-day period (prone images)



5.4.2.3 Colonising patterns of NTHi in presence of *M. catarrhalis*

The colonising pattern of luminescent NTHi within the pharynx, when co-inoculated with *M. catarrhalis* is shown in **Figure. 5.5**. At day 1 post-bacterial inoculation, a low level of luminescent NTHi cells was visible within the anterior nares and pharynx in all animals. Upon termination and exposure of the nasal cavity on day 7, there was a

distinctive distribution of luminescent NTHi cells in the nasal cavities of all mice at the distal and proximal ends of the nasopharynx **[Figure. 5.6(A)]**. This pattern of localisation within the nasal cavity was also seen when NTHi was infected alone (Cohort 2) **[(Figure. 5.3(B)]**, although on a lower scale. No luminescent NTHi cells were detected in the bullae in this group where there was a co-infection with *M. catarrhalis*.

Figure 5.5: Cohort 3- Detection of luminescent NTHi 86-028NP/pKMLN-1 when co-inoculated with *M. catarrhalis* 1857. This figure shows the luminescent NTHi cells within the pharynx when co-inoculated with *M. catarrhalis*. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. The day represents the time point post nasal inoculation with the luminescent bacterium (day 0).

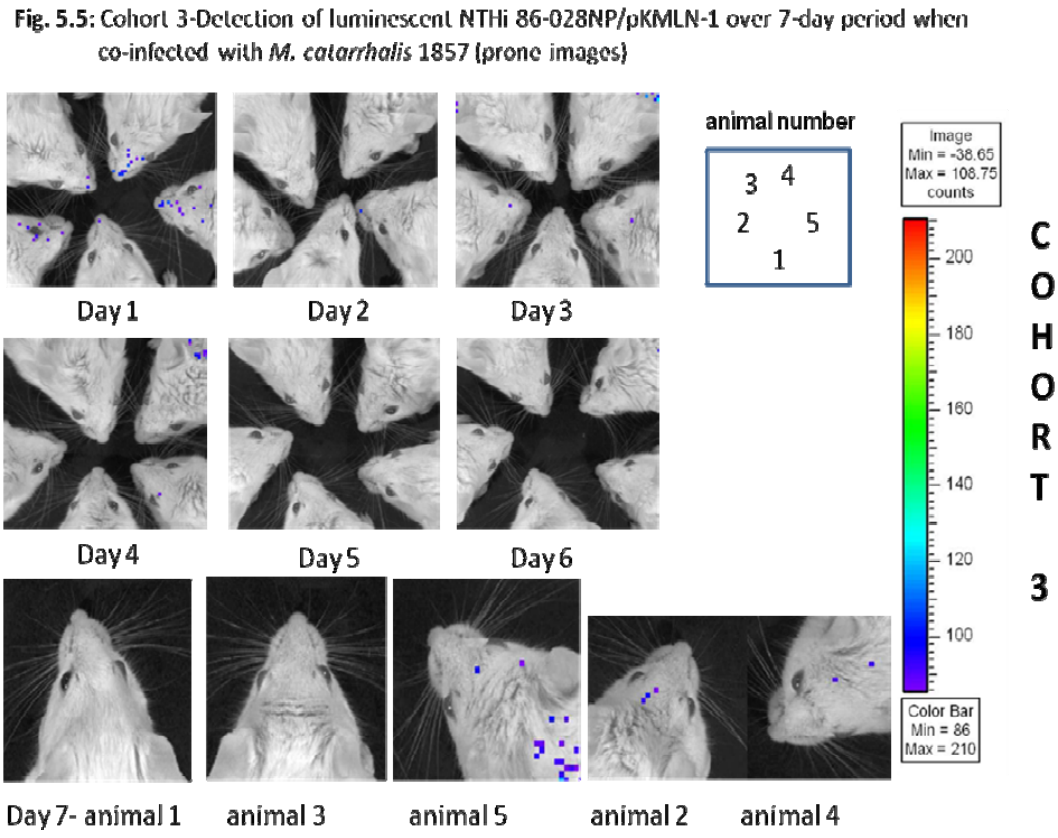
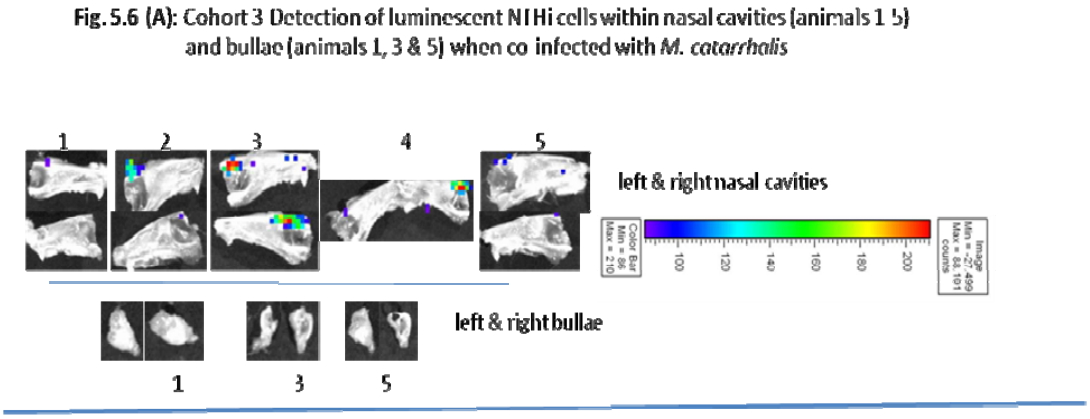
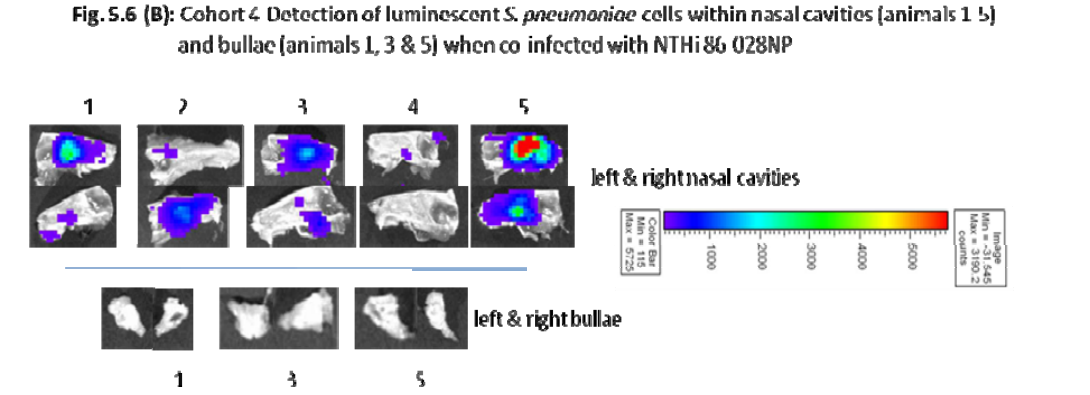


Figure 5.6: Cohorts 3 & 4- Detection of luminescent NTHi 86-028NP/pKMLN-1 (A) and *S. pneumoniae* 19F (B) in the nasal cavities and bullae when co-inoculated with *M. catarrhalis* or NTHi, respectively. These figures show the luminescent NTHi when co-inoculated with *M. catarrhalis* (A) and *S. pneumoniae* 19F cells when co-inoculated with NTHi (B), within the nasal cavities (mice 1-5) and bullae (mice 1, 3 and 5). The colour bars indicate the relative luminescent signal intensities.





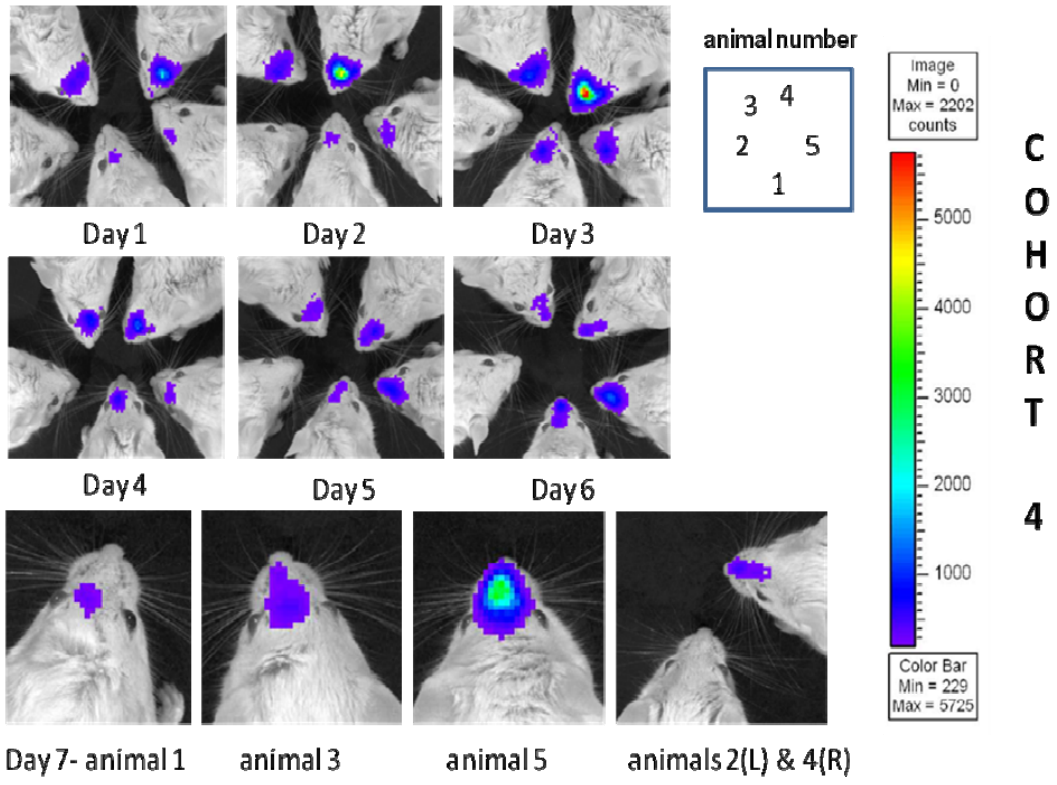
5.4.2.4 Colonising patterns of *S. pneumoniae* in presence of NTHi

The ability of *S. pneumoniae* to establish a colonisation site distal to the intranasal inoculation site when co-infected with NTHi is shown in **Figure. 5.7**. The higher intensity and more extensive luminescent signal was evident in 4/5 animals throughout the infection period. Based on the luminescent intensities, pneumococcal colonisation when co-inoculated with NTHi **(Cohort 4, Figure. 5.7)** was much more extensive and

appeared to achieve a higher colonisation concentration level than when inoculated alone **(Cohort 1, Figure. 5.2)**. The distribution of luminescent *S. pneumoniae* cells was seen throughout the nasal cavities in most of the animals. Despite this strong signal, luminescent *S. pneumoniae* cells were not detected in the bullae **(Figure. 5.6B)**. Excision of the nasal cavities clearly shows the differences in the *S. pneumoniae* colonisation pattern of the nasal cavity with that exhibited by NTHi and when in a co-inoculation situation with another bacterium **(Figure. 5.6A and B)**. In addition, there was a distinct difference in the extent of distribution and intensity of *S. pneumoniae* when inoculated concurrently with NTHi than when inoculated alone **(Cohort 1, Figure. 5.3A).**

Figure 5.7: Cohort 4-Detection of luminescent *S. pneumoniae* 16F when co-inoculated with NTHi 86-028NP. This figure shows the luminescent *S. pneumoniae* cells within the pharynx when co-inoculated with NTHi. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. The day represents the time point post nasal inoculation with the luminescent bacterium (day 0).

Fig. 5.7: Cohort 4-Detection of luminescent *S. pneumoniae* 19F over 7-day period when co-infected with NTHi 86-028NP (prone images)



5.4.2.5 Colonising patterns of *S. pneumoniae* in presence of NTHi & *M. catarrhalis*

In the presence of both NTHi and *M. catarrhalis*, *S. pneumoniae* was seen colonising the anterior nasal region (nasal tips) and 4/5 animals showed strong luminescent signals within the pharynx **Figure. 5.8**. The strong luminescent signal expressed by *S. pneumoniae* when co-inoculated with NTHi and *M. catarrhalis* was consistently intense until day 5 post-inoculation and then appeared to slightly diminish **(Figure. 5.8)**. The luminescent cells of *S. pneumoniae* were seen more extensively throughout the nasal cavity when co-inoculated with NTHi and *M. catarrhalis* **[Cohort 5, Figure. 5.9]**, as opposed to locating within the specific niche area when inoculated alone **[Cohort 1, Figure. 5.3(A)]**. Although the localisation of *S. pneumoniae* within the nasal cavity was similar in both Cohort 4 and 5, the relative luminescent intensity expressed by *S. pneumoniae* was higher in Cohort 5. Overall, the relative luminescent intensity expressed by *S. pneumoniae* when co-inoculated with both NTHi and *M. catarrhalis* **(Figure. 5.8)** was greater in comparison with Cohort 1 (*S. pneumoniae* alone, **Figure. 5.2**) followed by Cohort 4 (*S. pneumoniae* plus NTHi) **(Figure. 5.7)**. No luminescence was detected in the bullae.

Figure 5.8: Cohort 5-Detection of luminescent *S. pneumoniae* 19F when co-infected with NTHi 86-028NP and *M. catarrhalis* 1857. This figure shows the luminescent *S. pneumoniae* cells within the pharynx when co-inoculated with NTHi and *M. catarrhalis*. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. The day represents the time point post nasal inoculation with the luminescent bacterium (day 0).

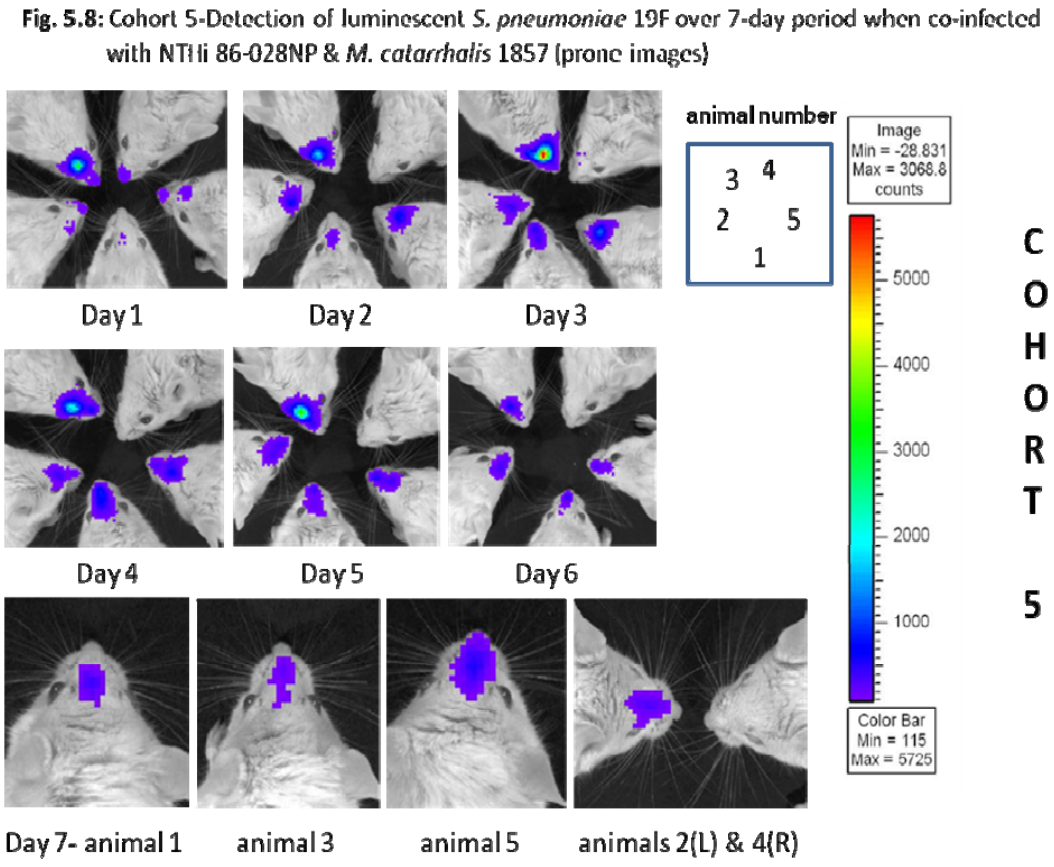
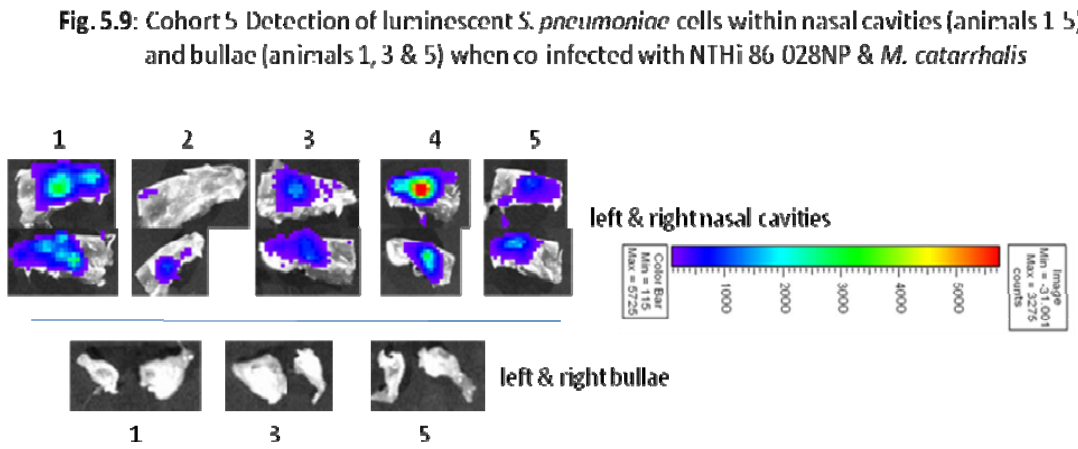


Figure 5.9: Cohort 5- Detection of luminescent *S. pneumoniae* 19F in the nasal cavities and bullae. This figure shows the luminescent *S. pneumoniae* 19F cells within the nasal cavities (mice 1-5) and bullae (mice 1, 3 and 5) when co-inoculated with *M. catarrhalis* and NTHi 86-028NP. The colour bars indicate the relative luminescent signal intensities.



5.4.3 Sequential bacterial infection

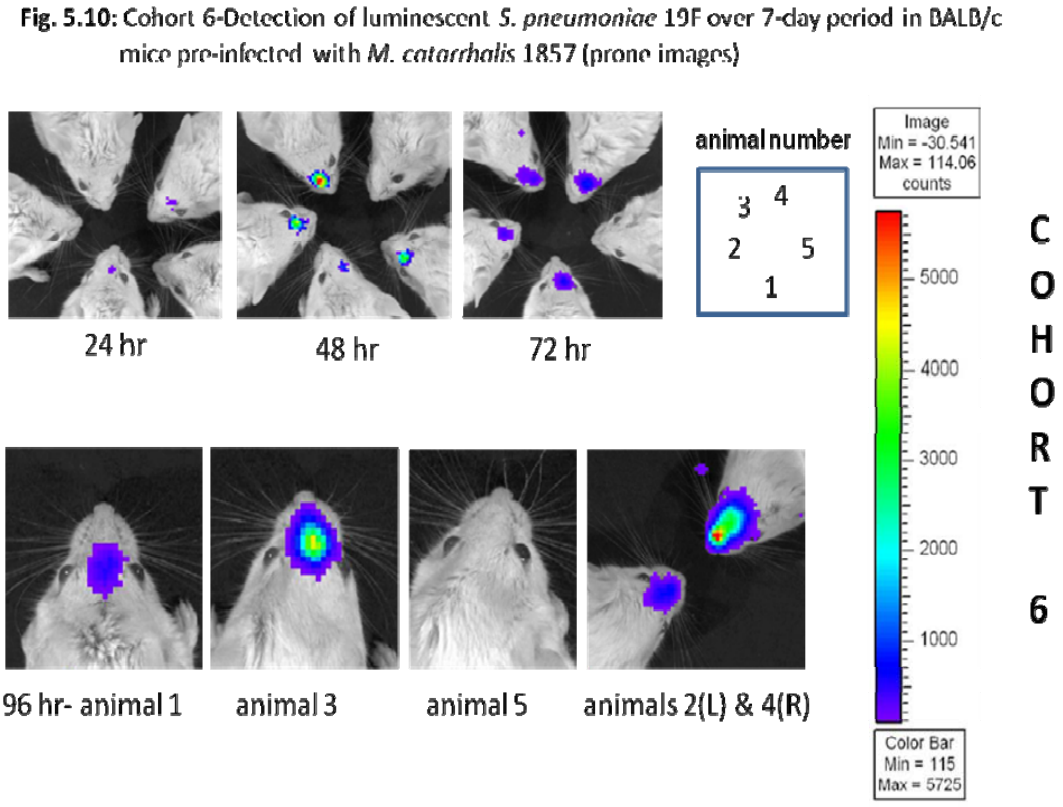
The ability of bacteria *S. pneumoniae* and NTHi to colonise following pre-existing colonisation by *M. catarrhalis* in the nasopharynx is shown in **Figure. 5.10-5.12**. Three days after the initial *M. catarrhalis* inoculation, cohorts 6 and 7 received a secondary nasal inoculation of *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1, respectively. Twenty-four hours after this secondary intranasal bacterial inoculation, cohorts 6 and 7 were imaged each day to assess localisation of the luminescent *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1 until 96 hours post the second bacterial inoculation.

5.4.3.1 Colonising patterns of *S. pneumoniae* in mice pre-inoculated with *M. catarrhalis*

Based on the relative luminescent intensities, the ability of *S. pneumoniae* to establish a colonisation site distal from the intranasal inoculation site was observed within 24 hours post-infection with *S. pneumoniae* in mice pre-colonised with *M. catarrhalis* **(Figure.**

5.10). The intensity of luminescent signal was increasing up to the end point at 96 hours post-inoculation in 4/5 animals. The luminescent signal expressed by *S. pneumoniae* within the pharynx when inoculated alone **(Cohort 1, Figure. 5.2)** was much lower when compared with the intensity expressed in the presence of the pre-existing *M. catarrhalis* **(Cohort 6, Figure. 5.10)**. The localisation of *S. pneumoniae* cells within the nasal cavities was also different between these cohorts. A strong luminescent signal was expressed by *S. pneumoniae* in the presence of the pre-existing *M. catarrhalis* (**Cohort 6, Figure. 5.12A)**. The luminescent signal was comparatively lower in the single *S. pneumoniae* infection group **(Cohort 1, Figure. 5.3A)**. In addition, a low level of luminescence was also observed within the left bulla of animal 1 **(Figure. 5.12A).**

Figure 5.10: Cohort 6-Detection of luminescent *S. pneumoniae* 19F in BALB/c mice pre-inoculated with *M. catarrhalis* 1857. This figure shows the luminescent *S. pneumoniae* cells within the pharynx in mice that had been nasally inoculated with *M. catarrhalis* 3 days earlier. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. Time points represent the time elapsed since nasal inoculation of the luminescent *S. pneumoniae.*



5.4.3.2 Colonising patterns of NTHi in mice pre-inoculated with *M. catarrhalis*

Based on the relative luminescent intensities, pre-nasal inoculation with *M. catarrhalis* did not seem to have any effect on NTHi colonisation. There was no luminescence observed within the pharynx following inoculation with the luminescent NTHi **(Figure. 5.11)**. However, a low level of luminescence was observed in the distal and proximal

ends of the nasal cavity in some animals **(Figure. 5.12B)**. There was no real difference between the luminescent levels expressed by NTHi within the pharynx and nasal cavities in the cohort 7 groups than when inoculated alone **(Cohort 2, Figure. 5.4),** suggesting that any affect of increased intensities observed in combination cohorts identified above were not associated with a synergistic action between NTHi and *M. catarrhalis*.

Figure 5.11: Cohort 7-Detection of luminescent NTHi 86-028NP/pKMLN-1 in BALB/c mice pre-inoculated with *M. catarrhalis* 1857. This figure shows the luminescent NTHi cells within the pharynx in mice that had been nasally inoculated with *M. catarrhalis* 3 days earlier. The top-right corner of the figure shows the orientation of the animals used while imaging and the colour bars indicate the relative luminescent signal intensities. Time points represent the time elapsed since nasal inoculation of the luminescent *S. pneumoniae*.

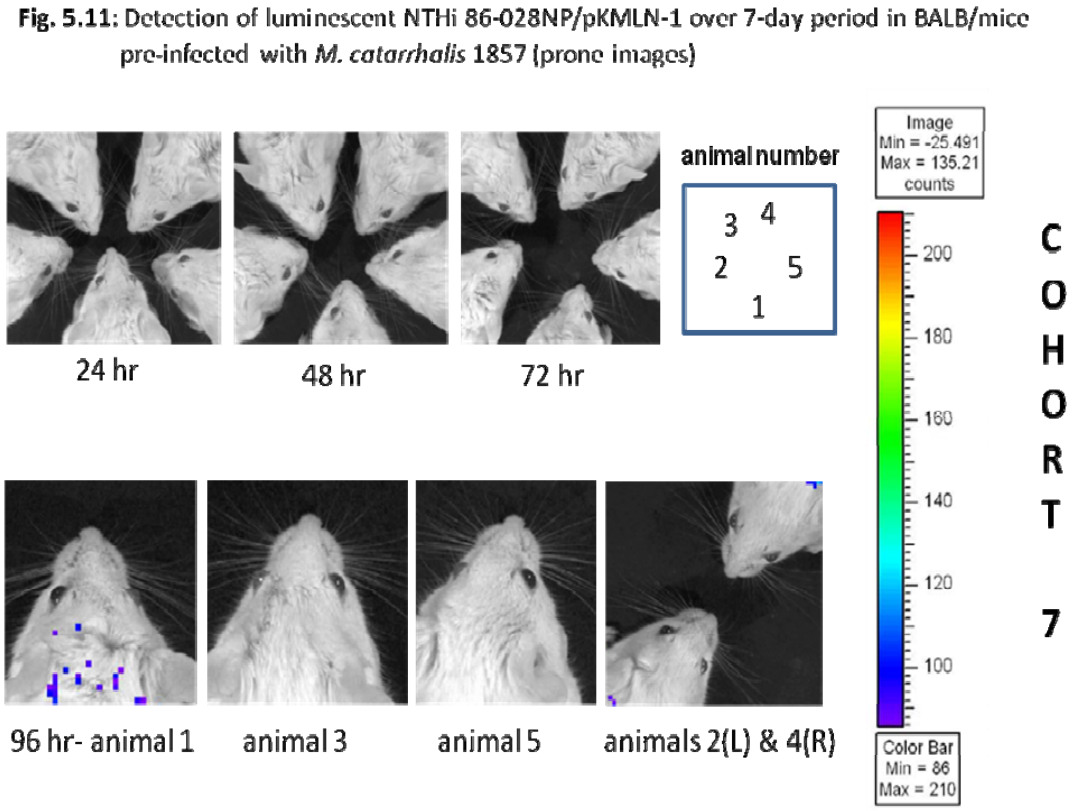
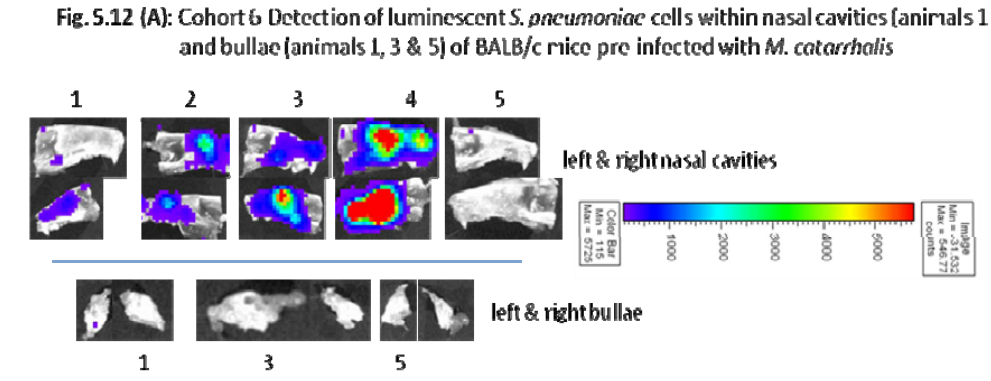
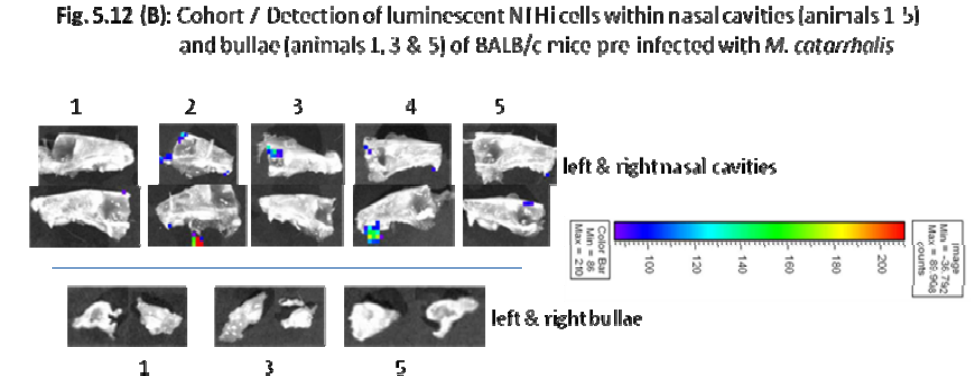


Figure 5.12: Cohorts 6 & 7- Detection of luminescent *S. pneumoniae* 19F (A) and NTHi 86-028NP/pKMLN-1 (B) in the nasal cavities and bullae of BALB/c mice previously inoculated with *M. catarrhalis* 1857. These figures show the luminescent *S. pneumoniae* and NTHi cells within the nasal cavities (mice 1-5) and bullae (mice 1, 3 and 5) in mice that had been nasally inoculated with *M. catarrhalis* 3 days prior to receiving the luminescent bacteria. The colour bars indicate the relative luminescent signal intensities.





5.4.4 Bacterial recovery in nasal washes, bullae washes, nasopharyngeal tissues, left and right middle ear epithelium

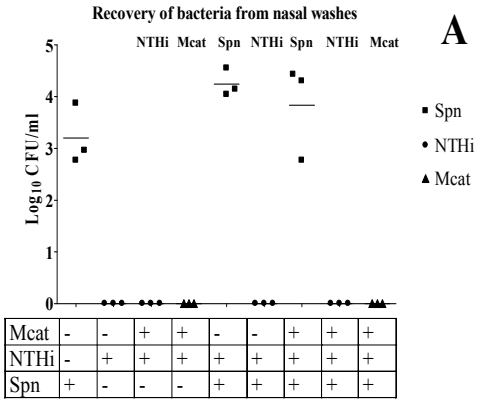
The bacteriology was performed on the nasal washes, bullae washes, and tissue homogenates such as nasopharyngeal tissues, left and right middle ear mucosal epithelium collected from the 3 mice in each cohort after imaging. The remaining 2 mice from each cohort were used for imaging only and have been kept for future histology sections for examination of the nasopharyngeal and the middle ear mucosae. The tissues were weighed before homogenisation and the recovery of bacteria was expressed as CFU/mg of tissue. The weights of these tissues are provided in the appendix **(Appendix B.8)**. The recovery of bacteria from nasal and bullae washes was expressed as CFU/ml.

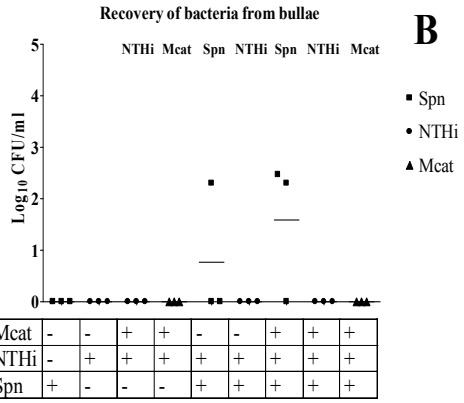
The recovery of bacteria when infected alone or in one of the bacterial combinations is shown in **Figure. 5.13**. Overall, more bacteria were recovered from the nasal washes and nasopharyngeal tissue homogenates **(Figure. 5.13A and 5.13C)**.

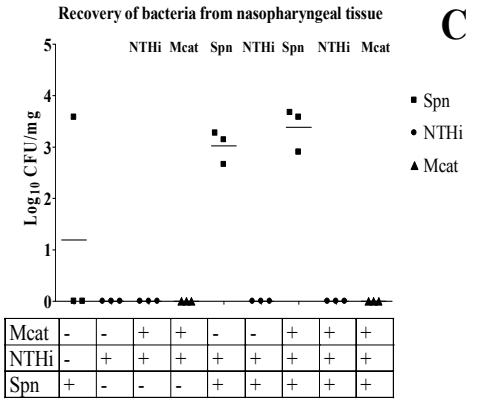
The recovery of bacteria from the sequential colonisation groups is shown in **Figure. 5.14**. The recovery of *S. pneumoniae* in the nasal washes and nasopharyngeal tissue was similar when inoculated alone and to when inoculated into mice three days after they had received *M. catarrhalis* **(Figure. 5.14 A and 5.14C)**. NTHi was not recovered using culture methods both when inoculated alone or in the presence of another bacterium **(Figure. 5.14A-5.14E)**. Even though bacteriology was performed on a subset of the mice and some of these did not show luminescence, the results from **Figure. 5.3B, 5.6A and 5.12B** clearly showing luminescent NTHi in the nasal cavities of mice used in these culture assessments confirms that bacterial culture are underestimating the presence of bacteria at these sites.

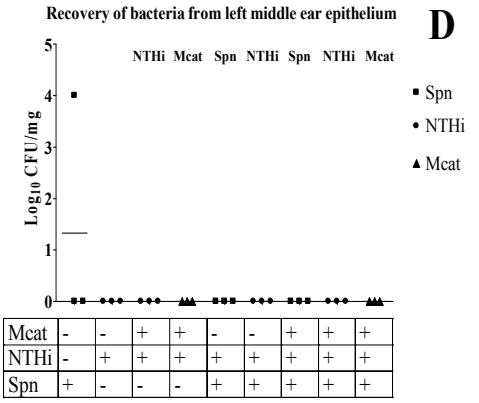
Figure 5.13: Recovery of bacteria when inoculated alone and in bacterial combination cohorts. Single and concurrent combinations were collected on day 7 following bacterial inoculation. The bars represent the mean $\log_{10}$ CFU of the bacteria recovered from A] nasal washes, B] bullae washes, C] nasopharyngeal tissue, D] left middle ear epithelium, and E] right middle ear epithelium (n=3 per group). The table indicates the bacteria when infected alone or the specific bacterial combination; and the bacterium recovered is indicated at the top.

Recovery of bacteria when inoculated alone and in bacterial combination cohorts









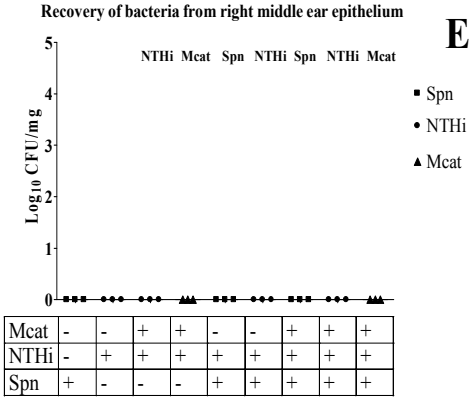
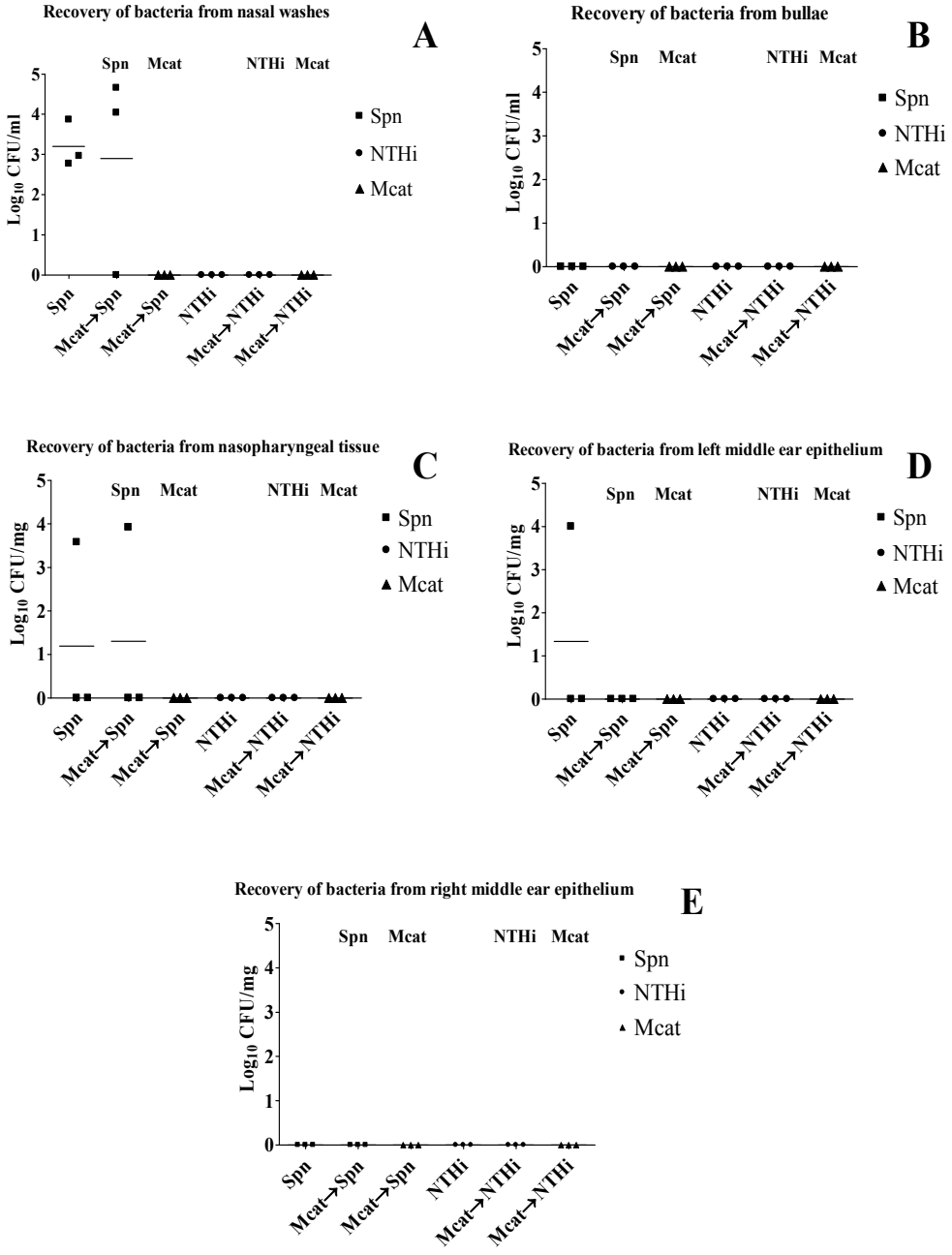


Figure 5.14: Recovery of bacteria from various lavage and tissue homogenates following sequential bacterial intranasal inoculation. *S. pneumoniae* and NTHi were nasally inoculated three days after the intranasal inoculation with *M. catarrhalis*. The bacterium in combination was compared against its recovery when inoculated alone. The bars represent the mean $\log_{10}$ CFU of the bacteria recovered from A] nasal washes, B] bullae washes, C] nasopharyngeal tissue, D] left middle ear epithelium, and E] right middle ear epithelium (n=3 in each group). The arrow points from the first bacterium to the second infecting bacterium; Mcat→Spn, Mcat followed by Spn, and the recovery of each bacterium is indicated at the top.

Comparative recovery of bacteria following sequential intranasal bacterial inoculation



5.4.5 Correlation between bacteriology, relative luminescent intensities and otoscopy

The development of nasopharyngeal colonisation and OM was compared by assessing the results obtained by bacteriology (recovery of bacteria from bullae washes on appropriate culture media), biophotonic imaging (luminescence expression) and otoscopy [signs of inflammation (including fluid in the bulla) and/or tympanic membrane perforation and/or membrane opacity]. The number of animals that developed OM based on these methodologies is listed in **Table 5.1**. It is evident that the traditional approach of diagnosing OM by otoscopy cannot be easily replaced by any of the other methodologies as the majority of mice showed signs of changes either within the bullae or tympanic membrane, indicative of developing OM. The otoscopy images of a selection of middle ear images that showed evidence of perforation, opacity and inflammation are shown in **Figure. 5.15**.

The conventional culture methods enabled recovery of bacteria from the bullae washes, especially in co-infected groups. Although bacteria were recovered from bullae washes by culture method, it was not necessarily always observed using the luminescent imaging technology. This is not surprising as the organisms expressing the *lux* operon emit light in the presence of oxygen and flavin mononucleotide ($FMNH_2$). The light emitted can penetrate tissues and is detected externally in a qualitative and quantitative manner, as seen in this study. As the availability of oxygen is almost non-existent in the bullae, the chances of detecting luminescence *in vivo* are very limited.

Table 5.1: Comparison of detection of OM as assessed using the different analysis methods. The results indicate the total number of mice that were detected as developing bacterial OM. The incidence of OM was based on either the recovery of bacteria from bullae washes or luminescence emitted or otoscopy.

Cohort	**Number of animals that developed OM** [a]		
	Bacteriology [b]	**Relative luminescent intensity** [c]	[d] **Otoscopy**
Virus only control	N/A	0/5	0/5
S. pneumoniae 19F	0/3	1/3	4/5
NTHi 86-028NP/pKMLN-1	0/3	1/3	5/5
NTHi 86-028NP/pKMLN-1 + *M. catarrhalis* 1857	0/3	0/3	5/5
S. pneumoniae 19F + NTHi 86-028NP	1/3	0/3	4/5
S. pneumoniae 19F + NTHi 86-028NP + *M. catarrhalis* 1857	2/3	0/3	4/5
M. catarrhalis 1857 followed by *S. pneumoniae* 19F	0/3	1/3	4/5
M. catarrhalis 1857 followed by NTHi 86-028NP/pKMLN-1	0/3	0/3	2/5

[a] The presence of OM was based on the recovery of any of the bacteria tested in the bullae washes by conventional culture methods, luminescent signal expressed by the luminescent bacterial strains tested in the bullae and signs of inflammation, perforation or opacity observed in the middle ear by otoscopy.

[b] Recovery of any of the bacteria from the bullae washes using conventional bacterial culture methods.

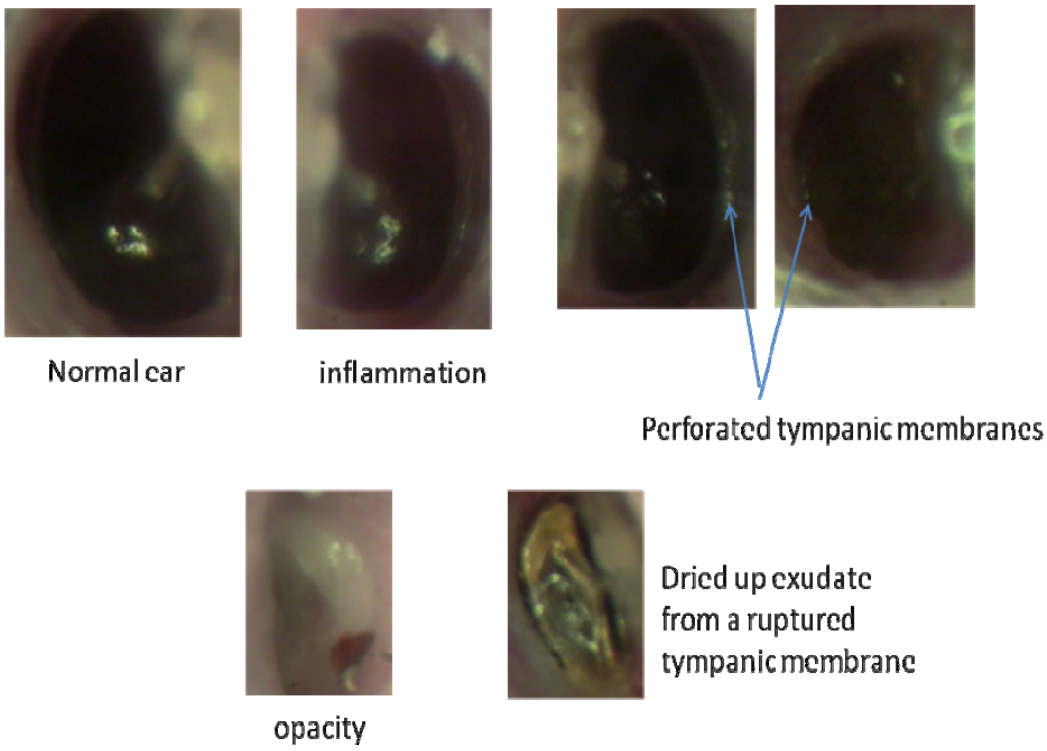
[c] Signal intensity expressed by luminescent bacterial strains *S. pneumoniae* 19F and NTHi 86-028NP/pKMLN-1 in the bullae (either right or left bullae).

[d] The presence of inflammation and/or tympanic perforation and/or membrane opacity in either of the middle ear (left and right) was used to indicate the presence of OM in those mice.

N/A- Bacteriology not performed.

Figure 5.15: Otoscopy images showing different signs of OM. The figure shows the otoscopy images down the ear canal showing abnormal conditions leading to development of OM.

Fig. 5.15: Different signs of OM developed in BALB/c mice



5.5 DISCUSSION

This study has clearly demonstrated that there are specific changes to the dynamics of colonisation patterns in the nasopharynx in the polymicrobial environment and that there is localisation of bacteria within the pharynx and the middle ear specific to the bacterial species and the co-colonising bacterium. The results have also shown that the acquisition of a new bacterium in the nasopharynx does affect the colonisation of the second bacterium, and in the case of *S. pneumoniae* resulted in it more efficiently colonising when *M. catarrhalis* had already colonised the nasal cavity (shown in Chapter 2). This study was designed as a consequence of the significant findings in Chapter 2 that showed the impact of concurrent nasal inoculation of *M. catarrhalis* with *S. pneumoniae* on the incidence and severity of pneumococcal OM. Real time imaging using luminescent bacteria to investigate the kinetics of bacterial ascension to the middle ear, nasal co-colonisation patterns and distribution of bacteria within the nasal cavities and bullae was able to show significant differences between the cohorts in this study. The engineered strains of NTHi and *S. pneumoniae* and their luminescence expression under *in vivo* conditions have been demonstrated before (Jurcisek *et al.* 2007; McCullers & Bartmess 2003). Hence, these luminescent strains were included in this study. The NTHi 86-028NP/pKMLN-1 strain contained a reporter plasmid in which expression of the *lux* operon is under the control of the strong OMP P2 promoter (Mason *et al.* 2005), whereas, the promoter for the expression of *S. pneumoniae* 19F was unknown.

Using this bioluminescent imaging technology, other studies in animal models have identified differences in disease progression caused by various strains of pneumococci (Orihuela *et al.* 2003), evaluated the efficacy of antibiotics on pneumococcal infection in the lungs (Francis *et al.* 2001) and assessed the effect of an antiviral agent used to reduce the occurrence of a secondary infection, bacterial pneumonia, following infection with influenza virus and *S. pneumoniae* (McCullers & Bartmess 2003). In the context of the middle ear infection studies, *in vivo* experiments have monitored the progression of disease using NTHi alone (Novotny *et al.* 2005) and investigated a strategy to prevent

pneumococcal colonisation in the nasopharynx (McCullers *et al.* 2007). These studies have highlighted the importance of real time information using the bioluminescent imaging technology by providing an insight into disease progression caused by a single bacterium or different strains of the same bacteria and demonstrating the potential for future prophylactic treatments against these infections. This study has investigated nasopharyngeal colonisation patterns of different bacteria when infected alone, during co-infection and associated with sequential bacterial acquisitions and their effect on contributing to the development of OM. These studies aimed to provide insight into the complex interplay between bacterial pathogens in a polymicrobial environment and also provide information that could direct future studies to aid understanding of the differences in disease pathogenesis caused by bacteria such as *S. pneumoniae* and NTHi. This chapter has adapted the same approach demonstrated in the kinetics of bacterial ascension observed in the NTHi chinchilla model (Novotny *et al.* 2005) , and addressed the dynamics of nasal co-colonisation patterns and the effect of sequential bacterial infection on the incidence rate of OM in a BALB/c mouse model using luminescent NTHi 86-028NP/pKMLN-1 and *S. pneumoniae* 19F.

The mechanisms involved with the bacterial ascension from the nasopharynx to the middle ear through the ET as a result of an under-pressured middle ear space or due to a compromised ET are well known (Miyamoto & Bakaletz 1997; Sabirov & Metzger 2008). The kinetics of single bacterium ascension from the nasopharynx to the middle ear has been previously demonstrated in a chinchilla model using indirect immunofluorescent staining and electron microscopy (Miyamoto & Bakaletz 1997). The Miyamoto study (1997) demonstrated a possible mechanism through which NTHi might cause OM by adhering to mucous in the ET, as well as, to the nasopharyngeal epithelial cells that have been compromised by an adenovirus infection. This study used adenovirus infected mice and has demonstrated the differences in the nasopharyngeal bacterial colonisation when infected alone and during co-infection through direct, continuous monitoring of disease progression within the pharynx. The single infection with luminescent *S. pneumoniae* and NTHi showed preferential colonisation patterns in

the anterior region of the pharynx and the two bacteria localised in different niches within the nasal cavity, suggesting that the bacteria adhere to either certain cell types or cells expressing different receptor molecules present in different areas of the nasopharynx. This preferential adherence by NTHi to mucous contained in the ET floor region and not the middle ear epithelium surface has been previously demonstrated using frozen sections of chinchilla ET and middle ear mucosa *in vitro* (Miyamoto & Bakaletz 1996). In contrast, this study also detected the luminescent signal expressed by *S. pneumoniae* cells until day 5 when infected alone, whereas real time imaging was only detectable to day 1 for NTHi when infected alone. It is difficult to determine whether this is evidence of the dominating ability of *S. pneumoniae* to ascend the pharynx region and colonise more efficiently than NTHi, or whether the NTHi has a preference for anaerobic niches, thus suppressing the luminescence expression. Irrespective, the high recovery of *S. pneumoniae* from the nasal washes (using conventional culture methods) in this study also supports the suggestion that *S. pneumoniae* readily and extensively colonises the nasal cavity in this model.

Based on the relative luminescent intensities, pneumococcal colonisation when co-infected with non-luminescent NTHi and with both non-luminescent NTHi and *M. catarrhalis* differed from that observed with single infection by *S. pneumoniae*. When co-infected with NTHi, *S. pneumoniae* colonised in a distal location to the initial inoculation site **(Figure. 5.7)**, as opposed to colonising the nasal tip region when infected alone **(Figure. 5.2)**. This suggests that its ability to colonise the nasopharynx is more effective when in the presence of another bacterium. The observation of advantage during concurrent infection seemed to be selective for *S. pneumoniae* with NTHi or *M. catarrhalis* infection, as co-infection of luminescent NTHi and *M. catarrhalis* **(Figure. 5.5)** did not produce similar results, and in fact the luminescence intensity expressed by NTHi was similar to that observed during the single infection with NTHi. *S. pneumoniae* was able to rapidly ascend and colonise the pharynx region when in a bacterial combination of all three and this appeared stronger (as observed with relative luminescence) and for longer, as evidenced by the day 5 results **(Figure. 5.8)**, as

opposed to day 3 when co-infected with NTHi only **(Figure. 5.7)**. These findings suggest the existence of a complex interplay between the bacteria and the host in a polymicrobial environment facilitating the ability to establish colonisation and potentially cause an infection. The dominance of *S. pneumoniae* seen during co-infection in the pharynx region (as observed by strong luminescent signals) and higher recovery of *S. pneumoniae* (using culture methods) from the nasal washes in the co-infection cohorts **(Figure. 5.13)**, confirms the previous observations in Chapter 2. The Chapter 2 and this study used different respiratory viruses, Sendai and adenovirus, and different strains of each bacterium, thus suggesting that these observations might not be virus or bacterial strain specific.

The differences in results using difference analytical methods highlight the importance of multiple measures in these studies. The visualisation of the bacteria alone is also not sufficient, but it did indicate the general location of the bacteria. Both culture and visualisation methods are probably underestimating the presence of bacteria, particularly NTHi, and these may be residing within the sub epithelial mucosal layers in the nasopharynx. These findings provide additional insight into colonisation patterns that may also exist in children and adults. It is known that high nasopharyngeal colonisation and carriage rates in infants and young children, and particularly in certain high risk communities, are a predisposing factor for OM (Faden *et al.* 1997; Leach *et al.* 1994). The observation that the bacteria have the ability to colonise differently when alone and during concurrent colonisation could inform studies that investigate nasopharyngeal colonisation patterns, particularly those in studies in children to assess colonisation with multiple strains of bacteria (Leach *et al.* 1994; Smith-Vaughan *et al.* 2006). The relevance of these findings to nasopharyngeal collection techniques warrants further investigation.

The high incidence rate of OM in infants and young children has significantly increased the number of visits to the doctor for diagnosis while increasing the financial burden (Alsarraf *et al.* 1999; Bondy *et al.* 2000). The introduction of the pneumococcal

conjugate vaccine has not had a major impact on the overall incidence rate of OM (Palmu *et al.* 2008) and the USA which had one of the highest coverage rates for serotypes in this vaccine is now reporting significant evidence of serotype replacement (Pichichero & Casey 2007). Moreover, the treatment of OM with frequent use of antibiotics poses a threat to increasing bacterial drug resistance, thereby making the treatment options limited. This could be overcome with newer treatment alternatives using bacteriophage lytic enzymes or lysin. Lysin is produced by bacteriophages and is used to digest the bacterial cell wall for the release of progeny bacteriophages. Moreover, purified lysin has been shown to cause bacterial death, especially gram-positive bacteria (Fischetti 2005). This approach has been tested on *S. pneumoniae* and was shown to reduce or eliminate pneumococcal colonisation in an experimental mouse model (McCullers *et al.* 2007). The McCullers study (2007) monitored the ability of lysin to eliminate pneumococcal colonisation and suggested an alternative strategy to treat AOM. The effect of purified lysin to eliminate or reduce pneumococcal colonisation in the presence of NTHI and *M. catarrhalis* could add further insight to the development of new approaches to treat AOM, as these commensals along with *S. pneumoniae* are equally involved in the development of OM.

Another important finding in this study was the ability of *M. catarrhalis* to predispose the nasal cavity to pneumococcal colonisation but not NTHi colonisation. This finding is consistent with the co-infection data determined by culture in Chapter 2. The increased colonisation by *S. pneumoniae* in the nasopharynx was also observed in Chapter 4 as a result of pre-colonisation by *M. catarrhalis* by only one day. These findings support the modelling study that identified a positive association between these bacteria in children, at both the microbe-microbe and host-microbe levels in children less than 2 years of age (Jacoby *et al.* 2007). The previously known association between these bacteria along with the high nasopharyngeal carriage rates of *S. pneumoniae* and *M. catarrhalis* (49% and 50%, respectively) in Aboriginal children is well recognised (Watson *et al.* 2006) and the results in the current study support the significance of these carriage rates as a contributor to OM in these children. In addition, the findings in this

study that *S. pneumoniae* rapidly ascends into the pharynx (24 hr post-infection) and multiplies within the sub epithelial mucosal layer of the pharynx when introduced either concurrently or with sequential to another bacterium. This suggests that acquisition of new strains in colonised individuals may also be a significant factor contributing to the incidence rate of OM. Along with the real time monitoring of pneumococcal infection progression in the presence of *M. catarrhalis*, this study has identified significant differences in the bacterial colonisation patterns providing a new dimension to the understanding of the dynamics of bacterial colonisation that may be associated with susceptibility to OM.

5.6 CONCLUSION

In conclusion, this study has shown that the polymicrobial environment in the nasopharynx has a significant impact on colonisation and is a significant contributor to the induction of OM. Real time monitoring of infection progression was able to discern differences in colonisation patterns for the luminescent bacterium and the ability of NTHi and *S. pneumoniae* to localise in different niches within the nasal cavity. Of importance in this study was the increased ability of pneumococci to colonise in the nasopharynx in the presence of *M. catarrhalis* and NTHi supporting a positive association between the bacteria which probably contributes to an increase in pneumococcal OM.

CHAPTER 6

Investigation of microbe-host dynamics involved in airway colonisation leading to respiratory infections

6.1 ABSTRACT

The human respiratory tract is colonised by multiple species of bacteria at any given time. The bacterial adherence to various mucosal and epithelial surfaces is considered as an important step in its colonisation. Bacteria such as *S. pneumoniae, H. influenzae* and *M. catarrhalis* are well known to cause respiratory tract infections in humans. Moreover, respiratory viruses are recognised as major triggers in enhancing bacterial adherence, colonisation and translocation through the epithelial barrier. This study hypothesised that the co-presence of different microbes would affect bacterial adherence, a factor associated with the virulence of the bacteria. This study investigated how different microbes interact with each other and the host to cause infection in a polymicrobial environment. *In vitro* cell culture models; A549 (human lung epithelial cell line) and BEAS-2B (human bronchial epithelial cell line) infected with respiratory virus (Adenovirus 5) and the above mentioned bacteria were used in this study. The bacterial adherence was measured following infection of both cell lines with either single bacteria or combinations of bacteria in the presence or absence of adenovirus. The results of this study demonstrated that the infection of adenovirus with single bacterium enhanced the adherence of *M. catarrhalis* to BEAS-2B and *S. pneumoniae* to both BEAS-2B and A549 cell line ($p<0.001$). Moreover, the presence of adenovirus significantly increased the bacterial adherence of both, *S. pneumoniae* and *M. catarrhalis* to the A549 cell line, when co-infected together ($p<0.001$). In addition, the presence of NTHi in combinations with *S. pneumoniae* and *M. catarrhalis* affected the ability of these bacteria to adhere to the cells, an observation that warrants further investigation. In conclusion, *S. pneumoniae* and *M. catarrhalis* when infected alone showed greater ability to adhere to the bronchial epithelial cell line and when co-infected together their adherence was higher on a lung epithelial cell line.

6.2 INTRODUCTION

The bacterial colonisation of the human nasopharynx is known to begin soon after birth (Brook 2005; Rynnel-Dagoo & Agren 2001). The nasopharyngeal commensals usually

consist of *S. viridans,* anaerobic *Streptococci*, Diphtheroids, *Neisseria* species etc (Murphy 2000). Apart from the normal microflora, pathogens like *M. catarrhalis,* NTHi and *S. pneumoniae* also occupy a microflora niche and inhabit the respiratory tract (Garcia-Rodriguez & Martinez 2002). Under normal circumstances, the lower respiratory tract is a sterile environment and bacterial colonisation is not observed due to the host's natural defence mechanisms. The airway epithelial cells are known to play a dual role in offering a protective barrier against a number of bacteria and contribute towards triggering the innate immune system to respond to infectious agents. Within the respiratory tract, mucociliary clearance mechanisms, production of various pro-inflammatory mediators and antimicrobial peptides all contribute to the elimination of pathogenic bacteria (Bals & Heimstra 2004; Van Alphen 1996). In the compromised host, the presence of damaged epithelium, chronic inflammatory situations or other mechanisms that have compromised the normal integrity and function of the respiratory system provide pathogens with an ideal location for the establishment of bacterial infection (Van Alphen 1996). Under these circumstances, the host's natural defence mechanisms are less capable of managing encounters with new bacteria that can often then out compete other normal microflora (Wilson *et al.* 2002).

The epithelial lining of the nasopharynx provides a niche for the disease causing microbes, which upon inhalation establishes the contact with the host necessary to colonise and/or establish an infection (Bals & Heimstra 2004). The most important step in bacterial colonisation of mucosal surfaces is undoubtedly the mandatory adherence by the infecting bacteria. A range of surface exposed bacterial adhesins facilitate the localisation of the bacteria by attaching to the various receptor molecules present on mucosa, epithelial cells and other cell types (Hakansson *et al.* 1996). Furthermore, the infecting bacteria express a variety of virulence factors such as cytotoxins, enzymes, polysaccharides and proteins which facilitates its ability to colonise, survive and cause infection within the host (Hammerschmidt 2006; Van Alphen 1996). The various bacterial proteins responsible for mediating adherence of bacteria to various mucosal surfaces are well documented (Barenkamp *et al.* 2002; Hammerschmidt 2006; Jedrzejas

2001; Karalus & Campagnari 2000; Mitchell 2000; Schweda *et al.* 2000). In many bacterial URTI, viral infection is a well recognised trigger for secondary bacterial infections. The respiratory viruses assist bacterial colonisation, adherence and facilitate passage of bacteria through the epithelial barrier and subsequent establishment of an infection (Hament *et al.* 1999). Many studies have reported the increased adherence of bacteria to human epithelial cells following an infection with a respiratory virus (Avadhanula, V. *et al.* 2006; Hakansson *et al.* 1994; Hament *et al.* 2004).

The factors associated with microbial interactions, including adherence, with the host are often identified using *in vitro* cell cultures as a model because of their ease of manipulation, cost-efficiency and reduction in the usage of experimental animals. The transformed cell lines used in most of the *in vitro* cell culture studies may not be a complete representation of an individual cell type or respiratory epithelium as a whole. This could result in differential expression of baseline or stimulated surface receptors to which the bacteria may attach and initiate adherence (Avadhanula, V. *et al.* 2006; Hakansson *et al.* 1996). In a polymicrobial environment it is important to understand the extent of and mechanisms by which different bacteria interact with each other and also with the host to cause disease. Speculation of how the concurrent presence of different bacteria affects their adherence has not been fully investigated. This study addresses the hypothesis that different bacteria may interact or compete with each other when colonising the host that alters their ability to adhere. The results presented in chapter 5 showed enhanced nasal colonisation by *S. pneumoniae* in the presence of NTHi and *M. catarrhalis*, whereas NTHi was not affected by the presence of *M. catarrhalis.* This study aimed to investigate these microbial interactions between each other and human airway epithelial cells to better understand microbe-host dynamics associated with nasopharyngeal colonisation that could be contributing to various respiratory infections.

6.3 MATERIALS AND METHODS

6.3.1 Bacteria and virus infections

The bacterial strains used in this study and their preparation have been previously described in Chapter 2 (Section 2.3.1). Based on the optical density at 405 nm, the starting concentrations of each bacterium were adjusted to 10^7 CFU/ml and 10^{10} CFU/ml. The higher bacterial concentration was included to determine whether bacterial adherence to epithelial cells was dosage dependent. Fifty µl of each concentration was used to infect the cell lines. The resulting infectious dose for each bacterium was 10^5 CFU/well and 10^8 CFU/well, respectively. For double and triple bacterial combinations, equal volumes of the required bacterial suspensions were mixed and 50 µl was added to the designated wells. The human respiratory adenovirus-5 (kindly provided by Dr Jim Buttery), a clinical isolate from Royal Children's Hospital, Melbourne, Australia was used in this study. The starting concentration of adenovirus was adjusted to 10^9 PFU/ml. Fifty µl of this concentration was used for pre-viral infection of the cell lines. The resulting infectious dose for adenovirus was 10^7 PFU/well.

6.3.2 Epithelial cell culture

A549 (lung epithelial cell line) and BEAS-2B (bronchial epithelial cell line) were grown in RPMI 1640 containing L-Glutamine (Gibco BRL, Invitrogen), supplemented with heat-inactivated 10% Fetal Calf Serum (FCS) (Cambrex, Lonza Australia Pty. Ltd.) and 100 units of penicillin/ml, 100 µg/ml of streptomycin (Gibco BRL, Invitrogen). Both the cell lines were used between passages 10-35 and grown to 90-95% confluence in 75 cm^2 tissue culture flasks (Sarstedt, Germany) and subsequently cultured in a 12-well tissue culture plate (Orange Scientific, Belgium), unless otherwise stated.

6.3.3 Standardisation of cell monolayer infection

The cell monolayer infection protocol was standardised using all the above mentioned bacteria. The ability of each bacterium to adhere to both A549 and BEAS-2B cells was tested at 5 min, 10 min, 15 min, 30 min, 60 min and 90 minutes. The time point at which

each bacterium showed the highest adherence was chosen for the cell line infection protocol. The cell monolayer infection protocol was standardised using 10^5 CFU of each bacterial suspension/well. The cell monolayer infection protocol described by Holm *et al.* was used (Holm *et al.* 2003).

Briefly, both A549 and BEAS-2B epithelial cells were bulk seeded into 12-well tissue culture plates (Orange Scientific, Belgium) at a concentration of 5 x 10^5 cells per well. The tissue culture plates were then incubated overnight at 37^0C with 5% CO_2 until 90-95% confluence was observed. The tissue culture plates were carefully washed with Dulbecco's PBS (GIBCO-BRL, Invitrogen), to remove any non-adhered epithelial cells. Duplicate wells of the cell monolayers were infected with 10^5 CFU of each bacterium. The tissue culture plates were then centrifuged at 3000 x g for 5 minutes, to facilitate contact between bacteria and the epithelial cells and 1 ml of RPMI medium without antibiotics and FCS was added to each well. The plates were incubated for the time points indicated for the specific cell line at 37^0C with 5% CO_2. The non-adherent bacteria were removed by gentle washing (3-4 times) with 1 ml of Dulbecco's PBS. The epithelial cells containing adhered bacteria were released using 200µl of 0.025% trypsin-EDTA (GIBCO BRL, Invitrogen). The trypsinisation was stopped by adding 1ml of Dulbecco's PBS, and the cells containing bacteria were collected in sterile 1.5 ml eppendorfs tubes. The total number of viable bacteria adherent to each cell line was determined by serial dilutions on appropriate culture media and expressed as a percentage adherence. The percentage adherence of each bacterium was determined as the number of viable bacterial cells adherent to the epithelial cells relative to the original inoculum added to each well.

6.3.4 Quantitation of adenovirus

The concentration of adenovirus was determined using the lytic plaque assay (Hakansson *et al.* 1994), with a few modifications. Briefly, A549 cells were bulk-seeded in 6-well tissue culture plates (Greiner bio-one, Germany) at a concentration of 1 x 10^6 cells per well. The tissue culture plates were then incubated overnight at 37^0C with 5%

CO_2 until 90-95% confluence was observed. The confluent 6-well tissue culture plates were gently washed with Dulbecco's PBS to remove any non-adhered epithelial cells. The adenovirus suspension was serially diluted in sterile PBS from 10^{-1} to 10^{-12} per milliliter and 150 µl of each dilution was added into the designated wells in duplicates and incubated for 1 hour at 37^0 C with 5% CO_2. Sterile 1.8% bacteriological agar (Oxoid Ltd., Hampshire, England) was prepared and maintained at 45^0 C. The plates were then washed with Dulbecco's PBS and 2 ml of sterile overlay medium containing 4 parts of RPMI 1640–supplemented with L-glutamine, antimycotic and antibiotic solution, and 25mM $MgCl_2$, 1 part of FCS and 5 parts of melted 1.8% bacteriological agar was added into each well. The addition of $MgCl_2$ is known to enhance plaque formation (Williams 1970). The plates were left at room temperature for approximately 10 minutes to allow solidification of the agar and further incubated at 37^0 C with 5% CO_2 for 5-7 days. The plates were then stained with 0.1 volume of 5 mg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution (Sigma Aldrich Ltd.) for 3 hours at 37^0 C. The epithelial monolayer appeared blue/black and the plaques were visualised as clear areas. The concentration of virus was expressed as PFU/ml following the enumeration of lytic plaques.

6.3.5 Experimental groups

In this study, A549 and BEAS-2B epithelial cell lines were bulk-seeded into 12-well tissue culture plates at a concentration of 5×10^5 cells per well and the effect of bacterial adherence on both adenovirus infected and non-virus infected epithelial cells was investigated. The following experimental groups were used to infect both, adenovirus infected and non-virus infected A549 and BEAS-2B cell lines:

A] Single bacterium: a) *M. catarrhalis*; b) NTHi; c) *S. pneumoniae* alone

B] Double bacterial combinations: a) *M. catarrhalis* + NTHi; b) *M. catarrhalis* + *S. pneumoniae*; and c) NTHi + *S. pneumoniae*

C] Triple bacterial combination: *M. catarrhalis* + NTHi+ *S. pneumoniae*

6.3.6 Infection of cell monolayers

The adherence assay was performed as above for the standardisation experiments (Section 6.3.3). Briefly, 10^7 PFU of adenovirus/well was added to the sets of A549 and BEAS-2B cell monolayers for adenovirus infection and incubated for 1 hour at 37^0 C with 5% CO_2. These wells were then gently washed with Dulbecco's PBS and 1 ml of RPMI medium (without antibiotics) containing 1% FCS was added to each well and they were incubated overnight. The non-adhered epithelial cells from both adenovirus infected and non-virus infected cells were removed by gentle washing with Dulbecco's PBS. The bacteria were added to duplicate designated cell monolayers at 10^5 or 10^8 CFU/ml. The plates were centrifuged at 3000 x g for 5 minutes to facilitate contact between the bacteria and the epithelial cells and 1 ml of RPMI medium without antibiotics and FCS was added to each well. The A549 and BEAS-2B tissue culture plates were incubated for 30 minutes and 90 minutes, respectively, at 37^0C with 5% CO_2 as determined by the standardisation experiment **(Figure. 6.1)**. The non-adhered bacteria from both virus infected and non-virus infected epithelial cell lines were then removed by gentle washing with Dulbecco's PBS. The adherent bacteria were then released upon trypsinisation and the percentage adherence of each bacterium was determined as the number of viable bacterial cells adherent to the epithelial cells relative to the original inoculum added to each well.

6.3.7 Statistical analysis

The nonparametric analysis for significance of the bacterial adherence to virus-infected and non-virus infected epithelial cell lines was analysed with two-way ANOVA using a Bonferroni post-hoc test ($p<0.001$).

6.4 RESULTS

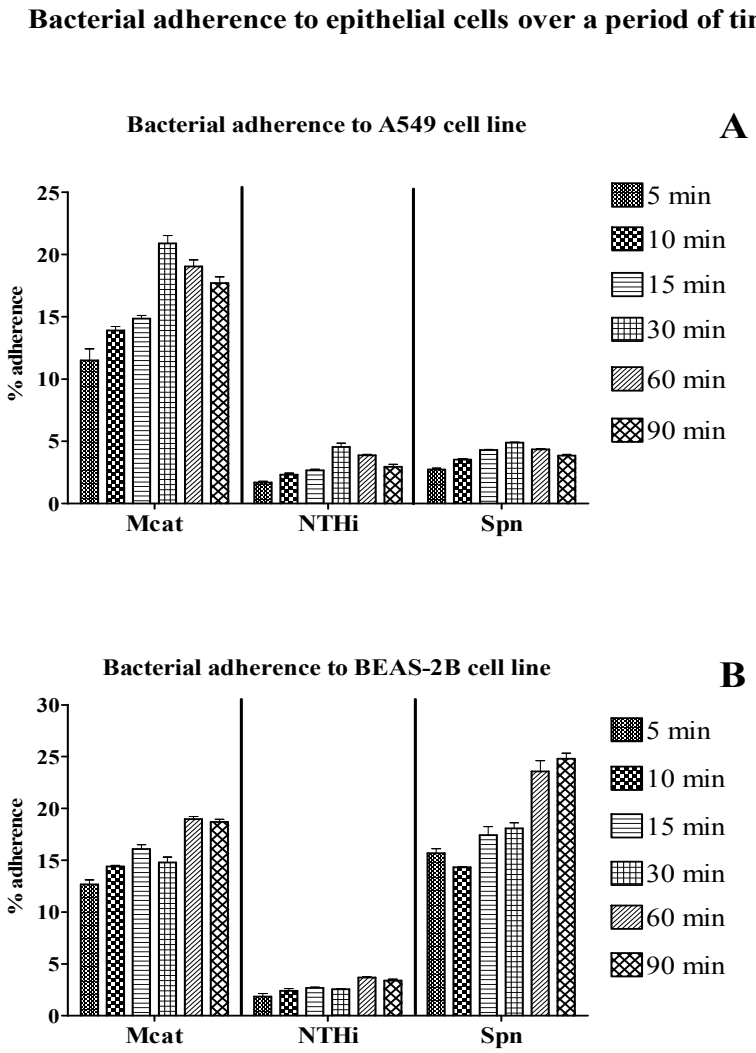
In this study, two different bacterial concentrations (10^7 CFU/ml and 10^{10} CFU/ml) were used to investigate bacterial adherence to epithelial cells. The percentage adherence of each bacterium to the different epithelial cell lines when infected alone, in different

combinations and in the presence or absence of adenovirus infection is shown in **Figure. 6.2-6.5**.

6.4.1 Establishment of adherence assay conditions

The ability of each bacterium to adhere to both A549 and BEAS-2B cells was tested at intervals from 5 min to 90 minutes. The time point at which each bacterium showed the highest level of adherence was chosen for the cell line infection protocol. Based on this standardisation, the highest bacterial adherence was observed at 30 minutes for A549 cells and 90 minutes for BEAS-2B cells **(Figure. 6.1A-B)**.

Figure 6.1: Bacterial adherence to epithelial cells. The results indicate the percentage adherence of each bacterium to the epithelial cells versus time. The bars represent the mean ± SEM of the percentage adherence of the indicated bacterium to A] A549 and B] BEAS-2B cells.



6.4.2 Bacterial adherence to A549 cells

The adherence levels of *M. catarrhalis* when infected alone using 10^7 CFU/ml was similar in both virus-infected and non-virus infected groups. However, the adherence of *S. pneumoniae* to the adenovirus infected cells was significantly greater than that

observed in the non-virus infected cells (# p<0.001) **(Figure. 6.2A)**. A synergistic relationship was observed between *M. catarrhalis* and *S. pneumoniae* when co-infected together which increased their adherence to the virus-infected cells as compared with the non-virus infected cells (# p<0.001) **(Figure. 6.2B)**. The adherence of both *M. catarrhalis* and *S. pneumoniae* when co-infected together was greater than when infected alone **(Figure. 6.2A-B)**. The adherence of *M. catarrhalis* when co-infected with NTHi was significantly higher to the virus-infected cells compared to the non-virus infected cells (* p<0.05) **(Figure. 6.2B)**. The bacterial adherence was low for all 3 bacteria when infected together and was not enhanced by adenoviral infection of the cells **(Figure. 6.2C)**.

At the higher bacterial concentration (10^{10} CFU/ml) the same trends in adherence was observed as for the 10^7 CFU/.ml. Adherence of *M. catarrhalis* and *S. pneumoniae* was significantly higher to the virus-infected cells when compared with the non-virus infected cells (*p<0.01 and # p<0.001, respectively) **(Figure. 6.3A)**. In addition to the synergism between *M. catarrhalis* and *S. pneumoniae* observed when co-infected using the higher bacterial concentration, adherence of *S. pneumoniae* to the virus-infected cells in combination with *M. catarrhalis* was significantly higher when compared with the non-virus infected cells (#p<0.001) **(Figure. 6.3B)**. In contrast, adherence of *M. catarrhalis* was similar in both virus-infected and non-virus infected A549 cells **(Figure. 6.3B)**.

Figure 6.2: Bacterial adherence to non-virus and adenovirus-infected A549 cells at 10^7 CFU/ml. The bars represent mean ± SEM of the percentage of bacteria adherent to the A549 cells when infected with A] a single bacterium (*M. catarrhalis*, NTHi or *S. pneumoniae*); B] double bacteria combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; or NTHi + *S. pneumoniae*), and, C] triple bacteria combination (*M. catarrhalis* + NTHi + *S. pneumoniae*) [*p<0.05, #p<0.001].

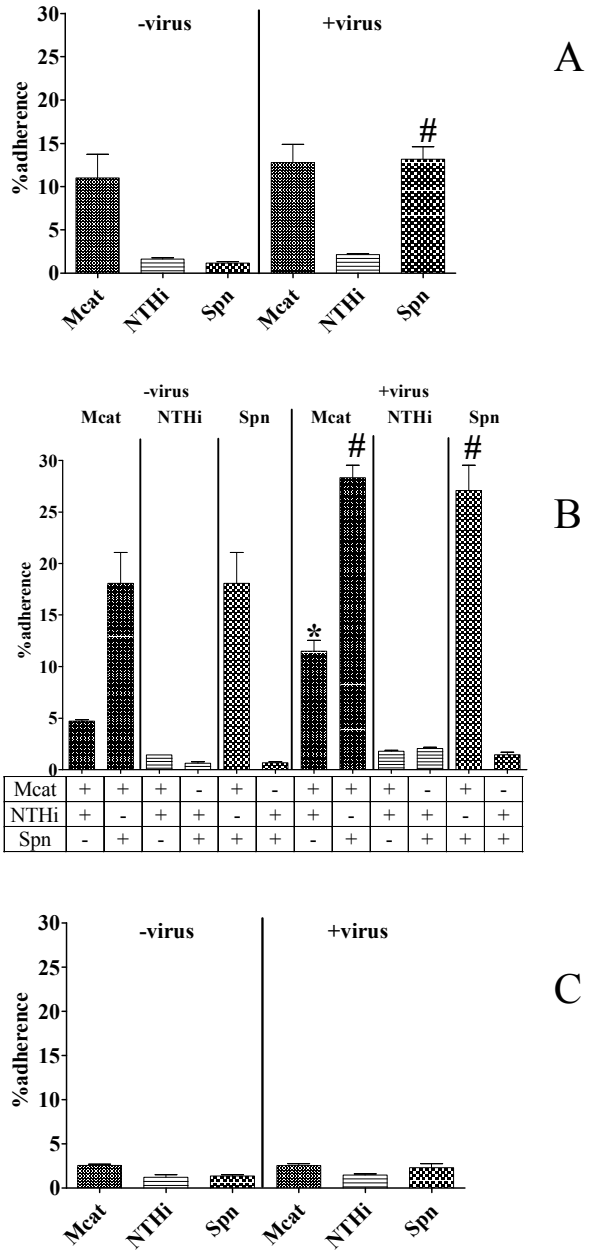
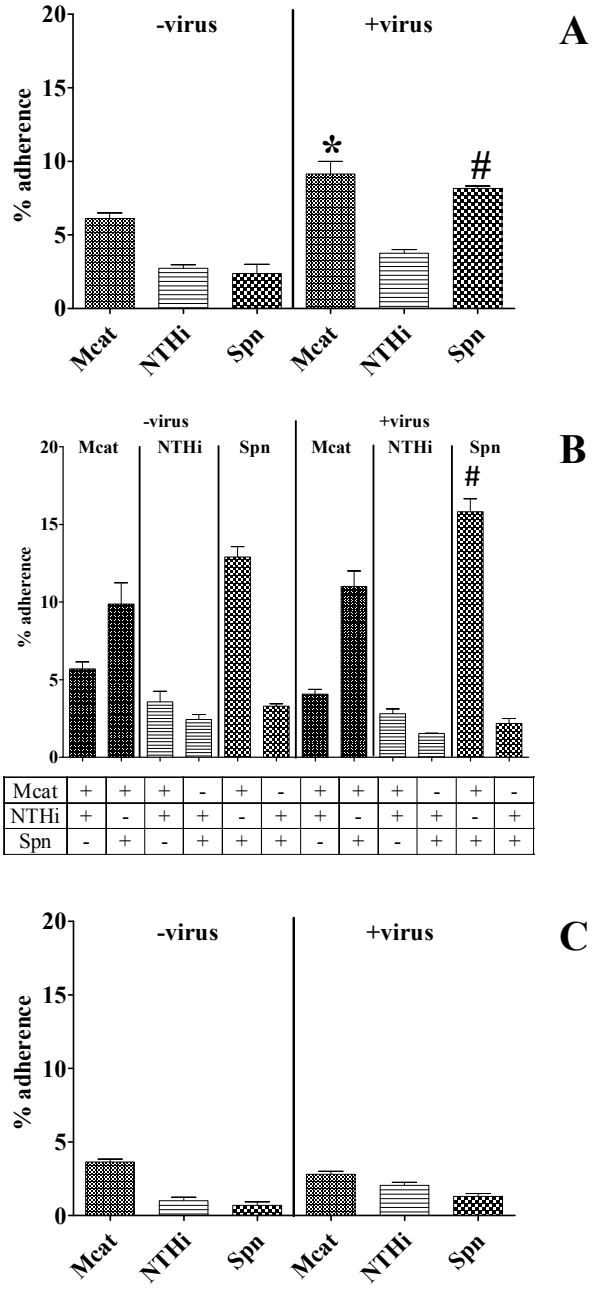


Figure 6.3: Bacterial adherence to non-virus and adenovirus-infected A549 cells at 10^{10} CFU/ml. The bars represent mean ± SEM of the percentage bacterial adherence to A549 cell line when infected with A] a single bacterium (*M. catarrhalis*, NTHi or *S. pneumoniae*); B] double bacteria combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; or NTHi + *S. pneumoniae*), and, C] triple bacteria combination (*M. catarrhalis* + NTHi + *S. pneumoniae*)[*p<0.01 #p<0.001].



6.4.3 Bacterial adherence to BEAS-2B cells

The adherence of *M. catarrhalis* and *S. pneumoniae* when infected alone using 10^7 CFU/ml was greater to virus-infected cells when compared with non-virus infected cells (# p<0.001) **(Figure. 6.4A)**. Moreover, the adherence of these two bacteria to BEAS-2B cells was higher when infected singly when compared to the percentage adherence to A549 cells **(Figure. 6.2-6.5)**. However, in the double and triple bacterial combination groups, bacterial adherence was lower in comparison with the single infection group **(Figure. 6.4B-C)**. At the higher bacterial concentration (10^{10} CFU/ml), the ability of bacteria to adhere to the BEAS-2B cells showed similar trends to that observed with the lower concentration (10^7 CFU/ml) **(Figure. 6.5A-C)**. Overall, bacteria were able to adhere more efficiently when infected alone and their adherence was greater to the virus-infected cells in comparison with the non-virus infected cells. The general percentage bacterial adherence to BEAS-2B cells line did not tend to increase with the higher infection dose of bacteria.

Figure 6.4: Bacterial adherence to non-virus and adenovirus-infected BEAS-2B cells at 10^7 CFU/ml. The bars represent mean ± SEM of the percentage bacterial adherence to BEAS-2B cells when infected with A] a single bacterium (*M. catarrhalis*, NTHi or *S. pneumoniae*); B] double bacteria combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; or NTHi + *S. pneumoniae*), and, C] triple bacteria combination (*M. catarrhalis* + NTHi + *S. pneumoniae*)[#*p*<0.001].

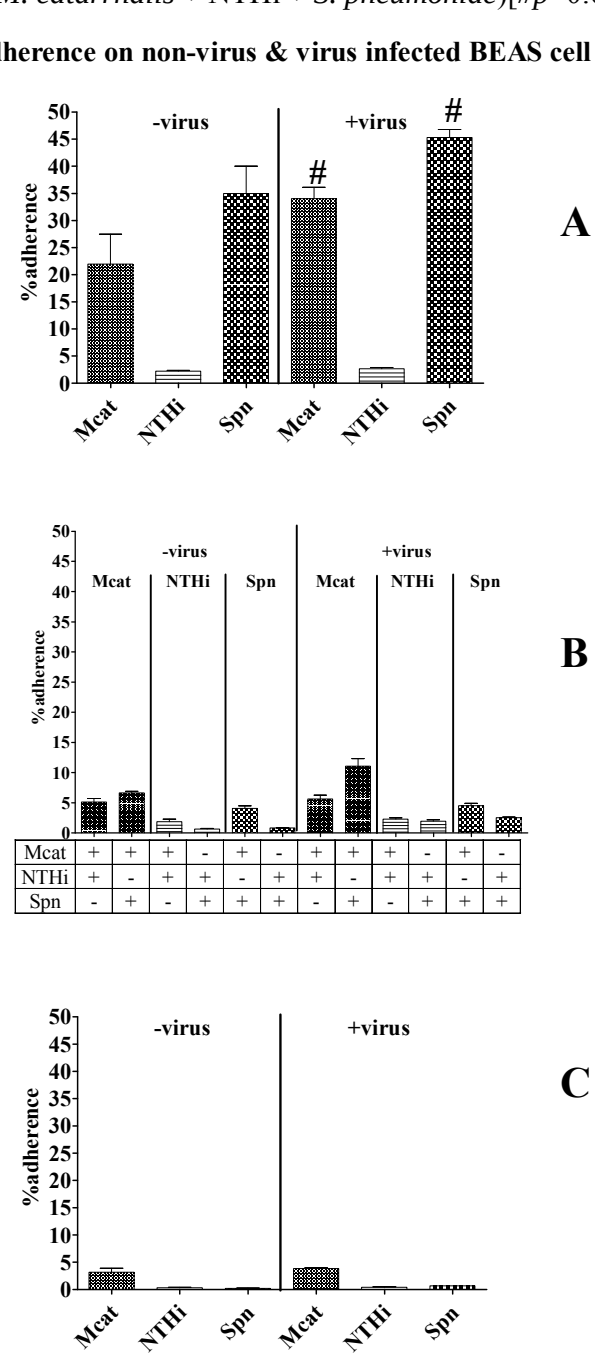
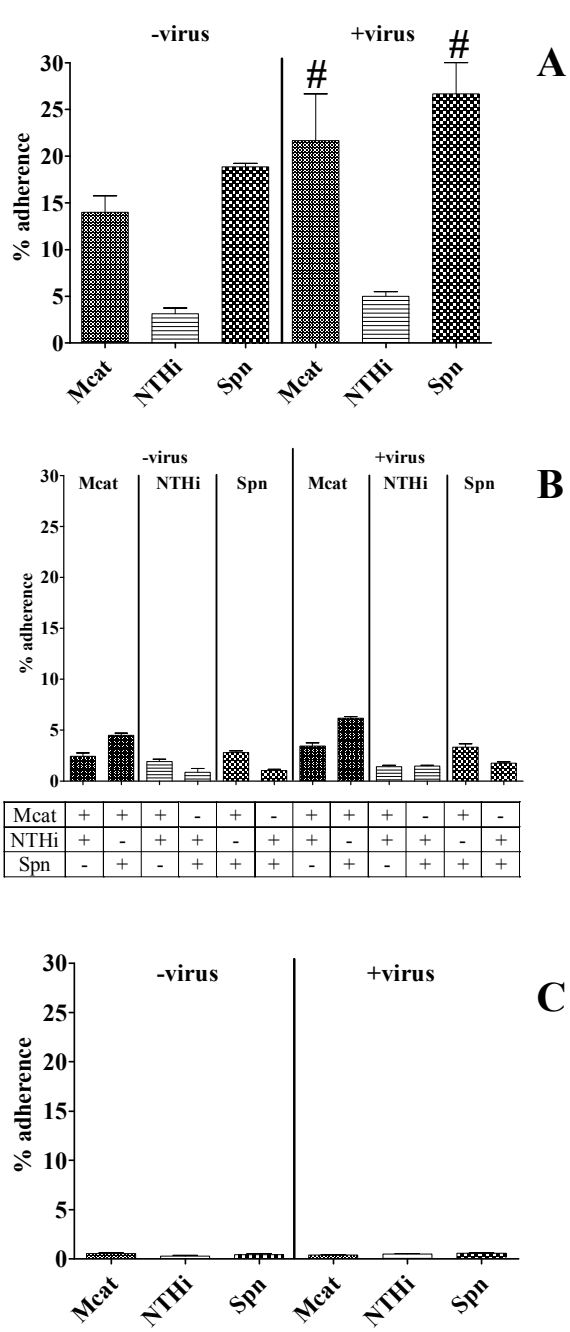


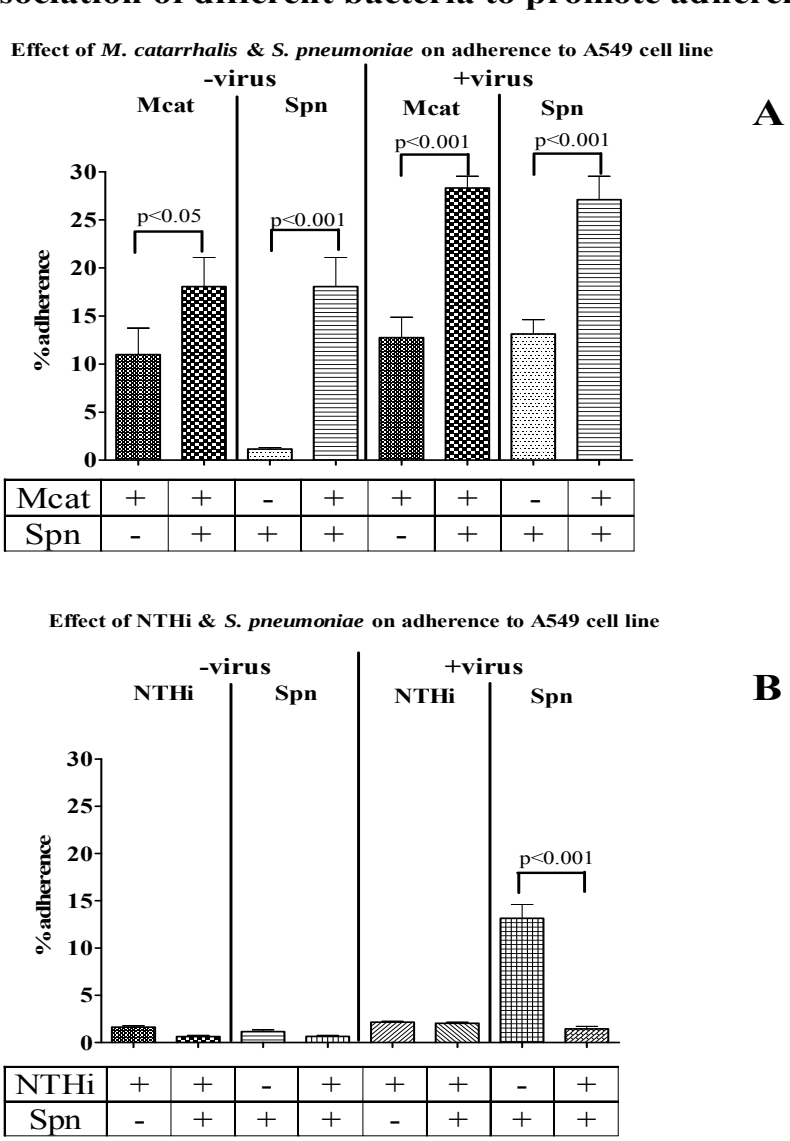
Figure 6.5: Bacterial adherence to non-virus and adenovirus-infected BEAS-2B cells at 10^{10} CFU/ml. The bars represent mean ± SEM of the percentage bacterial adherence to BEAS-2B cell line when infected with A] a single bacterium (*M. catarrhalis*, NTHi or *S. pneumoniae*); B] double bacteria combination (*M. catarrhalis* + NTHi; *M. catarrhalis* + *S. pneumoniae*; or NTHi + *S. pneumoniae*), and, C] triple bacteria combination (*M. catarrhalis* + NTHi + *S. pneumoniae*)[*#p*<0.001].



6.4.4 Association between different bacteria to promote adherence

The results in **Figure. 6.6** demonstrate the association between different bacteria in promoting adherence to the virus-infected and non-virus infected A549 cells. Importantly, it shows the impact of co-infection on enhancing adherence of *M. catarrhalis* and *S. pneumoniae*, and NTHi and *S. pneumoniae* in comparison with single bacterial infection **(Figure. 6.6A-B)**. In addition, it also shows that the ability of *S. pneumoniae* to adhere to virus-infected and non-virus infected A549 cells is significantly higher in presence of *M. catarrhalis* ($p<0.001$) than NTHi. In contrast, in the presence of NTHi, the adherence of *S. pneumoniae* to the virus infected cells was significantly reduced ($p<0.001$).

Figure 6.6: Association between bacteria in promoting adherence to A549 cells. Effect of *M. catarrhalis* and *S. pneumoniae* (A), and NTHi and *S. pneumoniae* co-infection (B), on adherence to virus-infected and non-virus infected A549 cells. The bars represent the mean ± SEM of percentage bacterial adherence when infected alone and in combination. Significance *p*-values are indicated with bars denoting the comparison.



6.5 DISCUSSION

The interaction between microbes and the host often begin at the mucosal surface of the nasopharynx. In most URTIs, viral infection is a well recognised trigger for secondary bacterial infections. Viral infections are known to cause damages to the respiratory epithelium, and mucociliary dysfunction leading to increased bacterial adherence. Various *in vitro* and *in vivo* studies have demonstrated this bacterial-viral synergy in promoting increased bacterial adherence (Hakansson *et al.* 1994; Patel *et al.* 1992). In addition, the selective role of respiratory viruses to predispose secondary bacterial infections has also been reviewed (Bakaletz 1995). The role of respiratory viruses in enhancing secondary bacterial infections was also demonstrated in the experimental OM animal model in Chapter 2. Furthermore, it also showed that the co-presence of bacteria such as *M. catarrhalis* and *S. pneumoniae* along with a viral infection increased the severity of middle ear infection. This was further demonstrated in Chapter 5 where there were significant changes to the nasopharyngeal colonisation by *S. pneumoniae* when concurrently or sequentially inoculated into the nose with another bacterium. These significant findings led to further exploration of the microbe-host dynamics involved in nasopharyngeal colonisation leading to various respiratory infections. The effect of bacterial interactions with each other and the host in a polymicrobial environment that could contribute to causing an infection was therefore investigated using an *in vitro* cell culture model. In addition, this study also investigated whether the bacterial concentration affected the interaction or competition to adhere.

This study demonstrated increased pneumococcal adherence to an adenovirus infected A549 cell line, when infected alone. In addition, there was a reproducible synergistic association between *M. catarrhalis* and *S. pneumoniae* that increased their adherence on both non-virus and virus-infected A549 cells which was further enhanced in the presence of adenovirus. The ability of adenovirus to enhance pneumococcal adherence has been previously reported (Hakansson *et al.* 1994). According to the Hakansson study (1994), the increased adherence was shown to be bacterial and viral strain specific. Adenovirus serotypes that are capable of causing respiratory tract infections

(types 1, 2, 3 and 5) enhanced adherence of capsular serotypes of *S. pneumoniae* to A549 cells. It was also suggested that adenovirus increased the expression of receptors on the A549 cells required for the binding of *S. pneumoniae*. Certain studies have reported increased expression of intracellular adhesion molecules (ICAM-1) following infection with adenovirus (Chang *et al.* 2002). Recently, binding of NTHi and *S. pneumoniae* to the glycoprotein of RSV was demonstrated *in vitro* on respiratory epithelial cells such as A549 and Chinese Hamster ovary epithelial cells (Avadhanula *et al.* 2007). The involvement of host cell platelet-activating factor receptor (PAF-r) and choline binding proteins in facilitating bacterial adherence and colonisation is also known (Hammerschmidt 2006; Kim & Weiser 1998). However, a recent study has shown that the enhanced pneumococcal adherence to influenza virus infected A549 cells was not mediated by PAF-r (McCullers *et al.* 2008). In addition, the McCullers study (2008) also demonstrated similar results *in vivo* in mice model.

Transformed cell lines used in *in vitro* studies have been suggested to have different number of stimulated surface receptors (Avadhanula, *et al.* 2006). In addition, the Avadhanula study (2006) also demonstrated that different respiratory epithelial cells responded differently to the same infecting agent. It is also known that different epithelial surfaces have different receptors that interact with certain types of bacteria. This study demonstrated increased levels of bacterial adherence on BEAS-2B cells compared to A549 cells when infected alone. In addition, *S. pneumoniae* often migrate from the nasopharynx and traverse bronchial epithelial cells to establish an infection. Hence, BEAS-2B cells could serve as a secondary site for attachment for pneumococci, supporting the high level of adherence observed in this study, especially with the single infection group.

In this study, the co-presence of different bacteria was shown to alter the dynamics of bacterial adherence to epithelial cells. Moreover, the adherence of NTHi to both A549 and BEAS-2B cells were almost similar, regardless of any bacterial combinations. In fact, the adherence of NTHi was lower in comparison with *M. catarrhalis* and *S.*

pneumoniae. The observation that the presence of NTHi appeared to reduce the adherence ability of the other bacteria to the epithelia cells in a number of combinations warrants further investigation. Since NTHi in general exhibited a low level of adherence, it would seem unlikely that the reduction was due to competition for the same cell receptor. The results suggest that NTHi binding to the epithelial cells may induce cell receptor changes that affect the ability of the other bacteria to adhere. In Chapter 5, the luminescent NTHi appeared to occupy a different niche to *S. pneumoniae* in the nasopharynx of the mice. Therefore, these observations warrant further investigation to determine their significance to host colonisation.

Phosphorylcholine (ChoP) in conjunction with LOS is known to enhance nasopharyngeal colonisation. Various mucosal pathogens expressing surface-exposed ChoP suggest that ChoP may contribute to the persistence or survival of several bacteria within this niche (Gillespie *et al.* 1996; Kolberg *et al.* 1997). In addition, *S. pneumoniae* is known to utilise ChoP within the cell wall teichoic acid, and expression of ChoP on the LOS of NTHi enables them to bind to the PAF-r on host cells (Cundell *et al.* 1995; Swords *et al.* 2000). Moreover, neuraminidase expressed by *S. pneumoniae* has been shown to desialylate the cell surfaces of *H. influenzae* and *N. meningitidis* (Shakhnovich *et al.* 2002). An *in vivo* experimental animal study has also suggested that the removal of sialic acid residues by neuraminidase could expose new carbohydrate residues that may serve as receptors for adherence of *S. pneumoniae* (Tong *et al.* 2001). These observations could suggest that different bacteria compete with each other for the available receptors and promote adherence and colonisation. *In vivo* and *in vitro* gene expression studies have been previously used to study host-microbe interactions reflecting on the specific host reactions to various pathogens (Chen *et al.* 2005; Ichikawa *et al.* 2000). Future experiments involving microarray technology could be used to investigate the complex interplay between bacterial pathogens and the host, especially in a polymicrobial environment, and the results could provide an insight to differences in the disease pathogenesis.

In this study, bacterial adherence of *M. catarrhalis* and *S. pneumoniae* to A549 cells was higher in the double bacterial infection group in comparison with single infection group. This could suggest that these bacteria are able to adhere to the respiratory epithelium more efficiently in the presence of each other, as opposed to when infected alone. This association was also shown to be dependent on the bacteria and epithelial cell type, as co-infection with NTHi and *S. pneumoniae* did not affect their adherence to both A549 and BEAS-2B cells. This type of association has also been demonstrated in a clinical setting involving children less than 2 years of age, in the context of nasopharyngeal carriage (Jacoby *et al.* 2007), and also in an experimental OM animal model in the context of an increased incidence of pneumococcal OM in the presence of *M. catarrhalis* (Chapter 2). This positive association between bacteria could suggest that co-occurrence of two pathogens within a host often create conditions conducive to adherence of another and contribute to the establishment of an infection. This is evident in the case of nasopharyngeal carriage, especially in Australian Aboriginal children where the carriage rates are as high as 49%, 50% and 41% for *S. pneumoniae*, *M. catarrhalis* and NTHi respectively (Watson *et al.* 2006). In contrast, this study demonstrated the association between *M. catarrhalis* and *S. pneumoniae* in the context of airway infections.

Another important observation in this chapter was the enhanced adherence of *M. catarrhalis* to adenovirus-infected BEAS-2B cells. Whilst the role of adenovirus in enhancing pneumococcal adherence to BEAS-2B cells has been previously reported (Adamou *et al.* 1998), no studies have reported the increased adherence of *M. catarrhalis* to BEAS-2B cells. However, in relation to OM, adenovirus has not been shown to predispose *M. catarrhalis* induced or pneumococcal induced OM in the experimental chinchilla model (Bakaletz *et al.* 1995; Tong *et al.* 2000a). The adenovirus used in this study is commonly involved with respiratory tract infections. The predisposition of *M. catarrhalis* infections by adenovirus in this study could have clinical relevance, especially in adults suffering from COPD, as *M. catarrhalis* along

with NTHi are the commonly isolated bacteria known to cause severe exacerbations in adults suffering from COPD.

6.6 CONCLUSION

In conclusion, this study has highlighted the synergism between *M. catarrhalis* and *S. pneumoniae* in enhancing their adherence to A549 cells. Moreover, the co-presence of different bacteria was shown to alter the dynamics of bacterial adherence to epithelial cells. The reduction in adherence of *M. catarrhalis* and *S. pneumoniae* when in specific combinations with NTHi warrants further investigation on how NTHi may be affecting the cell surface molecules required by the other bacteria. Bacterial adherence to the respiratory epithelium was found to be consistent at both concentrations, indicating that the observations were not due to competition for adherence molecules. The findings are in agreement with the data in the experimental mouse model in which the ability of bacteria to cause lower respiratory infections was not affected by increased bacterial numbers (Chapter 3). The ability of the same infecting agent to adhere differently to lung and bronchial epithelium could also suggest the involvement of different mechanisms of adherence to cells at these sites.

CHAPTER 7

Cytokine responses by respiratory epithelia in response to polymicrobial infection *in vitro*

7.1 ABSTRACT

The respiratory infections caused by commensals such as *M. catarrhalis*, NTHi and *S. pneumoniae* are often characterised by inflammatory responses generated by recruitment of white blood cells following microbial interaction with the epithelial cells of the host. Moreover, the differences in the inflammatory cytokine responses during early stages of infection are also considered crucial in the development of a chronic inflammatory state. The above mentioned bacteria are capable of causing both upper and lower respiratory tract infections. This study was based on a hypothesis that the pulmonary burden could vary across the different compartments of the respiratory tract, leading to the induction of different cytokine responses. Previous studies have significantly contributed to the understanding of the inflammatory responses associated with respiratory infections. This study aimed to investigate the cytokine responses generated by both lung epithelial (A549) and bronchial epithelial (BEAS-2B) cell lines when infected with different combinations of the above mentioned bacteria. This study found increased levels of IL-6 released by BEAS-2B compared to A549 cells and a synergistic inflammatory response (IL-6 release) by BEAS-2B cells when co-infected with *M. catarrhalis* and *S. pneumoniae*. In addition, it also showed that a pre-viral infection did not augment the release of cytokines.

7.2 INTRODUCTION

Bacteria such as *S. pneumoniae*, NTHi and *M. catarrhalis* are among the many commensals that reside within the human nasopharynx and known to cause respiratory tract infections. The importance of these bacteria as causes of exacerbations in COPD patients is well known (Bandi *et al.* 2001; Murphy *et al.* 2005b). Moreover, the burden of disease caused by *S. pneumoniae* in both young children and adults worldwide is well reported (Farha & Thomson 2005; Rudan *et al.* 2008).

Microbial interaction with the host often begins at the mucosal surface of the nasopharynx, therefore, bacterial adherence to the respiratory mucosa is an important

step towards the establishment of respiratory tract infections. The airway epithelial cells protect the host from various infectious agents by mechanisms such as the mucociliary functions on certain cells, induction of pro-inflammatory mediators that trigger host responses and production of antimicrobial peptides that help eliminate invading pathogens (Bals & Heimstra 2004). In the compromised host, the different virulence factors produced by bacteria contribute to the disease process by interacting with the host. These interactions can result in the production of inflammatory mediators such as interleukin-6 (IL-6), IL-1β, tumor necrosis factor alpha (TNF-α) and chemokines such as IL-8 and macrophage chemotactic protein 1 (MCP-1). The production of cytokines and chemokines along with the recruitment of white blood cells such as phagocytes, lymphocytes and dendritic cells are part of the inflammatory cascade associated with the host's response to infection (Clemans *et al.* 2000; Yoon *et al.* 2007).

In URTIs, the inflammatory response generated by the host to the above mentioned pathogens and the subsequent release of various cytokines such as IL-6, IL-8, IFN-γ, TNF-α, IL-1β and IL-10 in the middle ear fluid of children with OM are also well known (Skotnicka & Hassmann 2000; Skotnicka & Hassmann 2008). In these studies, there appeared to be no correlation between the clinical status of the children and the levels of the cytokines, suggesting that the duration time (persistence) of inflammation might be the important contributor to middle ear musosa pathology. Certain *in vitro* studies have also demonstrated the release of cytokines such as IL-1β, IL-6, IL-8 and TNF-α in response to different respiratory viruses that are commonly involved with LRTIs (Arnold *et al.* 1994; Terajima *et al.* 1997; Yoon *et al.* 2007). Studies of both respiratory viruses and bacterial infection have contributed to the development of understanding how the release of inflammatory mediators by respiratory epithelium contributes to the pathogenesis of LRTIs (Khair *et al.* 1996; Message & Johnston 2004). The induction of cytokines by the epithelial cells as a result of an interaction with different bacterial virulence factors of bacteria, such as LPS, toxins etc., are also well documented (Clemans *et al.* 2000; Fink *et al.* 2006; Khair *et al.* 1994). Commensal flora appear able to alter the levels of cytokine production and it was recently shown that

concurrent stimulation by *S. pneumoniae* and *H. influenzae* resulted in amplification of the pro-inflammatory responses (Ratner *et al.* 2005).

The differences in inflammatory cytokine responses during early stages of infection are considered crucial in the development of a chronic inflammatory state. In addition, certain pattern-recognition receptors like CD14 and TLRs are a part of the innate immune system and are present on phagocytic and epithelial cells and recognise the PAMPs such as lipopolysaccharide (Schulz *et al.* 2002). The bacteria *M. catarrhalis*, NTHi and *S. pneumoniae,* are nasopharyngeal commensals sharing common niches and are known to cause both URTIs and LRTIs. The invasion by these bacteria into the epithelial cells, thereby avoiding extracellular immune recognition by TLRs, is a well known strategy that enables them to colonise the respiratory tract (Slevogt *et al.* 2007).

The pulmonary burden caused by *M. catarrhalis,* NTHi and *S. pneumoniae* could be different in the various respiratory compartments. Therefore, in this study it is hypothesized that different cytokine responses may be generated by bronchial epithelium and alveolar epithelium when co-infected with these bacteria. The cytokines chosen in this study were TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and IL-12, whose importance in both URTIs and LRTIs have been identified in clinical studies. In general, most studies have investigated the release of specific cytokines by epithelial cells in response to a single bacterium and/or respiratory virus infection. The results in Chapter 6 indicated that there were differences in the adherence of these bacteria to both A549 and BEAS-2B cells when the bacteria were incubated with the cells alone or concurrent with the other bacteria. Both enhanced and reduced adherence patterns were observed (Chapter 6), suggesting differences in adherence mechanisms. This study aimed to investigate whether the differences in adherence observed in Chapter 6 corresponds to differences in the cytokine responses of the airway epithelia.

7.3 MATERIALS AND METHODS

7.3.1 Bacteria and virus infections

The bacterial strains used in this study and their preparations have been previously described in Chapter 2 (Section 2.3.1). Based on the optical density at 405 nm, the starting concentration of each bacterium was adjusted to 10^7 CFU/ml. Fifty µl of this concentration was used to infect the cell lines. The resulting infectious dose for each bacterium was 10^5 CFU/well. For double and triple bacterial combinations, equal volumes from appropriately concentrated bacterial suspensions were mixed and 50 µl was added to the designated wells. The human respiratory adenovirus-5 (kindly provided by Dr Jim Buttery), a clinical isolate from Royal Children's Hospital, Melbourne, Australia was used in this study. The starting concentration of adenovirus and the infectious dose used to infect the cell lines have been previously described in Chapter 6 (Section 6.3.1).

7.3.2 Epithelial cell culture

The cell culture conditions for the growth and maintenance of A549 and BEAS-2B cell lines have been previously described in Chapter 6 (Section 6.3.2).

7.3.3 Quantitation of virus

The concentration of virus was determined by using the lytic plaque assay (Hakansson *et al.* 1994), as previously described in Chapter 6 (Section 6.3.4).

7.3.4 Experimental groups

In this study, both A549 and BEAS-2B epithelial cell lines were bulk-seeded into 12-well tissue culture plates (Orange Scientific, Belgium) at a concentration of 5 x 10^5 cells/well. The following experimental groups were used to infect both non-virus infected and adenovirus infected A549 and BEAS-2B epithelial cells. The cell culture supernatants were collected at 12 hr and 24 hr post-infection with the bacteria and

screened for cytokines using the Bio-plex cytokine assay. The following infection groups were assayed:

A] Single bacteria: a) *M. catarrhalis*; b) NTHi; c) *S. pneumoniae* alone

B] Double bacterial combination: a) *M. catarrhalis* + NTHi; b) *M. catarrhalis* + *S. pneumoniae*; and c) NTHi + *S. pneumoniae*

C] Triple bacterial combination: *M. catarrhalis* + NTHi+ *S. pneumoniae*

7.3.5 Infection of cell monolayers

The cell monolayer infection protocol used has been previously described in Chapter 6 (Section 6.3.6). Following preliminary screening to determine the relevant incubation time for detection of the cytokines (data not shown) in this study the incubation time following bacterial infection in both adenovirus-infected and non-virus infected epithelial cell lines was increased to 12 hr and 24 hr. The cell culture supernatants were then carefully removed and stored in -80^0 C prior to screening for cytokines using the Bio-plex™ cytokine assay (Bio-Rad Laboratories, Inc., California, USA).

7.3.6 Cytokine analysis by Bioplex assay

The Bio-plex™ suspension array system (Bio-Rad Laboratories, California, USA) uses individually identifiable fluorescently dyed microspheres (beads) to perform a multiplex assay to quantitate levels of multiple cytokines from one sample. The following cytokines were included in the multiplex assay: TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and IL-12(p70). The assay was performed in 96-well filtration plates as per the manufacturer's instructions. Briefly, cytokine standards were prepared in the same cell culture medium that was used for culturing the cells and were assayed in duplicate. The anti-cytokine beads were added to each well and following the filter wash, cytokine standards and samples (cell culture supernatants) were added to the designated wells and incubated at room temperature for 30 minutes with continuous shaking. Following washing, biotinylated detection antibody was added to each well and incubated for further 30 minutes with continuous shaking. The filtration plate was washed again and streptavidin–phycoerythrin (PE) was added and incubated for 10 minutes with

continuous shaking. After further washes, the fluorescence intensity of the beads was measured using the Bio-Plex array reader and Bio-Plex Manager™ software, which automatically calculates the unknown concentrations from the standard curve.

7.4 RESULTS

7.4.1 Measurement of cytokines

The amount of the cytokines TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and IL-12(p70) released following bacterial infection of adenovirus and non-virus infected epithelial cell lines was assessed. Of these, the levels of IL-6 and IFN-γ (in some infection groups) on both A549 and BEAS-2B cell lines were considered. The levels of all other cytokines released were below the limits of detection for the assay.

7.4.2 Cytokines released following bacterial infection of A549 cells

The levels of different cytokines released following bacterial infection to adenovirus-infected and non-virus infected A549 cell lines are shown in **Figure.7.1-7.2 and Appendix C.1.1 to C.1.4**. In the single infection group, IL-6 was released at 12 hr from cells infected with *M. catarrhalis* and NTHi, but not *S. pneumoniae.* IL-6 was the only cytokine produced in any abundance from the A549 cells **(Figure.7.1-7.2 and Appendix C.1.1 to C.1.4)**. By 24 hours the levels were still elevated for *M. catarrhalis* infection, but were declining in the non-virus infected cells for NTHi and had reduced to below the detection limit in the virus infected cells.

In the double infection groups, the levels of IL-6 released were highest when infected with *M. catarrhalis* + *S. pneumoniae* followed by *M. catarrhalis* + NTHi in both virus and non-virus infected groups **(Figure. 7.1B)**. The amount of IL-6 produced was not markedly different from when *M. catarrhalis* was infected alone, therefore it is possible that the levels observed are predominantly due to a response to *M. catarrhalis.* In the non-virus infected cells, the NTHi + *S. pneumoniae* group had detectable levels of IL-6 released at both 12 and 24 hr, whereas in the virus-infected cells the much lower level

detected at 12 hr had returned to baseline by 24 hr. In the triple combination group, there were detectable levels of IL-6 produced at 24 hr post-infection in both sets of cells. IFN-γ was detected in the non-virus infected supernatants at 12 hr in the *S. pneumoniae*, and 24 hr in the *M. catarrhalis* infected cells only (**Figure. 7.2A)**. In the virus-infected cells all co-infection groups at 24 hr except *M. catarrhalis* + *S. pneumoniae* produced IFN- γ post-infection **(Figure.7.2B)**.

All the other cytokines were not produced at significant levels **(Appendix C.1.1 to C.1.4)**. TNF-α was just detectable in the non-virus NTHi group at 12 hr **(Appendix C.1.1)**; there was no detectable IL-10 **(Appendix C.1.2)** or IL-1β **(Appendix C.1.3)**; NTHi, *S. pneumoniae* and *M. catarrhalis* all stimulated just detectable levels of IL-12 (p70) at either 12 or 24 hr in non-virus infected cells, whereas only the *M. catarrhalis* group had a detectable level at 12 hr in the virus-infected cells **(Appendix C.1.4)**.

Figure 7.1: Levels of IL-6 released by non-virus and virus-infected A549 cells. The bars represent the median values of IL-6 released at 12 hr and 24 hr from A549 cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double or triple bacterial combinations (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-6 set by the instrument.

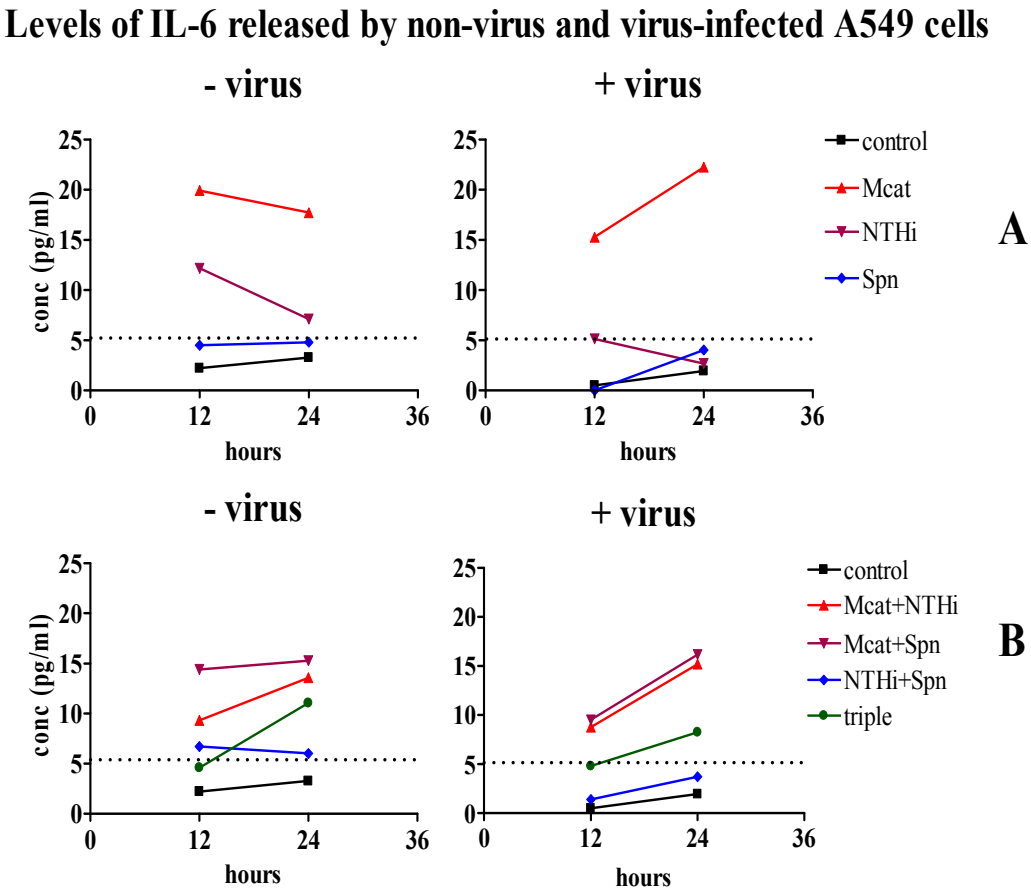
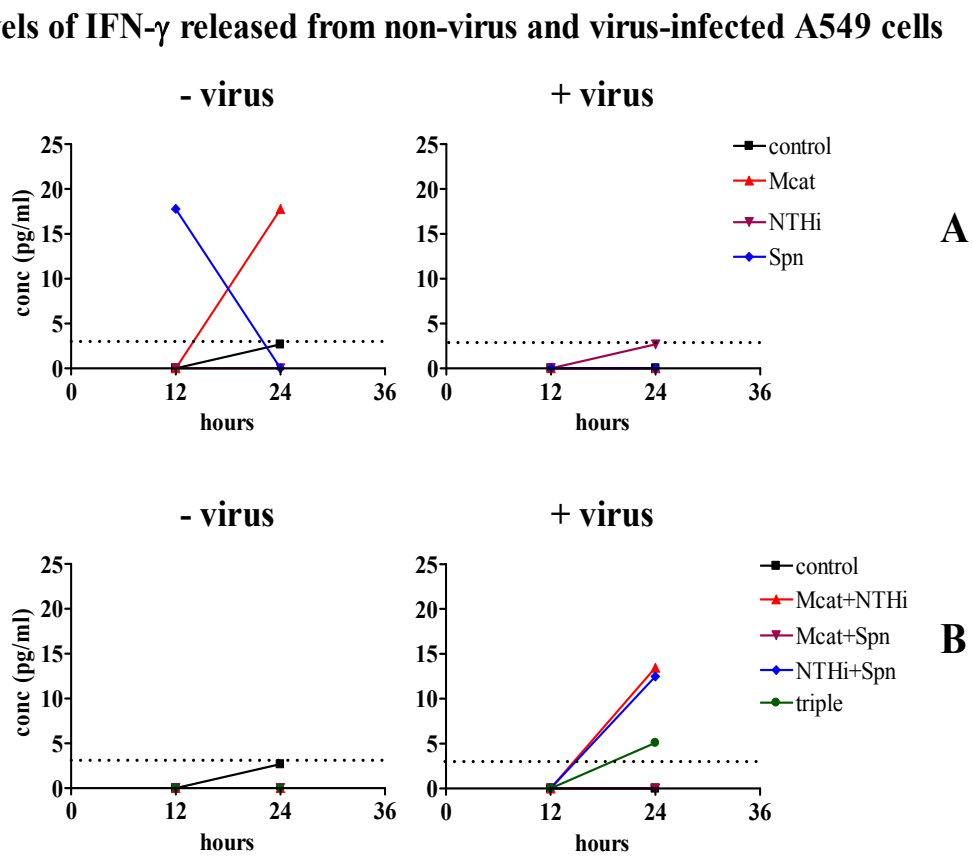


Figure 7.2: Levels of IFN-γ released from non-virus and virus-infected A549 cells. The bars represent the median values of IFN-γ released at 12 hr and 24 hr from A549 cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double or triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IFN-γ set by the instrument.



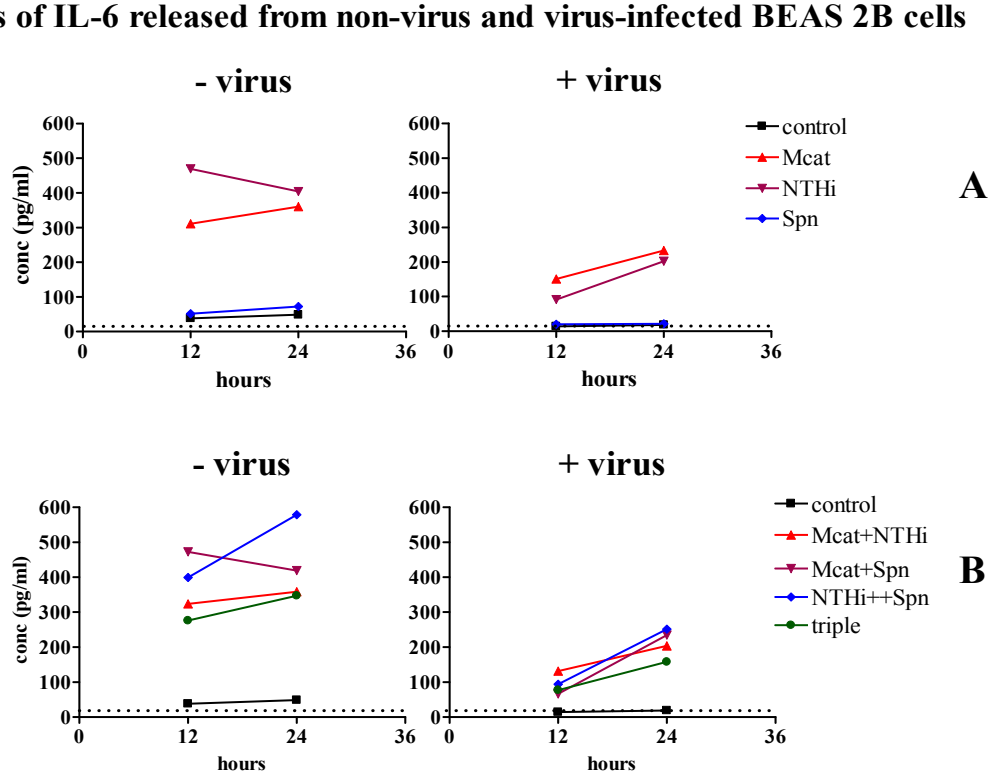
7.4.3 Cytokines released following infection of BEAS-2B cells

The measurement of different cytokines released following bacterial infection of adenovirus-infected and non-virus infected BEAS-2B cells is shown in **Figure.7.3 and Appendix C.2.1 to C.2.5**. As observed with A549 cell lines, IL-6 was the only cytokine

with recordable levels released by both virus-infected and non-virus infected BEAS-2B cells **(Figure.7.3)**, however, it should be noted that these cells constitutively expressed a low level of IL-6 in both virus- and non-virus infected groups. As a result, the concentration of IL-6 produced tended to be higher from the BEAS-2B cells than was observed from the A549 cells **(Figure.7.1 & 7.3)**. In contrast with A549 cell lines, when infected alone, IL-6 was highest in the NTHi infection group followed by *M. catarrhalis* and *S. pneumoniae* in the non-virus infected group in comparison with the virus-infected cells **(Figure. 7.3A)**. In the co-infection groups, the level of IL-6 release was higher when co-infected with *M. catarrhalis* + *S. pneumoniae* followed by NTHi + *S. pneumoniae* (12 hr post-infection) in the non-virus infected group in comparison with the virus-infected group **(Figure. 7.3B)**. The levels of IL-6 release in the non-virus infected group were almost similar at 12 hr and 24 hr post-infection in all groups except in the NTHi + *S. pneumoniae* co-infection group, in which higher levels of IL-6 were observed after 24 hr **(Figure. 7.3B)**. In the virus infected group, there were less noticeable differences between the combination groups with all producing high levels of IL-6.

IFN-γ was detected in the non-virus infected supernatants at 12 hr and 24 hr in the NTHi and *M. catarrhalis* infected cells only **[Appendix C.2.1 (A)]**. In the non-virus infected cells co-infection with *M. catarrhalis* + NTHi + *S. pneumoniae* produced IFN-γ at 12 hr **[Appendix C.2.1 (B)]**. All other cytokines were produced below the detectable levels **(Appendix C.2.2 to C.2.5)**.

Figure 7.3: Levels of IL-6 released from non-virus and virus-infected BEAS-2B cells. The bars represent the median values of IL-6 released at 12 hr and 24 hr from BEAS-2B cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double or triple bacterial combinations (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-6 set by the instrument.



7.5 DISCUSSION

The respiratory epithelium provides a niche for commensal bacteria and opportunistic pathogens to adhere and initiate colonisation, leading to an infection in most cases. The initial contact between the infecting bacterium and the respiratory epithelium often stimulates the release of several inflammatory mediators including cytokines and chemokines. These inflammatory mediators further amplify the process of inflammation

through activation and influx of white blood cells such as neutrophils, monocytes and macrophages (Clemans *et al.* 2000; Laberge & Bassam 2004). Many studies have demonstrated the release of cytokines *in vitro* in response to bacterial products such as lipopolysaccharides, pneumolysin, endotoxin and other bacterial gene products (Henderson *et al.* 1996; Khair *et al.* 1994). In addition, a recent study reported up-regulation of the genes that encoded for cytokines such as IL-6, IL-1β and chemokines such as IL-8 and CXCL1/2, in response to adherent pneumococci on Detroit 562 cells (Bootsma *et al.* 2007). This study has investigated the release of the cytokines IL-6, IL-10, IFN-γ, TNF-α, IL-1β and IL-12(p70) by A549 and BEAS-2B respiratory epithelial cells following infection with live bacteria in the presence or absence of an adenovirus co-infection. The study also investigated any differences in the pattern of cytokine response by respiratory epithelia when exposed to different bacterial combinations.

This study found IL-6 to be the common interleukin released by both A549 and BEAS-2B cells in response to different combinations of bacterial infection. IL-6 is produced by alveolar macrophages, fibroblasts, monocytes, lymphocytes and epithelial cells. Airway epithelial cells express certain pattern-recognition receptors such as TLRs that enables the innate immune system to recognise PAMPs used by pathogens, and enable cytokine production (Kato & Schleimer 2007). Airway epithelial cells expressing TLR2-6 recognises various PAMPs such as bacterial lipoproteins, peptidoglycan, teichoic acid, lipopolysaccharide, and viral dsRNA. The TLR initiates the activation of transcription factors such as NF-κβ and releases cytokines such as IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (Kato & Schleimer 2007; Prince *et al.* 2006).

The release of IL-6, IL-8 and TNF-α by human bronchial epithelial cells in response to *H. influenzae* endotoxin was shown to increase with the concentration of the endotoxin (Khair *et al.* 1994). In contrast, this study has demonstrated higher levels of IL-6 release from BEAS-2B cells in comparison with A549 cells in response to various bacterial combinations and a respiratory virus (adenovirus). The levels of TNF-α in this study were below the reportable range. The differences between these studies could be due to

use of different stimulant (endotoxin) and the greater concentration of the endotoxin in the Khair *et al.* study. Another study demonstrated the induction and gene expression of pro-inflammatory cytokines such as IL-6, IL-8, MCP-1, IL-1α, IL-1β and TNF-α from human respiratory epithelial cells in response to different strains of NTHi and different concentrations of NTHi LOS (Clemans *et al.* 2000). The Clemans study (2000) showed the induction of IL-6 to be strain dependent and the cytokine gene expression for both IL-6 and TNF-α was not affected by the increasing dose of NTHi LOS. In addition to bacterial LOS and endotoxins, certain outer membrane proteins and heat-killed bacteria have also been shown to stimulate the release of pro-inflammatory cytokines such as IL-6 and IL-8 (Fink *et al.* 2006). The Fink (2006) study demonstrated greater amounts of cytokine secretion (IL-6, IL-8 and IL-1β) in response to the OMPs of *M. catarrhalis* in comparison with heat-killed and non-agglutinating strains of *M. catarrhalis* strains. This could suggest the importance of OMPs binding to cellular receptors in the induction of an inflammatory response. The much higher levels of IL-6 release from *M. catarrhalis* infected A549 cells in the Fink study in comparison with this study could be due to the different *M. catarrhalis* isolates used in these two studies. All these studies: Clemans (2000), Fink (2006), Khair (1994) studies and results from this study have demonstrated that the induction of cytokines could depend on the epithelial cell type and stimulant used.

Studies have shown that the release of cytokines from respiratory epithelial cells peaks between 12 to 24 hr post-stimulation with bacteria, bacterial products, and also with different viruses (Clemans *et al.* 2000; Fink *et al.* 2006; Thorley *et al.* 2007; Yoon *et al.* 2007). This observation is also evident in this study, especially with the IL-6 release after stimulation of A549 and BEAS-2B cells with certain bacterial combinations **(Figure. 7.1B and 7.3B)**, and the IFN-γ release by non-virus infected A549 cells when stimulated with *M. catarrhalis* alone and virus-infected A549 cells with all co-infection groups **(Figure. 7.2)**.

The role of bacterial adherence in the stimulation of respiratory epithelial cytokine production has also been previously tested (Clemans *et al.* 2000). The Clemans study (2000) did not find any significant differences in IL-6 or IL-8 production by human tracheal epithelial cells when stimulated with adherent and non-adherent strains of NTHi. In contrast, increased IL-8 released by A549 cells in response to low-binding strains of *S. pneumoniae* as opposed to high-binding strains was recently demonstrated (Robson *et al.* 2006). The NTHi adherence to both virus-infected and non-virus infected A549 and BEAS-2B cells were very low in comparison to other bacteria (Chapter 6). However in this study, the IL-6 release from NTHi infected A549 was well above the detectable limit, and was the highest in the non-virus infected BEAS-2B cells (when infected alone). These conflicting results from the Robson (2006), Clemans (2000) and this study could suggest that the inflammatory response generated could depend on the bacteria and epithelial cell type, and bacterial binding to epithelial cells may not be the only process to initiate an inflammatory response. Enhanced bacterial adherence and colonisation following viral infection has been well recognised both *in vitro* and *in vivo* (Hakansson *et al.* 1994; Suzuki & Bakaletz 1994). The ability of a respiratory virus to enhance bacterial colonisation and adherence was found in chapters 2 and 6 of this thesis, respectively. However, the levels of cytokines released by both virus-infected A549 and BEAS-2B cells were generally lower than from the non-virus infected cells following bacterial infections. This could suggest that, under the testing conditions, the respiratory virus was less able to trigger the production of cytokines from the respiratory epithelia following bacterial infections, despite facilitating enhanced bacterial colonisation and adherence (Chapter 2 and 6).

Another interesting observation in this study was the ability of BEAS-2B cells to produce higher levels of cytokines than A549 cells. The release of IL-6 by BEAS-2B cells was approximately 15-20 folds higher than A549 cells following bacterial infections. The differential effects of cytokine release by BEAS-2B and A549 cells in response to lipopolysaccharide (LPS) has been previously demonstrated (Schulz *et al.* 2002). The Schulz study (2002) showed the effects of LPS and pattern-recognition

receptors such as CD14 and TLRs on the release of IL-6 and IL-8 by A549 and BEAS-2B cells. The differences observed implicated the requirement of different pathways leading to the induction of the cytokines. Recently, differential activation of cytokines and chemokine release by human alveolar macrophages and type II epithelial cells following LPS exposure has also been demonstrated (Thorley *et al.* 2007). The Thorley study (2007) showed that following LPS stimulation, alveolar macrophages secreted higher levels of IL-1β and TNF-α than type II epithelial cells, whereas, the levels of IL-6 release were higher in type II epithelial cells than alveolar macrophages. Although these studies demonstrated the difference using LPS, as opposed to live bacterial suspensions used in this study, the role of pattern-recognition receptors could explain the underlying mechanisms in induction of cytokines by different cell lines. Certain TLRs are involved in the recognition of specific bacterial products, for example, TLR-4 is involved with recognition of LPS from gram-negative organisms, whereas, TLR-2 recognises bacterial products from gram-positive organisms (Armstrong *et al.* 2004). The differences in the cytokines released by various infecting agents could also highlight the need to understand the underlying mechanisms involved with differential activation of cytokines.

This study also showed that the levels of IL-6 released from A549 cell lines following co-infection with *M. catarrhalis* and *S. pneumoniae* were much higher than that observed by *S. pneumoniae* infection alone. In contrast, the co-infection with *S. pneumoniae* and NTHi did not make any difference to the levels of IL-6 when compared with *S. pneumoniae* infection alone. The synergistic production of IL-8 *in vitro* has been well documented using *S. pneumoniae* and a NTHi strain (Ratner *et al.* 2005). Whilst, IL-8 was not included this study, the synergistic increase in IL-6 production by BEAS-2B cells, when co-infected with *M. catarrhalis* and *S. pneumoniae* in comparison with *M. catarrhalis* infection alone, especially in the absence of adenovirus, was demonstrated. This suggests differences in the priming of the inflammatory response during co-infection in comparison to single infection.

7.6 CONCLUSION

In conclusion, this study demonstrated that respiratory epithelia differ in their ability to produce IL-6 in response to same infecting agent or combination of agents. A synergistic increase in inflammatory response (IL-6 release) by bronchial cells was observed during co-infection with *M. catarrhalis* and *S. pneumoniae*. Although the respiratory virus enhanced bacterial adherence (Chapter 6), it did not augment cytokine production by respiratory epithelia. In addition, the inflammatory response generated by the respiratory epithelia may not entirely depend on the level of bacterial binding to the cells (Chapter 6).

CHAPTER 8

Final Discussion

FINAL DISCUSSION

This thesis has investigated the effect of polymicrobial infection on the induction of OM. The polymicrobial OM infection model was established in BALB/c mice, which is one of the first models to investigate all the predominant bacteria involved with OM and the affect of a respiratory virus (Sendai). This infection model allowed further identification of the various complexities associated with the infection such as: microbial interactions and host-bacterial relationships; dynamics involved in nasal colonisation; real-time co-colonisation patterns in the nasopharynx; and resulting differences in the inflammatory responses. Several animal models have been used to provide a better understanding of OM and the pathology associated with OM. Such studies have demonstrated the role of potential vaccine antigens against OM, inflammatory responses generated during OM, and bacterial-viral synergies using single bacterium and a virus, and are well reviewed (Bakaletz 2004; Lim *et al.* 2002; MacArthur & Trune 2006; Ryan *et al.* 2006; Sabirov & Metzger 2008; van der Ven *et al.* 1999). However, given the polymicrobial etiology of OM, no animal model has been established to investigate the underlying complex association between different bacteria that cause OM.

The experiments in this thesis were developed to better understand how the polymicrobial environment associated with nasopharyngeal carriage in children contributes to induction of OM. The significant findings in Chapter 2 were that the presence of multiple colonising bacteria in the nasal area, and pre-viral infection significantly increased the incidence and severity of OM. Importantly, the presence of *M. catarrhalis* as a co-colonising agent significantly enhanced pneumococcal OM in a polymicrobial environment. This is the first experimental evidence that demonstrated the potential importance of how the presence of *M. catarrhalis* in a polymicrobial environment could affect the disease state that appears to have been caused by other bacteria. The subsequent experiments were designed to further explore the effects of polybacterial infection on the incidence and severity of OM based on this finding.

The role of human respiratory viruses to predispose secondary bacterial infections is well known (Heikkinen & Chonmaitree 2003). In addition, human respiratory viruses do not always cause similar disease etiology in animal models, however the preferential ability of some respiratory viruses to predispose animals to bacterial OM has been demonstrated (Bakaletz *et al.* 1995; Heikkinen 2001). The human parainfluenza virus and the Sendai virus (used in this thesis in all animal experiments) belong to the same family of *Paramyxoviridae* (Bousse *et al.* 2006). In addition, Sendai virus also causes respiratory infections in its natural hosts (rodents). Therefore, the use of Sendai virus in this thesis was used as a prototype for studying human respiratory infections in the experimental BALB/c mouse model. The results of this thesis demonstrated that a pre-viral intranasal inoculation enhanced both single and co-bacterial infections (Chapter 2). In addition, the pre-viral infection along with increasing nasal bacterial load and time post-inoculation were all significantly important to increasing the incidence of pneumococcal OM (Chapter 3). A positive association between specific virus and bacteria, and different bacterial combinations has speculated upon the possibility of certain colonising patterns of bacteria, which could contribute towards establishment of an infection (Jacoby *et al.* 2007). The role of the acquisition of a new bacterium (*S. pneumoniae*) in the nasopharynx in the presence of a pre-existing nasal coloniser (*M. catarrhalis*) and a pre-viral infection resulted in a greater incidence of pneumococcal OM (Chapter 4). In addition to supporting the role of a respiratory virus in facilitating bacterial infection and causing OM, these results also demonstrate its ability to predispose bacterial infections in the presence of increasing bacterial numbers and as well as during sequential bacterial infections. The role of respiratory virus in microbe-host interactions and promotion of bacterial adherence to respiratory epithelia was demonstrated *in vitro* (Chapter 6). However, it did not augment cytokine production from respiratory epithelial cells *in vitro* (Chapter 7).

Another important observation in this thesis was the positive association between *M. catarrhalis* and *S. pneumoniae*. This association facilitated pneumococcal OM during co-infection experiments (Chapter 2) and sequential nasal bacterial inoculation (Chapter

4). The positive association between these two bacteria has been recently demonstrated using statistical modeling on the colonisation patterns between these two bacteria, suggesting preferential colonisation by *S. pneumoniae* in the presence of *M. catarrhalis* (Jacoby *et al.* 2007). This specific bacterial combination is of significant concern especially in Australian Aboriginal communities, as previous research has shown high bacterial nasopharyngeal carriage rates by these bacteria within 2 months of age (Leach *et al.* 1994; Watson *et al.* 2006), which increases the risk for recurrent OM. In addition, *M. catarrhalis* is also one of the earliest colonisers of the respiratory tracts of infants and Australian Aboriginal children have one of the highest and earliest colonisation rates in the world (Leach *et al.* 1994; Watson *et al.* 2006). The greater incidence of pneumococcal OM in the presence of *M. catarrhalis* (Chapter 2) and during pre-colonisation experiments (Chapter 4) could also suggest a detrimental effect of early *M. catarrhalis* colonisation on incidence rates of OM in these communities. The complications involved with untreated OM or recurrent OM with effusion lead to hearing loss, abnormality in speech, language, behavioural and cognitive development (Bluestone & Klein 1988; Haggard 1996), which could further lead to poorer educational and employment outcomes (Leach 1999).

Recently, high density nasal bacterial carriage has been linked to the persistence of OM in children (Smith-Vaughan *et al.* 2006). Therefore, the effect of bacterial concentrations in the nasal inoculum on the incidence of respiratory tract infections was investigated (Chapter 3). The incidence of both lower respiratory tract infections and OM caused by *S. pneumoniae* was found to be greater with the increasing nasal bacterial load. However, the increasing nasal bacterial carriage did not result in invasive lung infection by *S. pneumoniae*. The early onset of *M. catarrhalis* nasopharyngeal colonisation in Australian Aboriginal children (Leach *et al.* 1994), the positive association between *M. catarrhalis* and *S. pneumoniae* for clinical OM (Jacoby *et al.* 2007) and the results in this thesis (Chapters 2, 3 and 4) have provided an insight into factors that contribute to the existing burden of OM in children and more importantly in certain high-risk communities.

The higher bacterial colonisation rates of *S. pneumoniae*, NTHi and *M. catarrhalis* in children have been previously reported (Faden 2001; Leach 1999; Watson *et al.* 2006). The incidence rate and severity of infections caused by these bacteria when infected alone and/or in different combinations along with a respiratory virus have been investigated using lavage samples and culture methods (Chapters 2, 3 and 4). In contrast, the impact of the polymicrobial environment in the nasopharynx on the incidence rate of OM was shown using real-time monitoring of disease progression in adenovirus-infected BALB/c mice (Chapter 5). In addition, the results also demonstrated the differences in the colonising patterns by bacteria when infected alone and during co-infection, and localisation of different niches within the nasal cavity. The preferential adherence of NTHi to mucous contained in the ET floor region and not the middle ear epithelium has been demonstrated *in vitro* (Miyamoto & Bakaletz 1996). Previous studies have used the real-time monitoring of disease progression to explain the kinetics involved with ascension of single bacterium (Novotny *et al.* 2005), and different strains of pneumococci (Orihuela *et al.* 2003). In contrast, the results in Chapter 5 have demonstrated the real-time imaging of the kinetics of bacterial ascension involved, nasal co-colonisation patterns and distribution of bacteria within the nasal cavity and bullae, in a polymicrobial environment. In addition, it also showed an increased ability of pneumococci to colonise the pharynx and localise at a distal site to the initial inoculated site, in the presence of a pre-existing coloniser (*M. catarrhalis*) in the nasopharynx.

Studies using culture and PCR methods often underestimate the true bacteriology of OM and reporting false-positives in case of PCR studies and false negatives in the case of bacteriology. This is because most studies rely on interpreting the incidence of OM based on lavage samples and even though this technique offers relative incidence of OM, it fails to deliver any information regarding the bacteria residing in the nasal cavity and within middle ear tissues. These limitations were overcome to some extent with the use of *in vivo* biophotonic imaging technology, which detects the bioluminescence signal emitted by the luminescent bacteria (Chapter 5). Although the imaging

technology has great sensitivity to detect bioluminescence, there are certain limitations. The detection of OM in this study has been assessed using different analysis methods such as bacteriology (culture methods), relative luminescent intensity and otoscopy in Chapter 5, as well as with inflammatory responses (Chapters 2, 3, 4 and 5). The imaging technology successfully detected the bioluminescence signal from the cohorts that were culture negative, however also in some cases, culture methods detected the bacteria which could not be detected by bioluminescence. In contrast, the clinical representation of OM was observed through otoscopy in most of the animals irrespective of their bioluminescence and bacteriology results. This could suggest that the newer and modern technologies may have increased sensitivity in detecting pathogens *in vivo*, but the assessment of OM should be performed in conjunction with the prominent clinical representation observed by otoscopy.

The ability of *S. pneumoniae* to increase the severity of infection in the presence of other bacteria, and rapidly ascend the pharynx in the presence of a pre-coloniser (*M. catarrhalis*) in the nasopharynx was visualised with the aid of bioluminescence (Chapter 5). These findings highlight the underlying complex association between different bacteria that cause OM, and also corroborated the previously known positive association between *M. catarrhalis* and *S. pneumoniae* (Jacoby *et al.* 2007). The imaging results can also be linked to the early inflammatory response (as detected with nitric oxide levels in the middle ear) generated following co-inoculation with *M. catarrhalis* and *S. pneumoniae* and the slower (day 3) inflammatory response following single bacterium inoculation (Chapter 2). The rapid ascension of pneumococci across the nasopharynx in the imaging experiments was similar to the early onset of the inflammatory response observed. This finding could be linked to the persistent inflammation usually observed in children suffering from CSOM detected even in the absence of recoverable bacteria by culture methods. In the imaging experiments, day 3 was chosen for the second bacterial inoculation to coincide with the peak inflammatory response in the middle ear for the single inoculum groups (Chapter 2). The more rapid response in the sequential inoculated cohorts combined with the co-infection cohort supports the possibility of a

relationship between nasopharyngeal colonisation and inflammatory response generated in absence of recoverable bacteria. The high incidence and severity of OM has been previously interpreted using conventional culture methods and PCR methods (Faden *et al.* 1997; Hendolin *et al.* 1997; Smith-Vaughan *et al.* 2006). In contrast, the results in Chapter 5 have demonstrated the presence of bacteria such as NTHi and *S. pneumoniae* residing within and colonising certain niches in the nasal tissues, which could change the reported incidence rates.

The microbial interactions and host-bacterial interactions using an *in vitro* cell culture system provided a framework for investigating the microbe-host dynamics involved in airway colonisation. The burden of lower respiratory tract infections, such as the exacerbations in COPD and pneumonia has been well reviewed (Murphy *et al.* 2005b; Rudan *et al.* 2008). Therefore the effects of bacterial interactions with each other and host cells on the ability of the bacteria to adhere and induce release of cytokines from respiratory epithelial cells were investigated in Chapters 6 and 7. The *in vitro* finding of enhanced adherence of both *M. catarrhalis* and *S. pneumoniae* (when co-infected) to respiratory epithelial cells (Chapter 6) has provided a new insight to the previously identified positive association between these two bacteria (Chapters 2, 4 and 5). In contrast to enhancing pneumococcal OM when co-infected with *M. catarrhalis* (Chapters 2, 4 and 5), both *M. catarrhalis* and *S. pneumoniae* showed synergistic adherence to A549 cells (Chapter 6). Although this synergistic association was demonstrated *in vitro*, further clinical studies are warranted to investigate the potential risk of co-infection with *M. catarrhalis* and *S. pneumoniae* along with a respiratory virus involved in diseases such as COPD and pneumonia.

Bacterial adherence and colonisation is mediated by various adhesins, outer membrane proteins and cell wall polysaccharides that attach to the various receptor molecules present on host epithelial cells (Hammerschmidt 2006). In addition, bacterial binding to certain viral glycoproteins expressed on infected respiratory epithelial cells have been demonstrated *in vitro* (Avadhanula *et al.* 2007). The ability of several bacteria to bind to

fibronectin binding proteins and mediate bacterial adhesion and invasion of host cells have been previously reported (Hammarstrom 1999; Mongodin *et al.* 2002; Tan *et al.* 2005). The fibronectin binding proteins are one of the potential receptors for outer membrane proteins like Usp A and Hag (*M. catarrhalis*), and other mucosal pathogens (Tan *et al.* 2005). The binding of pneumococcal surface adhesin A to N-acetyl-glycosamine is known to promote its colonisation [as reviewed by (Bogaert *et al.* 2004)]. In addition, neuraminidase produced by respiratory viruses cleaves the sialic acid residue from glycolipids present on human lung tissues that then further expose various receptors for enhanced adherence of *S. pneumoniae* [reviewed by (Jedrzejas 2001)]. Various mucosal pathogens express surface-exposed ChoP suggesting its potential contribution to the persistence or survival of several bacteria in the nasopharynx (Gillespie *et al.* 1996; Kolberg *et al.* 1997). In addition, the ability of some bacteria to compete for same host cell receptor has been demonstrated (Shakhnovich *et al.* 2002). These studies have provided valuable information regarding the role of outer membrane proteins and surface adhesins of various bacteria in mediating adherence via binding of host cells receptors. Although this thesis did not investigate microbe-host relationships using specific receptor binding studies, the results in Chapter 6 have found significant alterations in bacterial adherence when co-infected in comparison with single bacterial infection. Importantly, the results have demonstrated the first reported synergistic adherence of *M. catarrhalis* and *S. pneumoniae* to A549 cells *in vitro*. Nevertheless, studies involving receptor-binding assays, competition assay and gene expression studies will be considered in the future for further exploration of the complex interplay between these microbes and the host in a polymicrobial environment.

Another important finding in Chapter 6 was the increased adherence of *M. catarrhalis* and *S. pneumoniae* to bronchial cells than the A549 cells, when infected alone. The ability of different respiratory epithelial cells to respond differently to the same infecting agent has been previously demonstrated (Avadhanula, V. *et al.* 2006). In a clinical setting however, upon inhalation the bacteria must traverse the bronchial epithelial cells and must get aspirated into the alveoli to cause infections such as pneumonia and

bronchitis. Therefore the bronchial epithelial cells could serve as transient secondary sites for attachment. However, during co-infection the bacterial adherence to bronchial cells was very low compared with the single infections. It has previously been reported that bacteria such as *S. pneumoniae* and NTHi compete for the same receptor (Shakhnovich *et al.* 2002). The bacterial competition might not be the explanation to the findings of Chapter 6, as NTHi adherence to both A549 and BEAS-2B cells was almost similar, regardless of any bacterial combinations. It could be possible that NTHi in a polymicrobial environment might have induced certain cell receptor changes that affected the ability of the other bacteria to adhere. In addition, results from Chapter 5 have shown NTHi localised in a different niche in comparison with *S. pneumoniae* in the nasopharynx of BALB/c mice. Another *in vitro* study has also demonstrated adherence of NTHi to the mucous in the ET floor region and not the middle ear epithelium (Miyamoto & Bakaletz 1996). These observations call for further investigation of the mechanisms involved in microbe-host colonisation.

The association between *M. catarrhalis* and *S. pneumoniae* observed in Chapters 2, 4, 5 and 6 has been consistent in the different studies suggesting that *M. catarrhalis* might predispose the host to respiratory infections. The results of this thesis have shown that *M. catarrhalis* is able to enhance pneumococcal OM (Chapter 2) during co-infection. In addition, it has also shown that pre-inoculation with *M. catarrhalis* facilitated pneumococcal OM (Chapter 4) and localisation of pneumococci in the pharynx (Chapter 5). *In vitro*, there was synergistic adherence of both *M. catarrhalis* and *S. pneumoniae* to alveolar cells in Chapter 6. These findings suggest that these two bacteria might create conditions conducive to each other to facilitate their adherence *in vitro*, however, it should be noted that there was limited recovery of *M. catarrhalis* from the middle ear washes *in vivo*. The *in vivo* results from Chapters 2, 4 and 5 were performed in BALB/c mice and *M. catarrhalis* is an exclusive human pathogen and is rapidly cleared by the innate immune system if the mice. This could be one of the reasons why there was limited recovery of *M. catarrhalis* from the middle ear washes in BALB/c mice.

In vitro studies have demonstrated the release of various inflammatory cytokines in response to different bacterial products such as lipopolysaccharides and endotoxins (Clemans *et al.* 2000; Fink *et al.* 2006). Recently, airway epithelial cells have been shown to express TLRs that enables the innate immune system to recognise bacterial products and activate cytokine responses (Kato & Schleimer 2007). The results of Chapter 7 have shown that different respiratory epithelia had different ability to produce IL-6 in response to the same infecting agent or combination of agents. In addition, it also demonstrated a synergistic increase in IL-6 production by bronchial cells in response to co-infection with *M. catarrhalis* and *S. pneumoniae*. The differential effects of the innate immune system on the release of cytokines like IL-6 and IL-8 have been previously demonstrated (Schulz *et al.* 2002; Thorley *et al.* 2007). The inflammatory response generated depends upon the innate immune system and recognition of various pathogen-associated molecular patterns like lipopolysaccharides by certain TLRs (Armstrong *et al.* 2004). The synergistic increase in inflammatory cytokine production (Chapter 7) also supports the findings by other studies (Ratner *et al.* 2005) indicating that bacterial combinations can amplify or alter the inflammatory responses in the host.

The role of respiratory virus, intranasal bacterial inoculation, and bacterial adherence in generating an inflammatory response (analysed by measuring nitric oxide levels, white blood cell counts and inflammatory cytokines) have been shown in Chapters 2, 3, 4 and 7. The inflammatory response (analysed by measuring nitric oxide levels, white blood cell counts and inflammatory cytokines) generated as a result of a pre-viral infection was much lower in comparison with bacterial infection (Chapters 2, 3 and 7). Respiratory viruses have been previously shown to promote bacterial adherence (Avadhanula, V. *et al.* 2006; Hakansson *et al.* 1994; Hament *et al.* 2004). Although the role of respiratory virus in enhancing bacterial colonisation, and mediating bacterial adherence was demonstrated in Chapters 2 and 6, it did not augment the cytokine production from respiratory epithelium *in vitro*. Previous studies have shown conflicting results about production of cytokines and bacterial binding to respiratory epithelial cells *in vitro* (Clemans *et al.* 2000; Robson *et al.* 2006). The results from Chapter 6 and 7 did

not find any relation between bacterial binding and cytokine production by both A549 and BEAS-2B cells. Although the NTHi adherence to both A549 and BEAS-2B cells were very low in comparison to other bacteria, the IL-6 released from both respiratory epithelial cells were well above the detectable limit and highest in the non-virus infected BEAS-2B cells (Chapter 7). The differential cytokine responses to bacterial infection could lead to future studies related to modulations of host's cellular immune system caused by different bacteria. These studies could also provide a link between nasopharyngeal colonisation and its effect on the host's immune system.

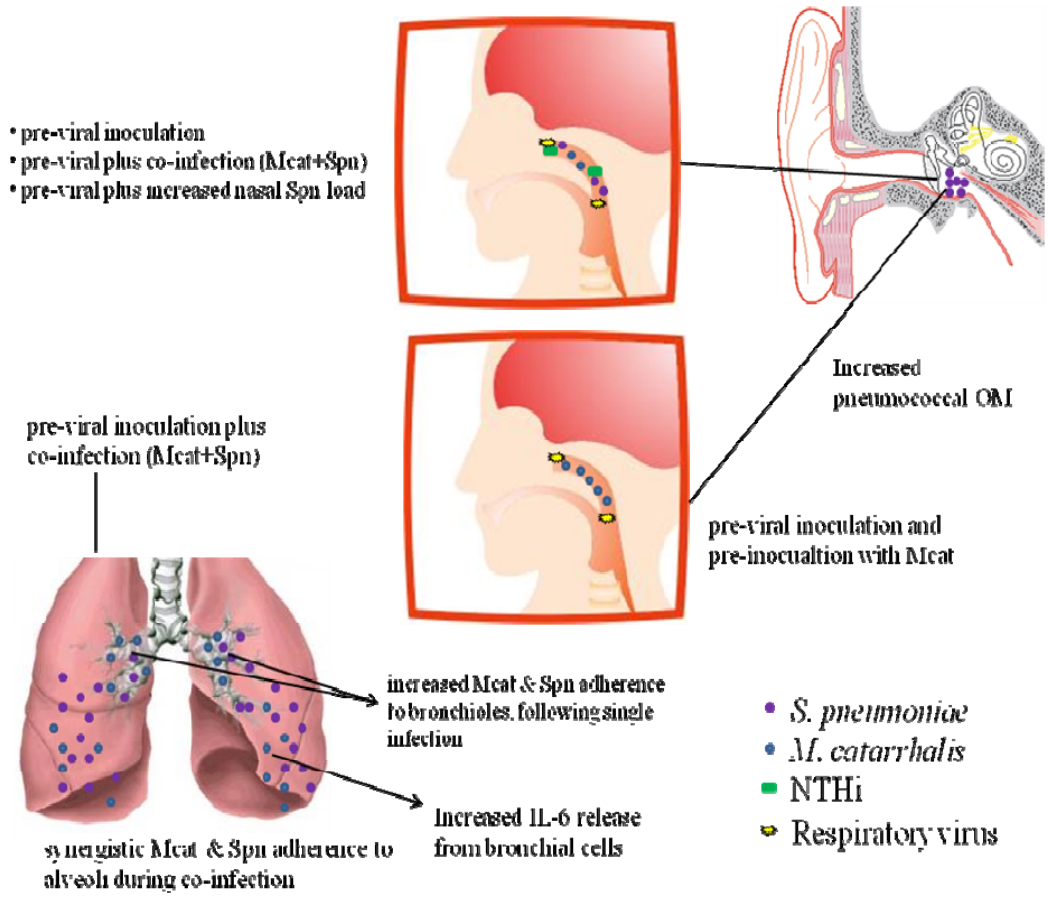
CONCLUSIONS

The thesis has established an experimental murine model that investigated the microbial interactions and host-bacterial relationships, dynamics of nasal colonisation patterns by different bacteria and the resulting differences in the inflammatory response involved in bacterial OM. The polymicrobial OM infection model involved study of the predominant bacteria associated with OM, that is *M. catarrhalis*, NTHi and *S. pneumoniae*, and a respiratory virus co-infection.

The key findings in this research were that the pre-viral infection, along with intranasal *M. catarrhalis* inoculation not only exacerbated pneumococcal OM, but also rapidly promoted pneumococcal colonisation at a site distal from its initial inoculation site. In addition, pneumococcal OM was also positively influenced by a pre-existing coloniser (*M. catarrhalis*) in the nasopharynx. In a polymicrobial environment different colonising patterns in the pharynx and localisation of different niches within the nasal cavity were observed by bacteria when compared with single bacterial infection. The positive association between *M. catarrhalis* and *S. pneumoniae* not only enhanced pneumococcal OM in BALB/c mice, but also facilitated synergistic adherence of both the bacteria to alveolar cells *in vitro*. The polybacterial infection *in vitro* altered the bacterial adherence to respiratory epithelium in comparison with single bacterial infection. In addition, synergistic increases in the production of IL-6 by bronchial cells

in response to co-infection with *M. catarrhalis* and *S. pneumoniae* were demonstrated *in vitro.*

Figure. 8.1: Findings of this research. The figure summarizes the significant findings of this thesis.



The cartoon figures of the human lungs, nasopharynx and the middle ear were downloaded from Source: Google Images (www.images.google.com.au, www.singhealth.com.sg, www.audilab.bmed.mcgill.ca and www.cancerline.com).

A concise summary of the findings of this research is shown in **Figure. 8.1**. The incidence of pneumococcal OM was influenced by an intranasal pre-viral infection and co-infection with *M. catarrhalis* and *S. pneumoniae*. A pre-viral infection followed by

pre-inoculation with *M. catarrhalis* also resulted in greater incidence rate of pneumococcal OM. In addition, a pre-viral infection followed by increased nasal pneumococcal load also enhanced pneumococcal OM. When infected alone, the adherence of *M. catarrhalis* and *S. pneumoniae* was greater to bronchial cells when compared to alveolar cells. However during co-infection, synergistic adherence of both *M. catarrhalis* and *S. pneumoniae* to alveolar cells was evident. A synergistic increase in the inflammatory response (IL-6 release) by bronchial cells was observed during co-infection with *M. catarrhalis* and *S. pneumoniae*.

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APPENDIX A

A.1 Bacterial isolates and growth conditions

A.1.1 *Moraxella catarrhalis* K65 and nontypeable Haemophilus influenzae 289

Standard growth conditions for *M. catarrhalis* K65 and nontypeable *H. influenzae* 289 were as follows: chocolate blood (Brain Heart Infusion) agar (BHI) at 37^0C with 5% CO_2 for growth on semi-solid media.

Preparation of chocolate blood (Brain Heart Infusion) agar plates

Chocolate BHI agar plates were prepared by dissolving 18.5g of BHI media (Oxoid Australia Pty. Ltd.) and 5g bacteriological agar no.1 (Oxoid Australia Pty. Ltd.) in 500ml of nanopure water. The media was sterilised by autoclaving for 20 minutes at 121^0C and was then maintained in a 56^0C water bath. Then, 25ml of defibrinated horse blood was added to the media and heated in a 100^0C water bath until the media turned slightly brown. The media was then allowed to cool in a 56^0C water bath again before pouring into sterile petri plates. After pouring into the plates, growth and sterility controls were done for each batch of media prepared and the plates were stored at 4^0C until further use.

A.1.2 *Streptococcus pneumoniae* serotype 14

Standard growth conditions for *S. pneumoniae* serotype 14 were as follows: Blood agar (BA) at 37^0C with 5% CO_2 for growth on semi-solid media.

Preparation of Blood agar plates

Blood agar plates were prepared by dissolving 20g of BA media (Oxoid Australia Pty. Ltd.) in 500ml of nanopure water. The media was sterilised by autoclaving for 20 minutes at 121^0C and was then maintained in a 56^0C water bath. Then, 25ml of defibrinated horse blood was added to the media and was poured into sterile petri plates. After pouring into the plates, growth and sterility controls were done for each batch of media prepared and the plates were stored at 4^0C until further use.

These bacterial isolates were used in all the *in vivo* and *in vitro* experiments except in Chapter 5. The bacterial isolates used in Chapter 5 were *M. catarrhalis* 1857, NTHi 86-028NP, NTHi 86-028NP/pKMLN-1 and *S. pneumoniae* 19F. *M. catarrhalis* 1857, NTHi 86-028NP and NTHi 86-028NP/pKMLN-1were grown on BBL™ Chocolate II Agar (Becton Dickinson), and *S. pneumoniae* 19F was grown on Trypticase™ Soy Agar supplemented with 5% sheep blood (Becton Dickinson) and containing 50 µg/ml of kanamycin.

Preparation of bacterial cell suspensions

Bacteria grown on appropriate growth media were harvested using an ethanol-dipped flamed glass slide and washes three times by centrifugation and resuspending in sterile PBS. After the washes, the concentration of each bacterium was adjusted to approximately 2 x 10^{10} CFU/ml, for *M. catarrhalis* and NTHi, and 1 x 10^{10} CFU/ml for *S. pneumoniae* using the optical density of the culture at 405nm and the following regression curves (previously developed) were used for appropriate bacteria:

M. catarrhalis	$y = 1.213x + 9.24$
	When $y = \log_{10}$ CFU/ml and $x = \log_{10} OD_{405}$
NTHi	$y = 1.249x + 10.118$
	When $y = \log_{10}$ CFU/ml and $x = \log_{10} OD_{405}$
S. pneumoniae	$y = 1.496x + 9.212$
	When $y = \log_{10}$ CFU/ml and $x = \log_{10} OD_{405}$

The samples were diluted if necessary to give an optical density close to 1 and the dilution factor was considered.

A.2 Media for *in vitro* cell culture experiments

A.2.1 Growth medium

RPMI 1640 containing L-glutamine (Gibco BRL, Invitrogen)

RPMI 1640 with glutamine	445ml
Heat-inactivated Fetal Calf serum (10%)	50ml
(Cambrex, Lonza Australia Pty., Ltd.)	
Antibiotic-Antimycotic solution (Gibco BRL, Invitrogen)	5ml

This medium was used for growing and passage of A549 and BEAS-2B cells.

A.2.2 Maintenance medium

RPMI 1640 containing L-glutamine (Gibco BRL, Invitrogen)

RPMI 1640 with glutamine	490ml
Heat-inactivated Fetal Calf serum (1%)	5ml
(Cambrex, Lonza Australia Pty., Ltd.)	
Antibiotic-Antimycotic solution (Gibco BRL, Invitrogen)	5ml

This medium was used to maintain adenovirus-infected A549 and BEAS-2B cells before infecting them further with bacteria.

A.2.3 Overlay medium

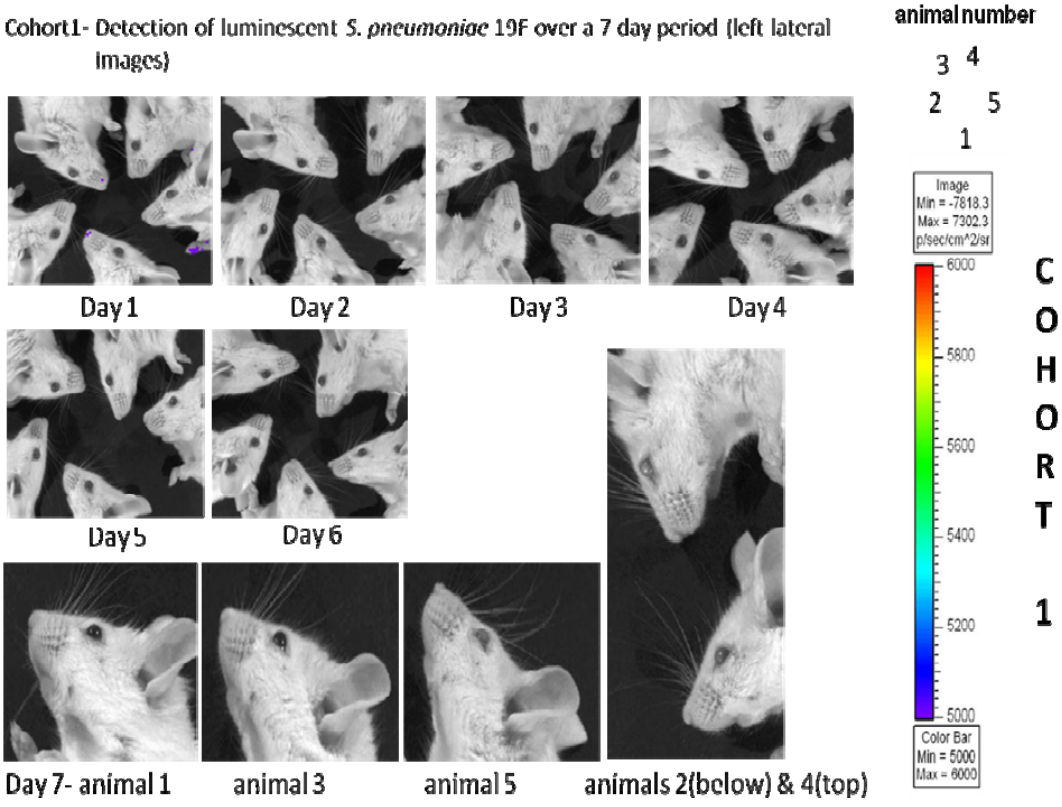
RPMI 1640 with glutamine (500ml), 25mM $MgCl_2$,	(4parts)
and antibiotic & antimycotic solution	
Heat-inactivated Fetal Calf serum (Cambrex, Lonza Australia Pty., Ltd.)	(1part)
1.8% melted agar (Oxoid Ltd., Hamshire, England)	(5parts)

This medium was used during quantitation of adenovirus. Two ml of this overlay medium was added to each well following adenovirus infection. The tissue culture plates were incubated for 5-7 days at 37^0C with 5% CO_2 and adenovirus concentration was expressed as PFU/ml after visualisation of plaques.

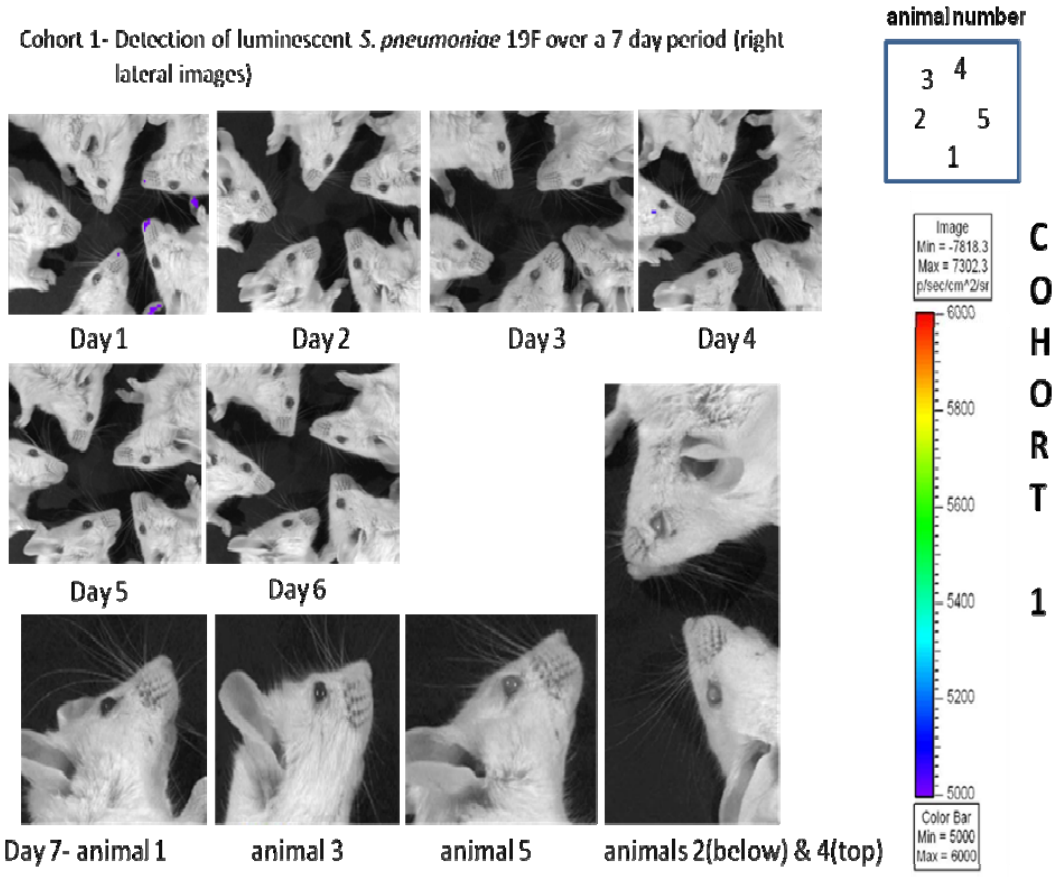
APPENDIX B

B.1 Cohort 1 (*S. pneumoniae* 19F alone)

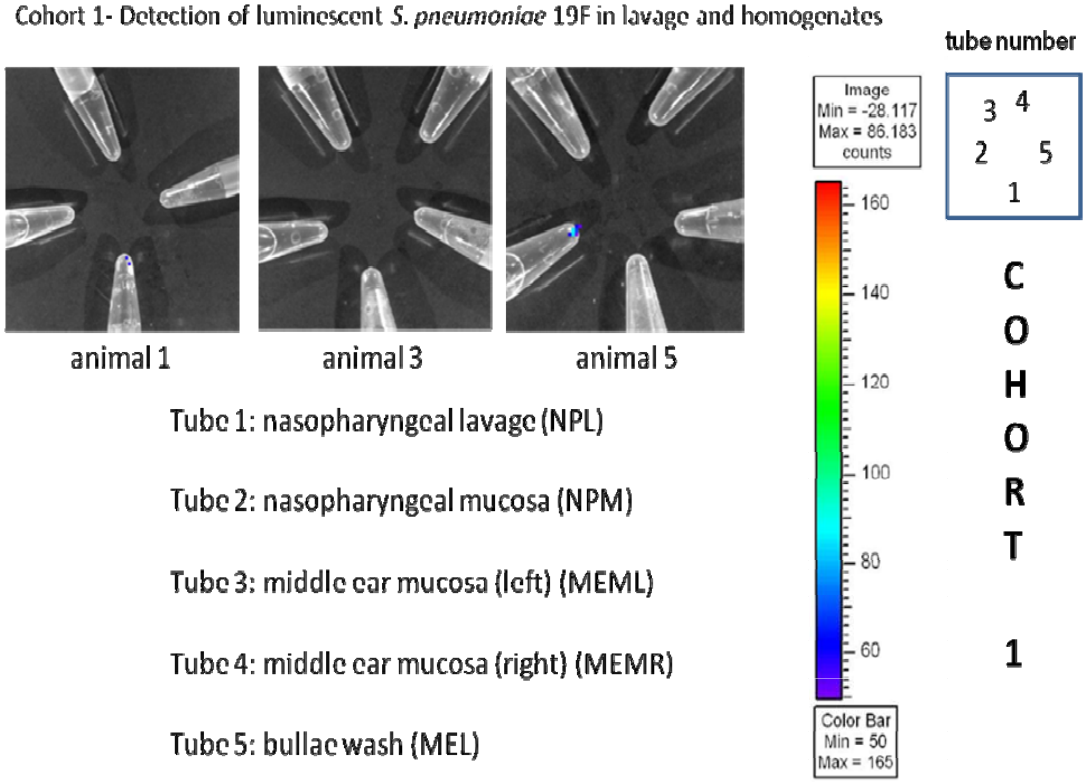
B.1.1 Detection of luminescent *S. pneumoniae* (left lateral images)



B.1.2 Detection of luminescent *S. pneumoniae* (right lateral images)

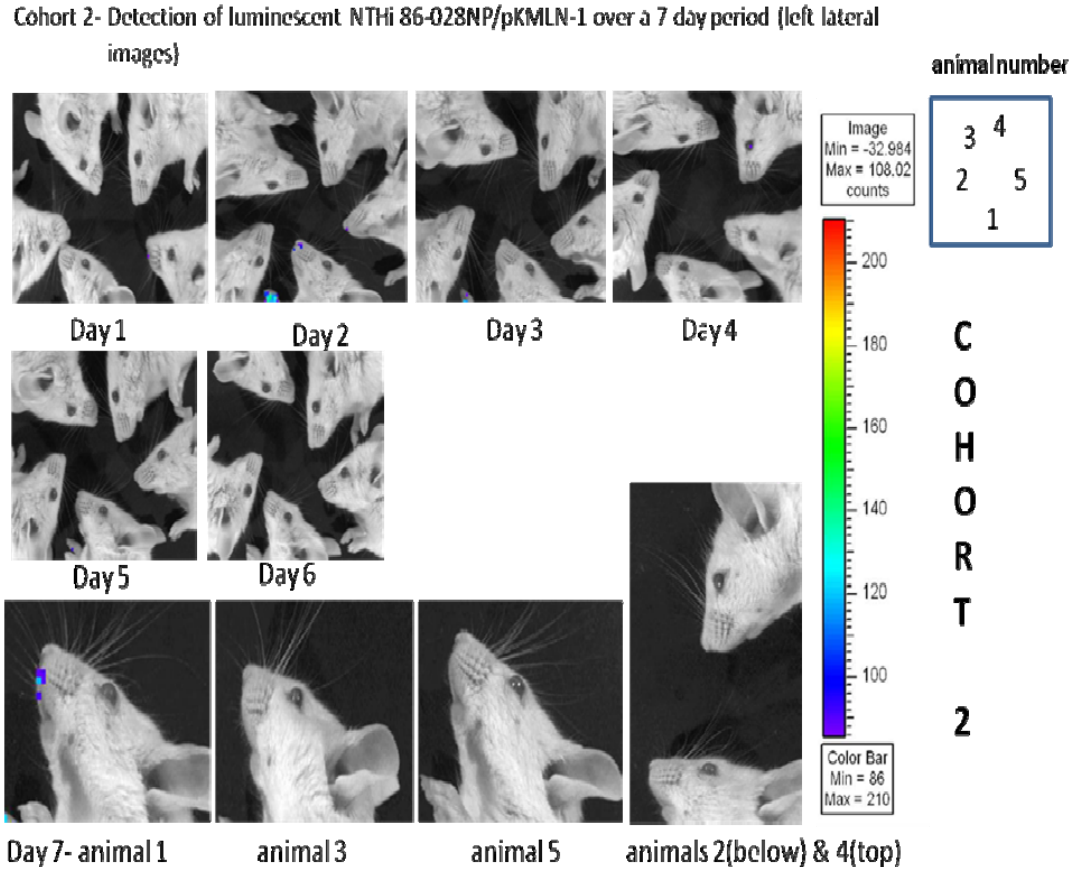


B.1.3 Detection of luminescent *S. pneumoniae* in lavage and homogenates

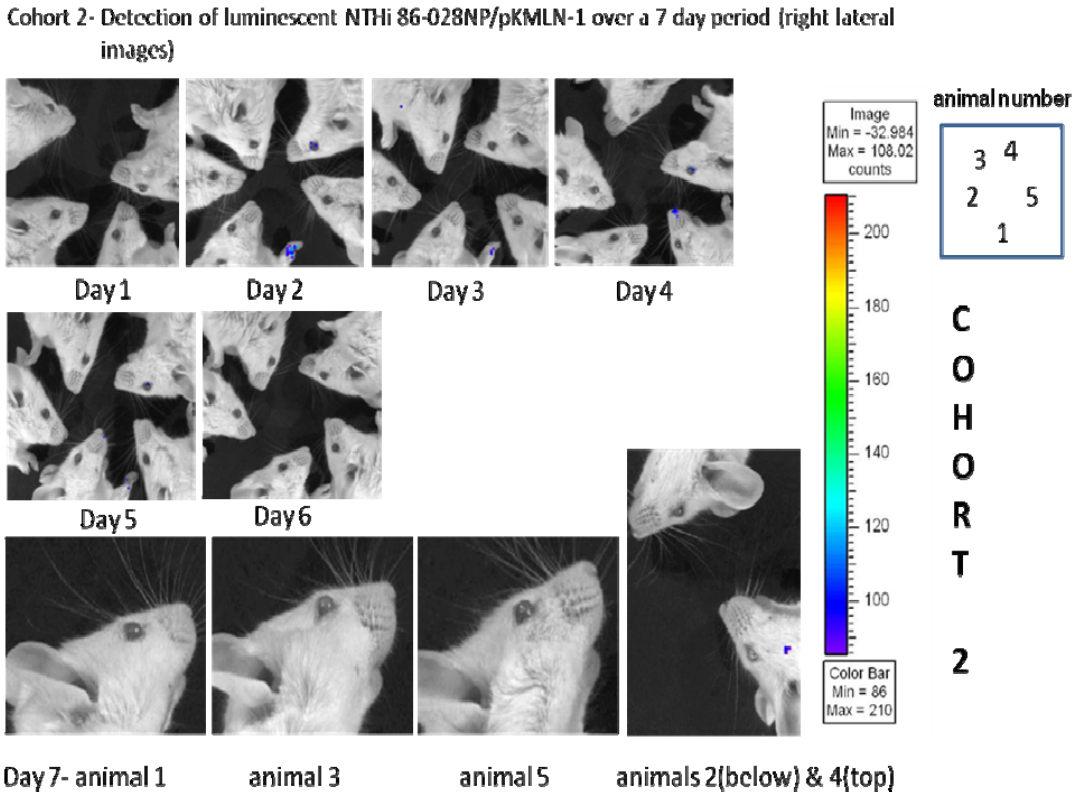


B.2 Cohort 2 (NTHi 86-028NP/pKMLN-1 alone)

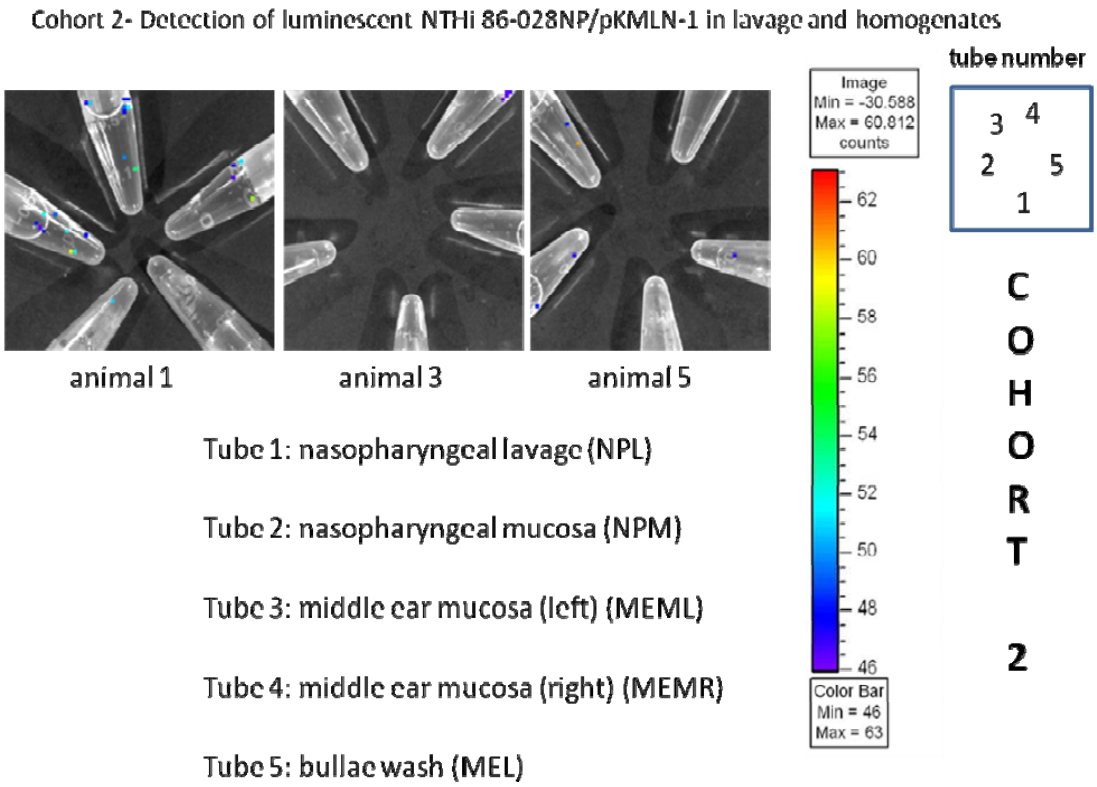
B.2.1 Detection of luminescent NTHi (left lateral images)



B.2.2 Detection of luminescent NTHi (right lateral images)

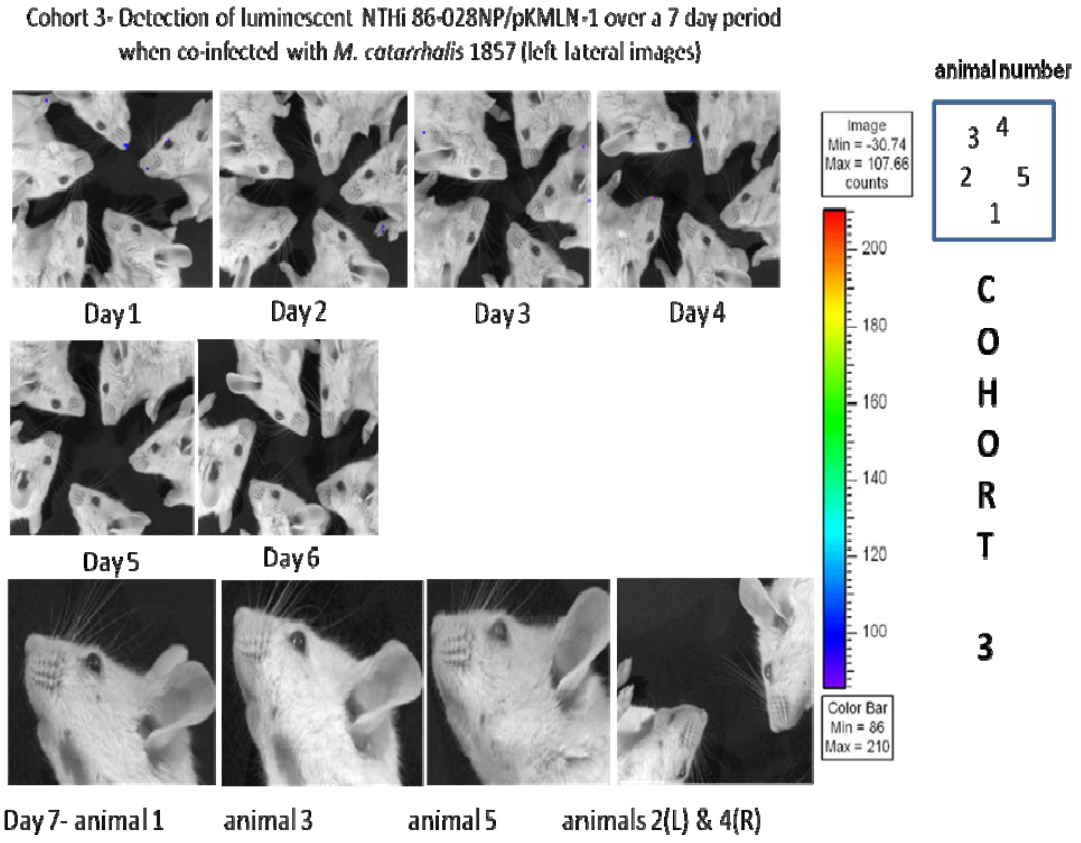


B.2.3 Detection of luminescent NTHi in lavage and homogenates

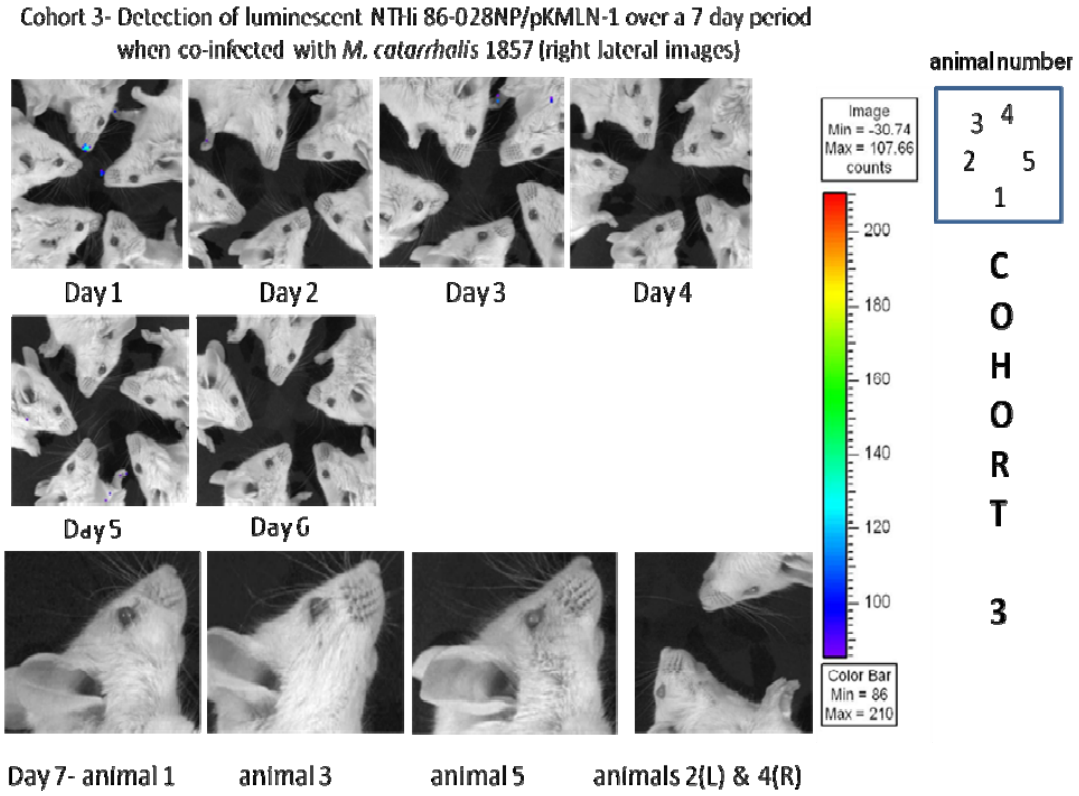


B.3 Cohort 3 (NTHi 86-028NP/pKMLN-1 + *M. catarrhalis* 1857)

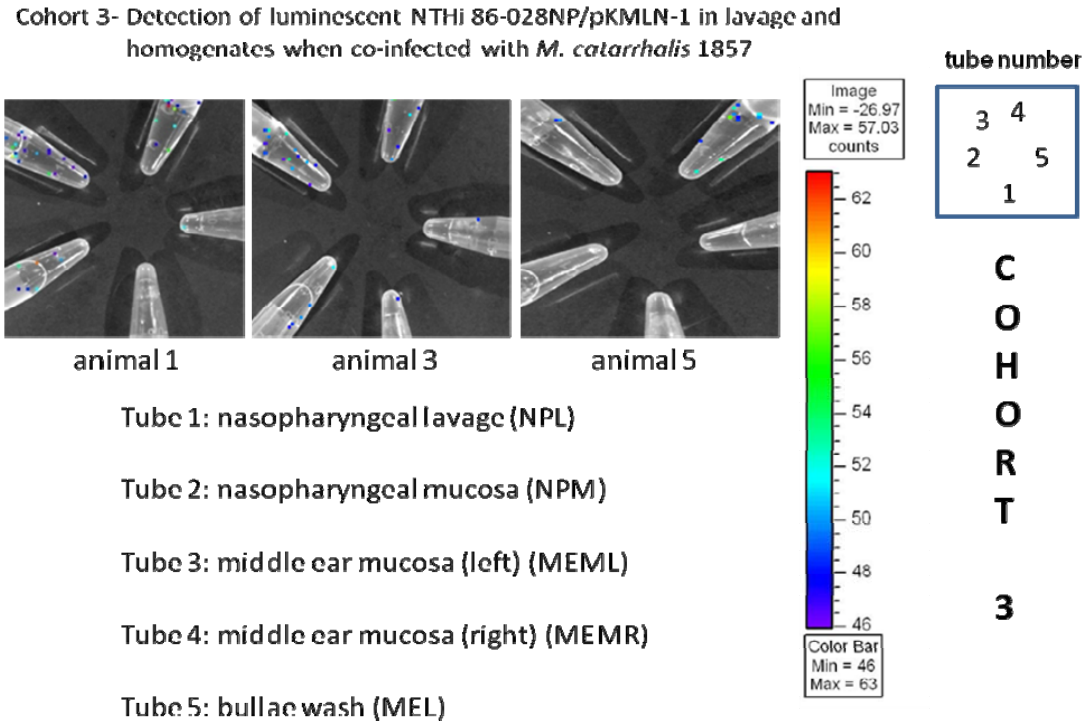
B.3.1 Detection of luminescent NTHi when co-infected with *M. catarrhalis* (left lateral images)



B.3.2 Detection of luminescent NTHi when co-infected with *M. catarrhalis* (right lateral images)

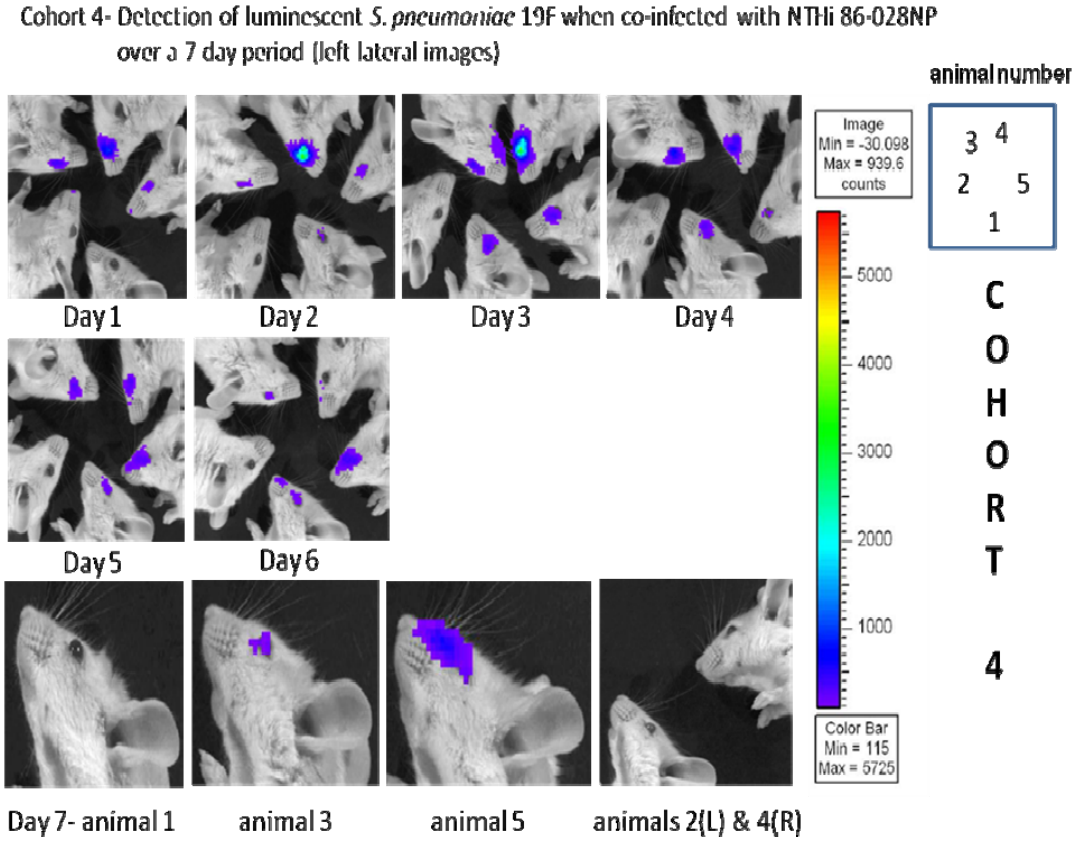


B.3.3 Detection of luminescent NTHi in lavage and homogenates when co-infected with *M. catarrhalis*

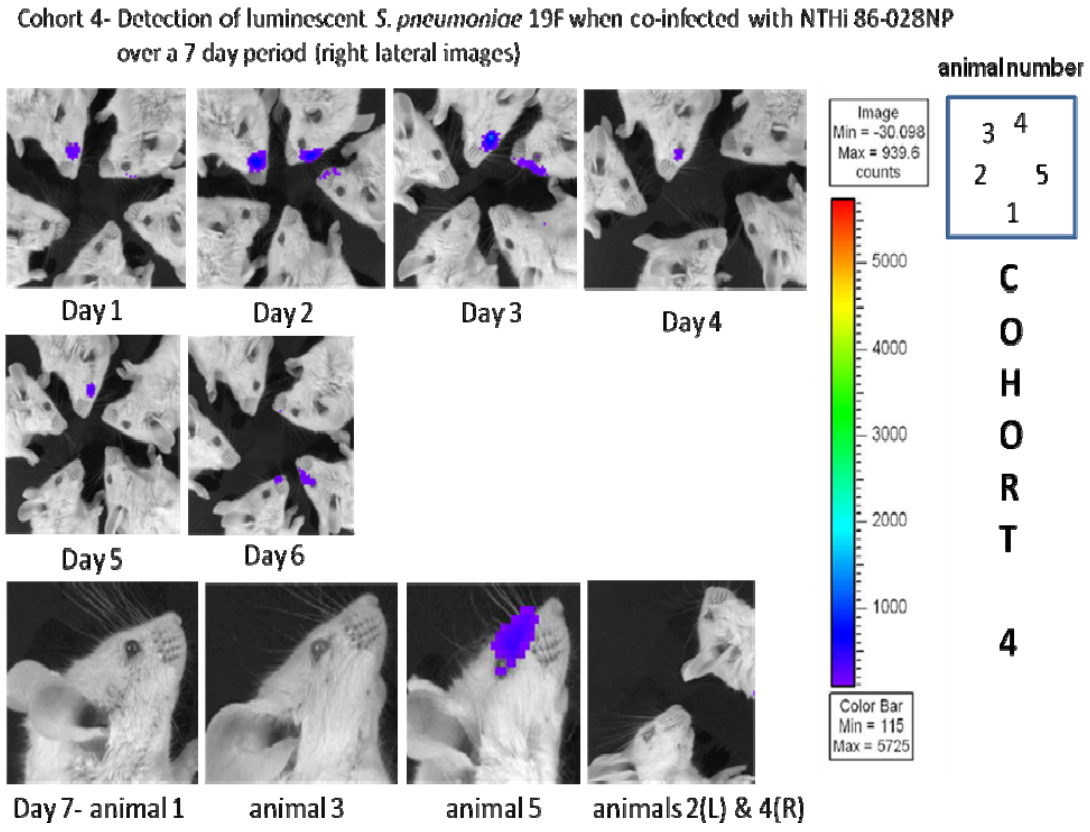


B.4 Cohort 4 (*S. pneumoniae* 19F + NTHi 86-028NP)

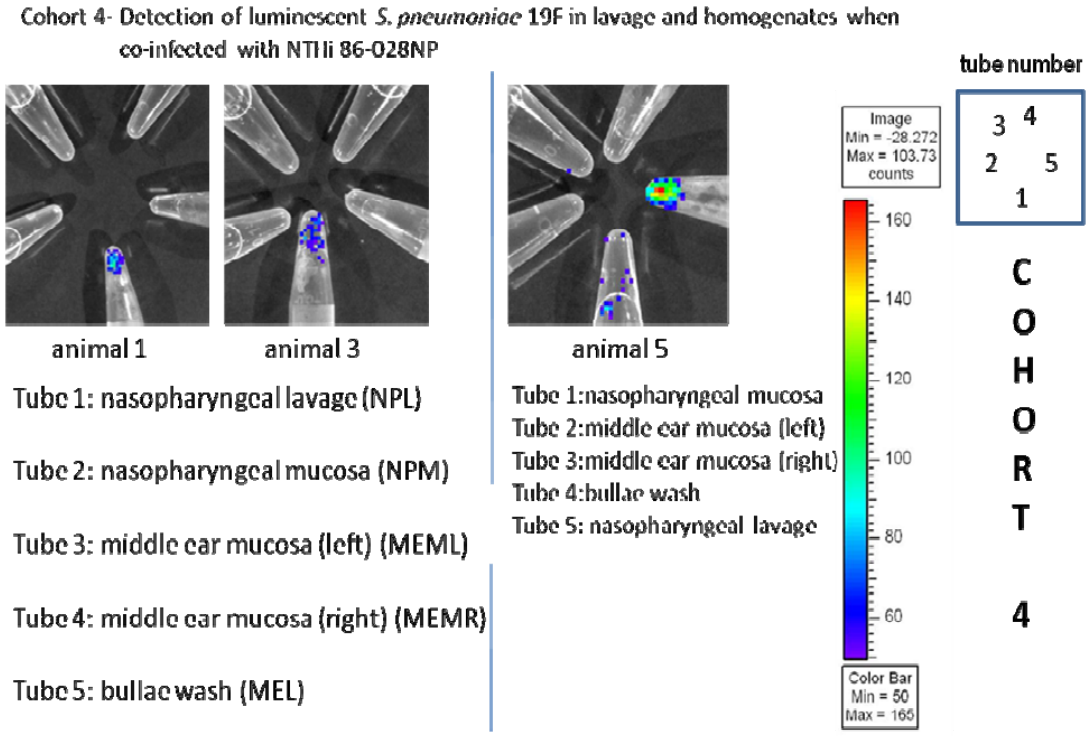
B.4.1 Detection of luminescent *S. pneumoniae* when co-infected with NTHi (left lateral images)



B.4.2 Detection of luminescent *S. pneumoniae* when co-infected with NTHi (right lateral images)

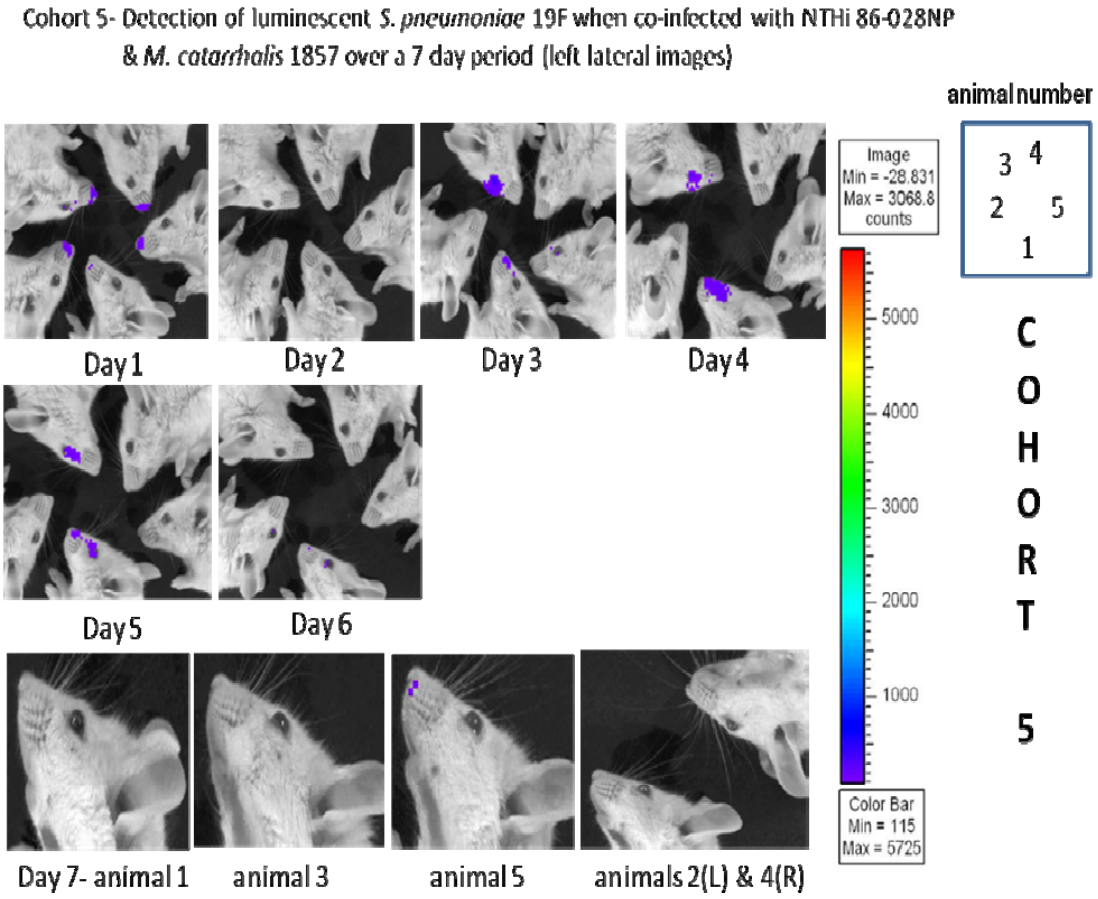


B.4.3 Detection of luminescent *S. pneumoniae* in lavage and homogenates when co-infected with NTHi

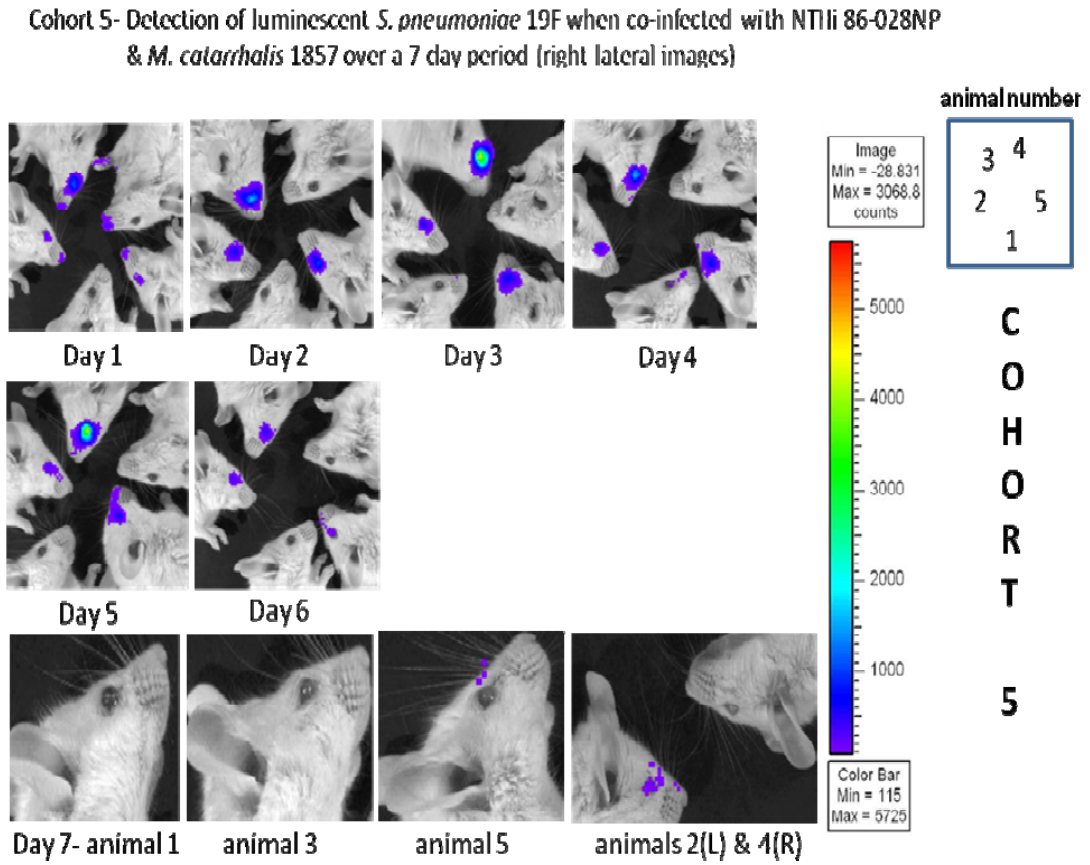


B.5 Cohort 5 (*S. pneumoniae* 19F + NTHi 86-028NP + *M. catarrhalis* 1857)

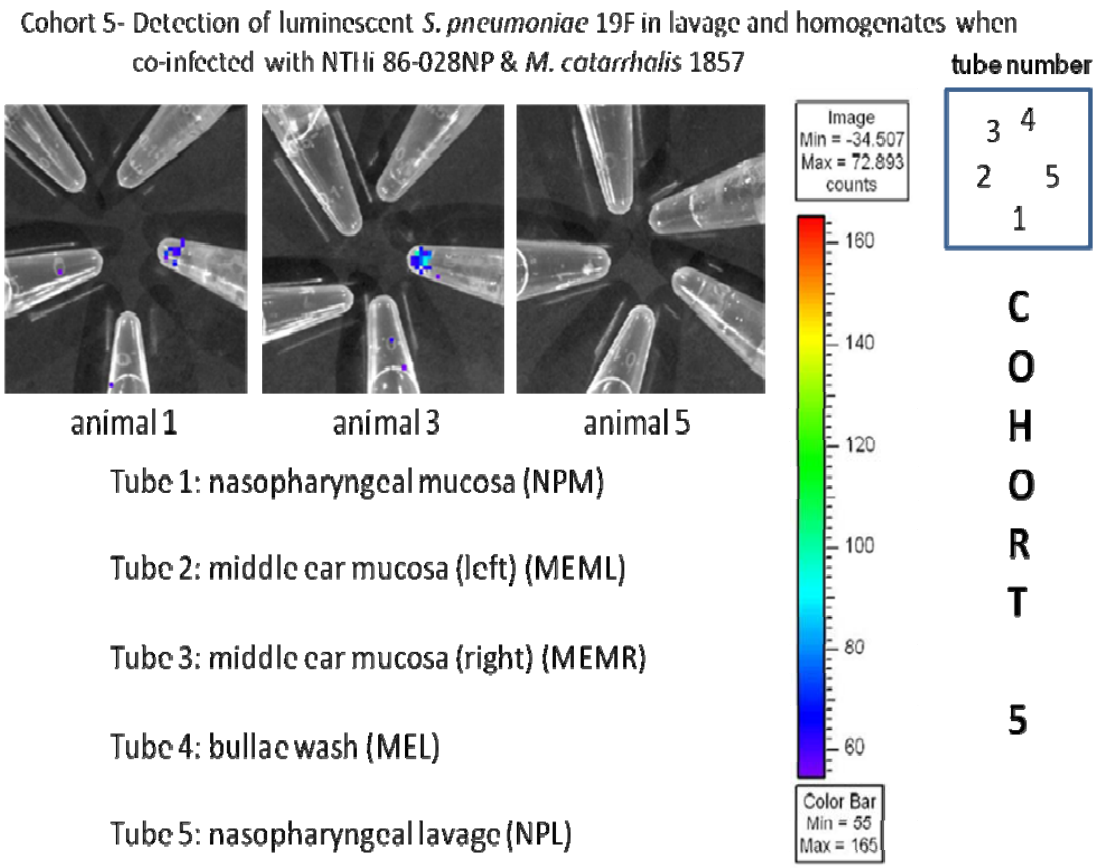
B.5.1 Detection of luminescent *S. pneumoniae* when co-infected with NTHi & *M. catarrhalis* (left lateral images)



B.5.2 Detection of luminescent *S. pneumoniae* when co-infected with NTHi & *M. catarrhalis* (right lateral images)

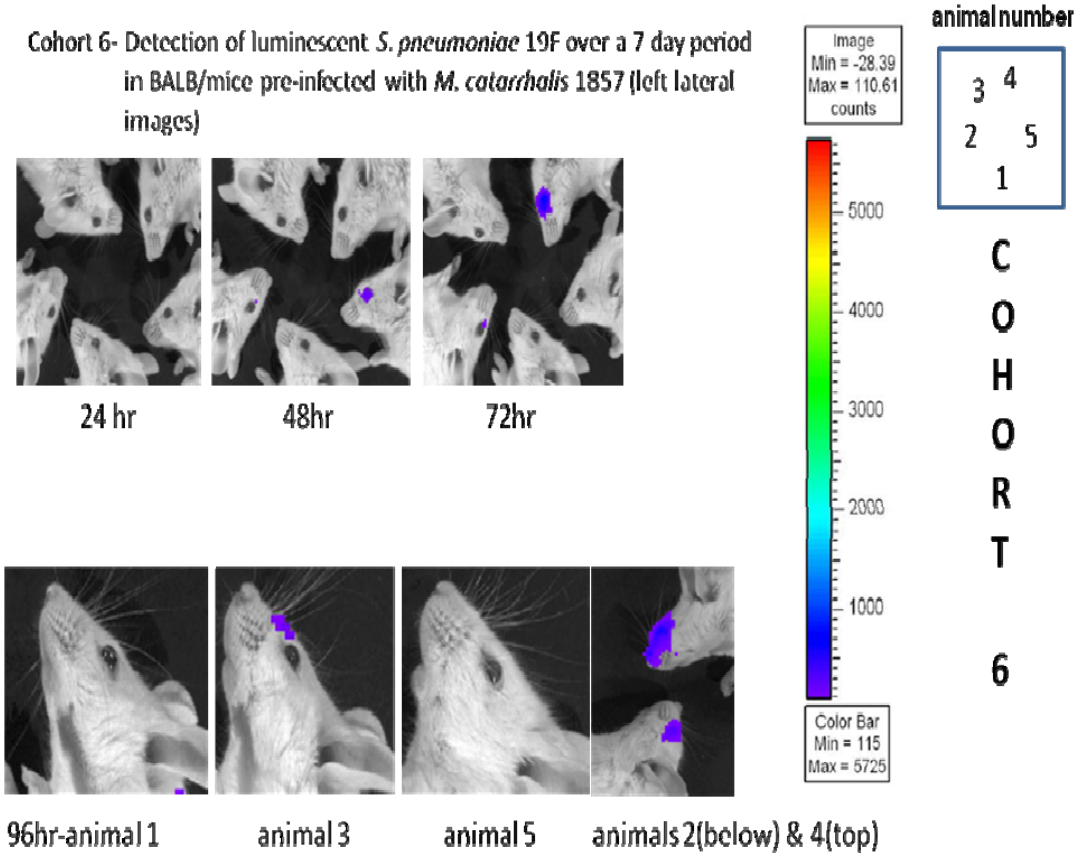


B.5.3 Detection of luminescent *S. pneumoniae* in lavage and homogenates when co-infected with NTHi & *M. catarrhalis*

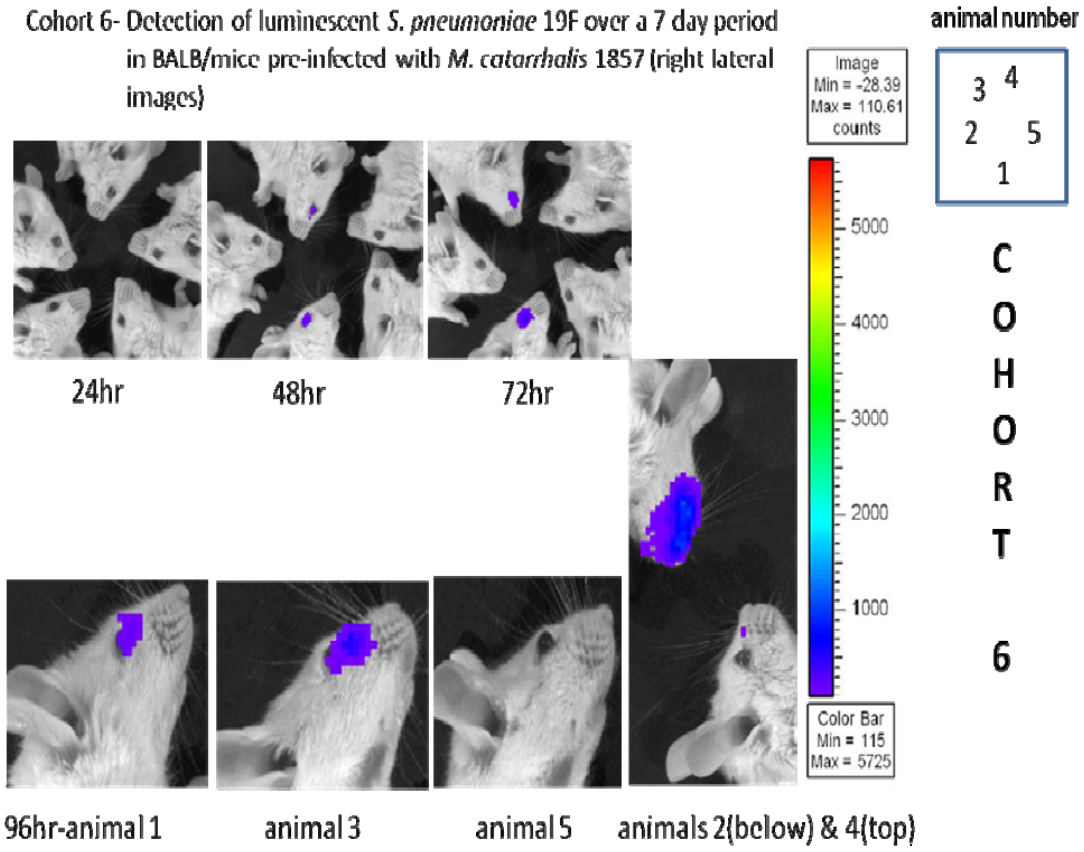


B.6 Cohort 6 (*S. pneumoniae* 19F in BALB/c mice pre-inoculated with *M. catarrhalis* 1857)

B.6.1 Detection of luminescent *S. pneumoniae* in BALB/c mice pre-inoculated with *M. catarrhalis* (left lateral images)

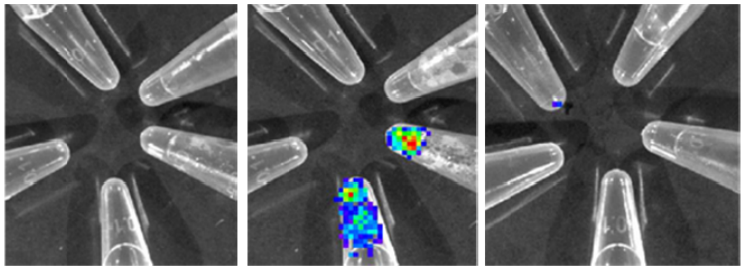


B.6.2 Detection of luminescent *S. pneumoniae* in BALB/c mice pre-inoculated with *M. catarrhalis* (right lateral images)

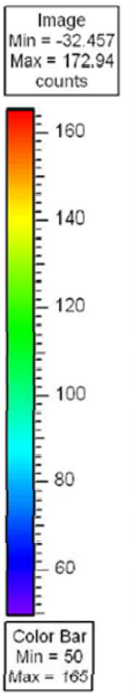


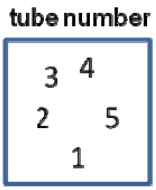
B.6.3 Detection of luminescent *S. pneumoniae* in lavage and homogenates when pre-inoculated with *M. catarrhalis*

Cohort 6- Detection of luminescent *S. pneumoniae* 19F in lavage and homogenates from BALB/c mice pre-infected with *M. catarrhalis* 1857



animal 1 animal 3 animal 5





COHORT 6

Tube 1: nasopharyngeal mucosa (NPM)

Tube 2: middle ear mucosa (left) (MEML)

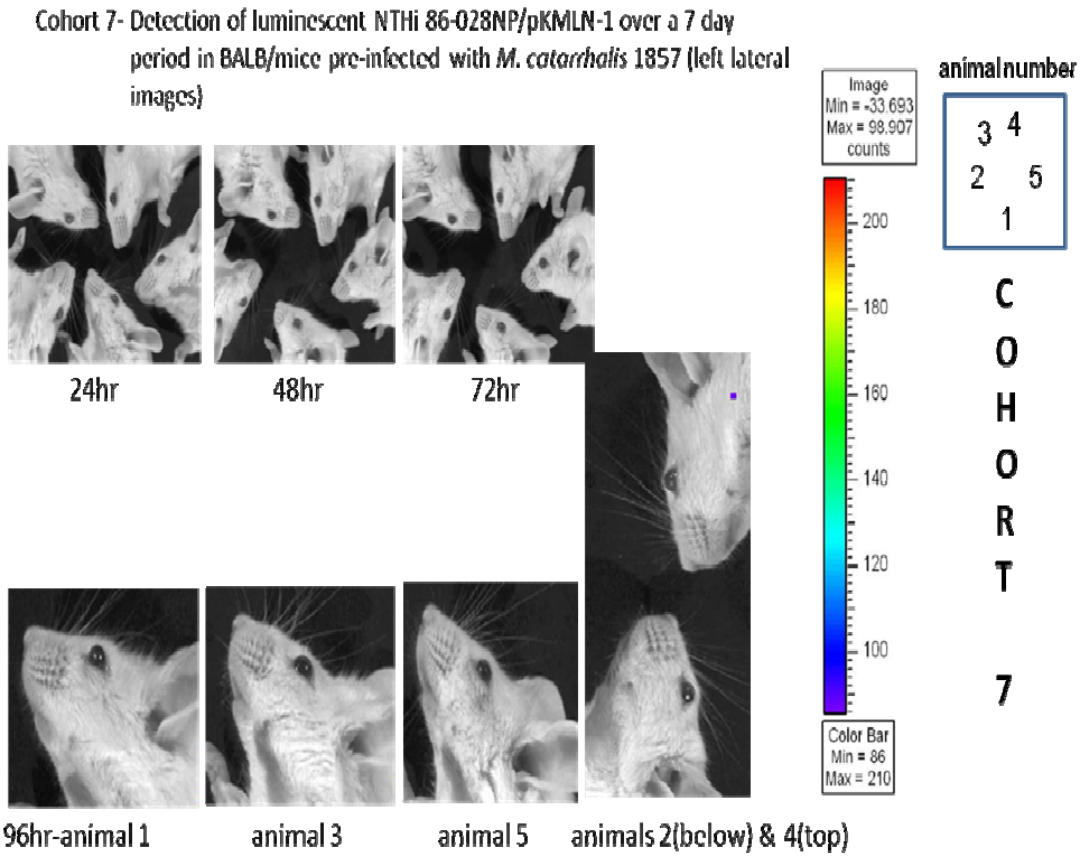
Tube 3: middle ear mucosa (right) (MEMR)

Tube 4: bullae wash (MEL)

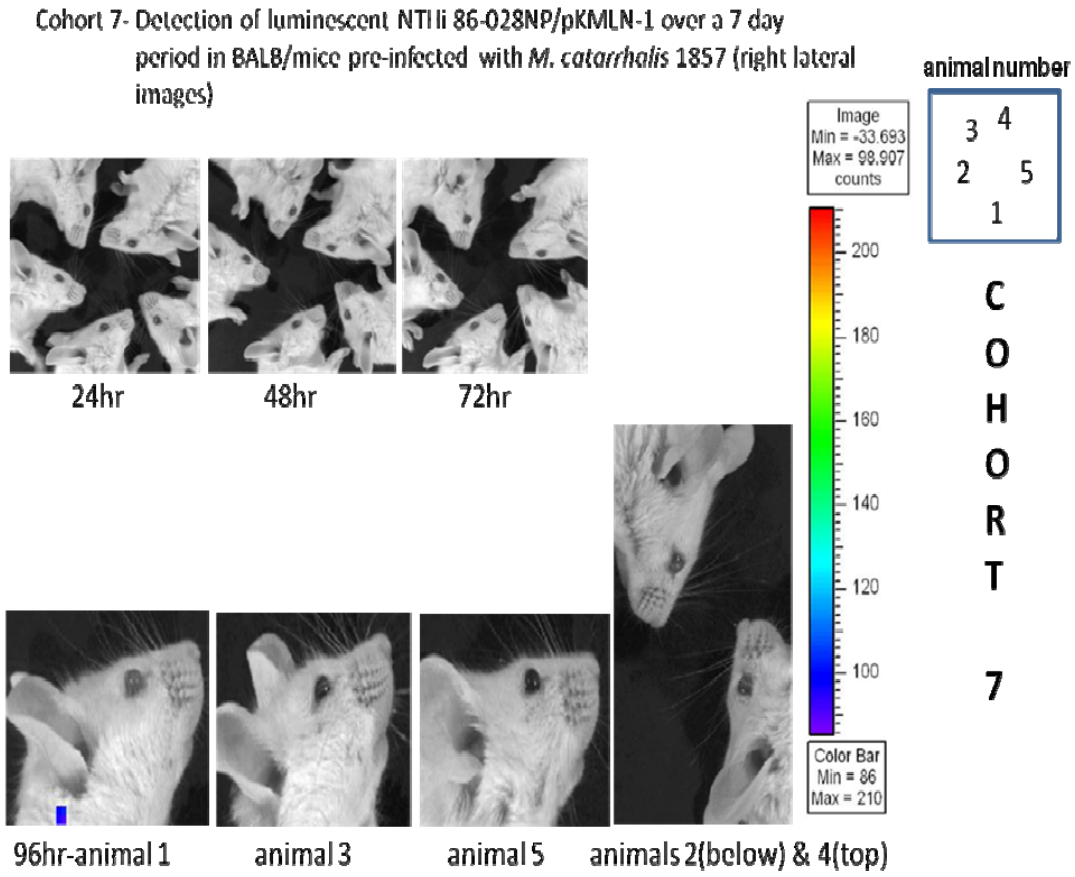
Tube 5: nasopharyngeal lavage (NPL)

B.7 Cohort 7 (NTHi 86-028NP/pKMLN-1 in BALB/c mice pre-inoculated with *M. catarrhalis* 1857)

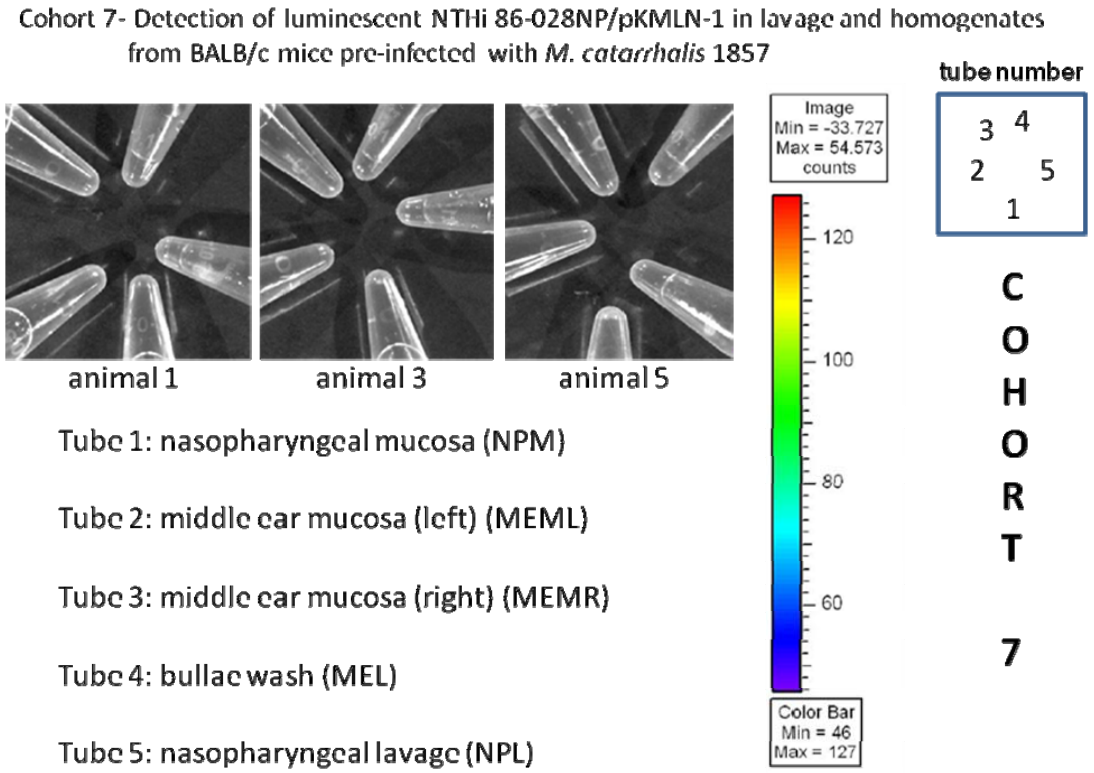
B.7.1 Detection of luminescent NTHi in BALB/c mice pre-inoculated with *M. catarrhalis* (left lateral images)



B.7.2 Detection of luminescent NTHi in BALB/c mice pre-inoculated with *M. catarrhalis* (right lateral images)



B.7.3 Detection of luminescent NTHi in lavage and homogenates when pre-inoculated with *M. catarrhalis*



B.8 Weights of various tissues before homogenisation

No.	Cohort	Tissue	Tissue weight (mg)
Animal 1	*S. pneumoniae* 19F	Nasopharyngeal mucosa	0.001
Animal 3			0.0012
Animal 5			0.0024
Animal 1		Left middle ear epithelium	0.0004
Animal 3			0.0003
Animal 5			0.0007
Animal 1		Right middle ear epithelium	Not enough tissue
Animal 3			0.0008
Animal 5			0.0018
Animal 1	NTHi 86-028NP/pKMLN-1	Nasopharyngeal mucosa	0.0017
Animal 3			0.0011
Animal 5			0.0004
Animal 1		Left middle ear epithelium	0.0005
Animal 3			0.0005
Animal 5			0.0007
Animal 1		Right middle ear epithelium	0.0006
Animal 3			0.0008
Animal 5			0.0010
Animal 1	NTHi 86-028NP/pKMLN-1 + *M. catarrhalis* 1857	Nasopharyngeal mucosa	0.0011
Animal 3			0.0022
Animal 5			0.0012
Animal 1		Left middle ear epithelium	0.0013
Animal 3			0.0005
Animal 5			0.0015
Animal 1		Right middle ear epithelium	0.0012
Animal 3			0.0003
Animal 5			0.0004
Animal 1	*S. pneumoniae* 19F + NTHi 86-	Nasopharyngeal	0.0022

Animal 3	028NP	mucosa	0.0018
Animal 5			0.0021
Animal 1		Left middle ear epithelium	0.0011
Animal 3			0.0008
Animal 5			0.0003
Animal 1		Right middle ear epithelium	0.0002
Animal 3			0.0006
Animal 5			0.0013
Animal 1	*S. pneumoniae* 19F + NTHi 86-028NP + *M. catarrhalis* 1857	Nasopharyngeal mucosa	0.0010
Animal 3			0.0013
Animal 5			0.0028
Animal 1		Left middle ear epithelium	0.0005
Animal 3			0.0004
Animal 5			0.0005
Animal 1		Right middle ear epithelium	0.0004
Animal 3			0.0003
Animal 5			0.0004
Animal 1	*M. catarrhalis* 1857 followed by *S. pneumoniae* 19F	Nasopharyngeal mucosa	0.0006
Animal 3			0.0026
Animal 5			0.0008
Animal 1		Left middle ear epithelium	0.0005
Animal 3			0.0006
Animal 5			0.0007
Animal 1		Right middle ear epithelium	0.0003
Animal 3			0.0005
Animal 5			0.0004

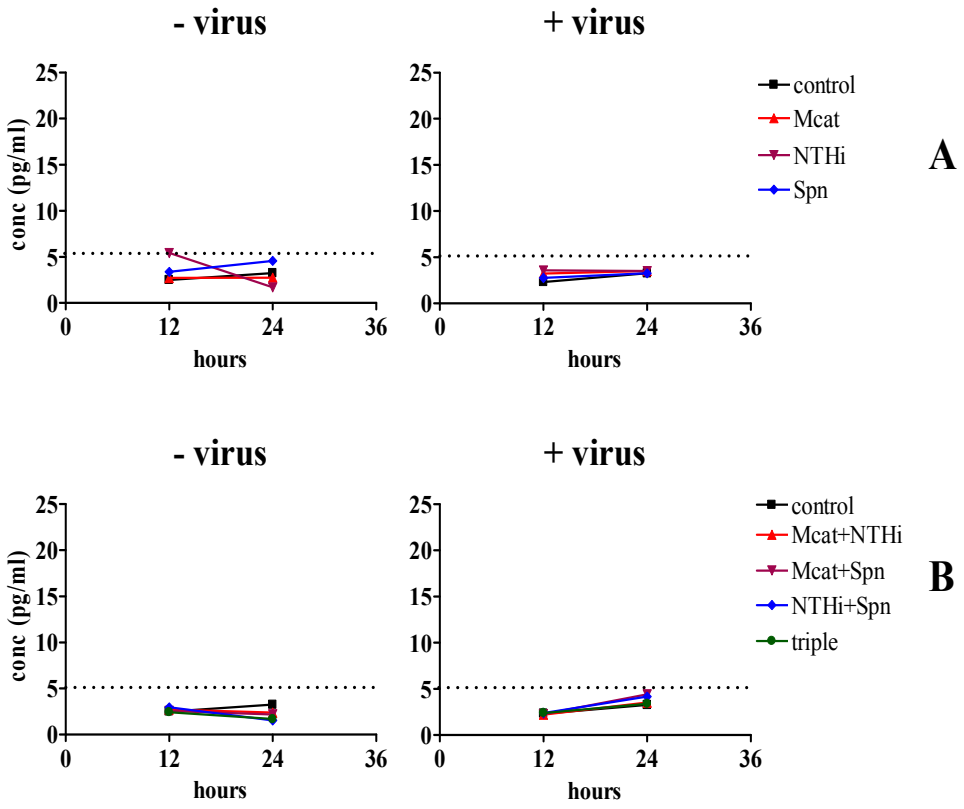
No.	Cohort	Tissue	Tissue weight (mg)
Animal 1	*M. catarrhalis* 1857 followed by NTHI 86-028NP	Nasopharyngeal mucosa	0.0015
Animal 3			0.0005
Animal 5			0.0033
Animal 1		Left middle ear epithelium	0.0007
Animal 3			0.0002
Animal 5			0.0002
Animal 1		Right middle ear epithelium	0.0006
Animal 3			0.0004
Animal 5			0.0004
Animal 1	Adenovirus only	Nasopharyngeal mucosa	-
Animal 3			-
Animal 5			-
Animal 1		Left middle ear epithelium	-
Animal 3			-
Animal 5			-
Animal 1		Right middle ear epithelium	-
Animal 3			-
Animal 5			-

Note: Minus (-) sign denotes that the tissues were not weighed but used for histology

APPENDIX C

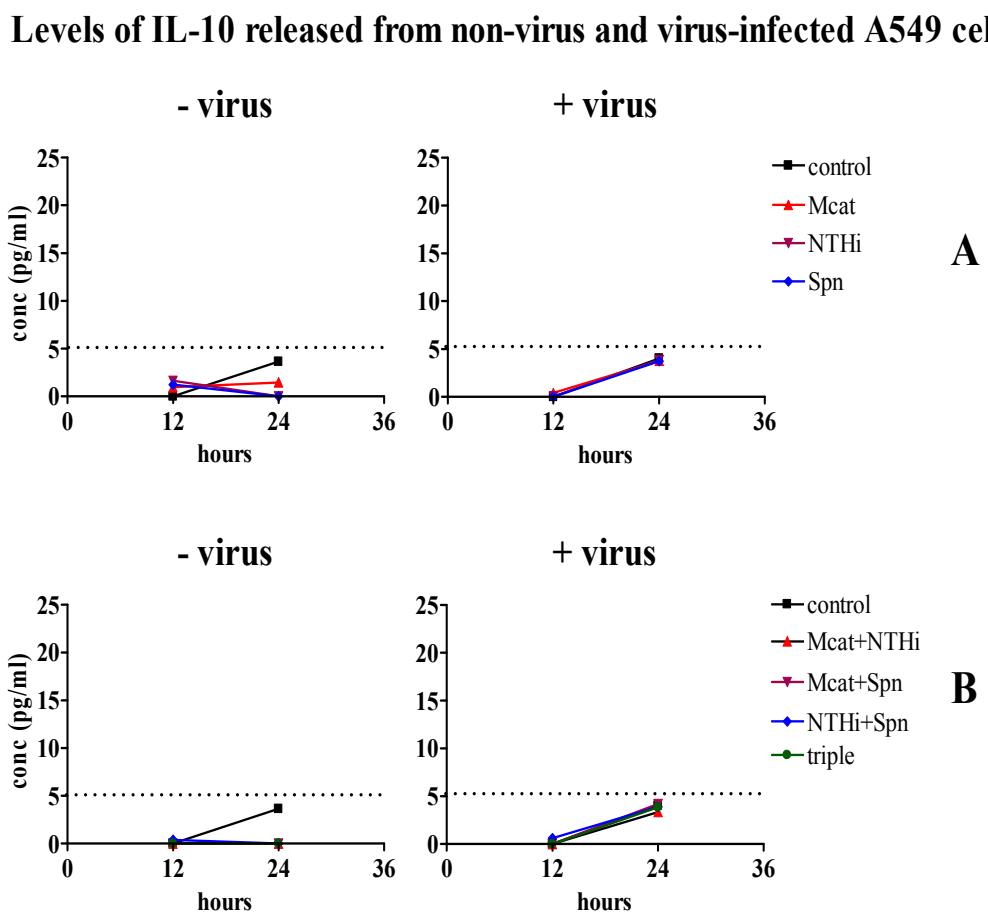
C.1 Cytokines released following infection of A549 cells

C.1.1 Levels of TNF-α released from non-virus and virus-infected A549 cells



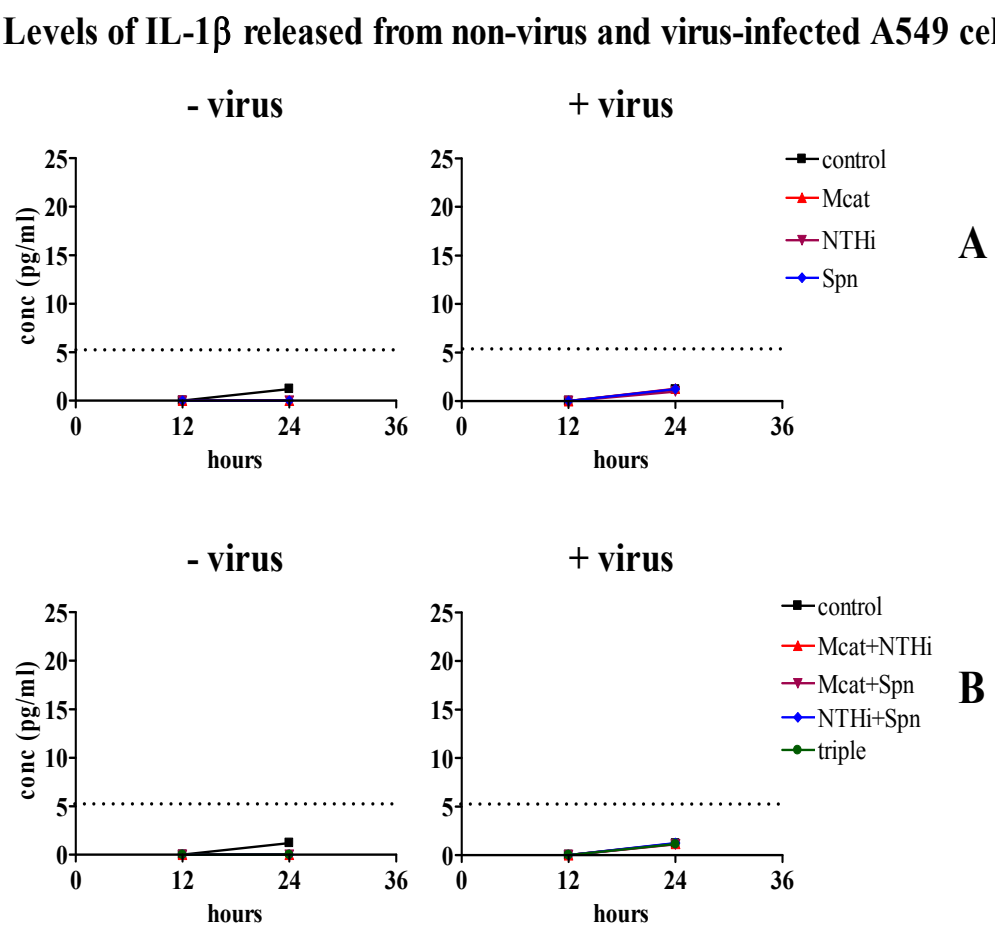
Levels of TNF-α released from non-virus and virus-infected A549 cells. The bars represent the median values of TNF-α released at 12 hr and 24 hr from A549 cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for TNF-α set by the instrument.

C.1.2 Levels of IL-10 released from non-virus and virus-infected A549 cells



Levels of IL-10 released from non-virus and virus-infected A549 cells. The bars represent the median values of IL-10 released at 12 hr and 24 hr from A549 cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-10 set by the instrument.

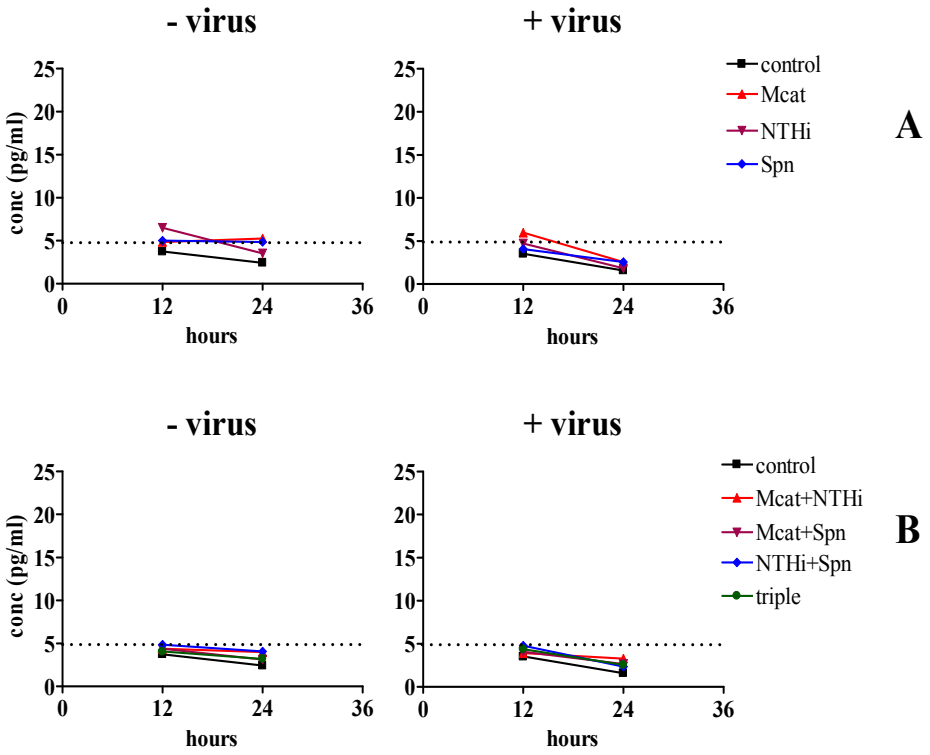
C.1.3 Levels of IL-1β released from non-virus and virus-infected A549 cells



Levels of IL-1β released from non-virus and virus-infected A549 cells. The bars represent the median values of IL-1β released at 12 hr and 24 hr from A549 cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-1β set by the instrument.

C.1.4 Levels of IL-12(p70) released from non-virus and virus-infected A549 cells

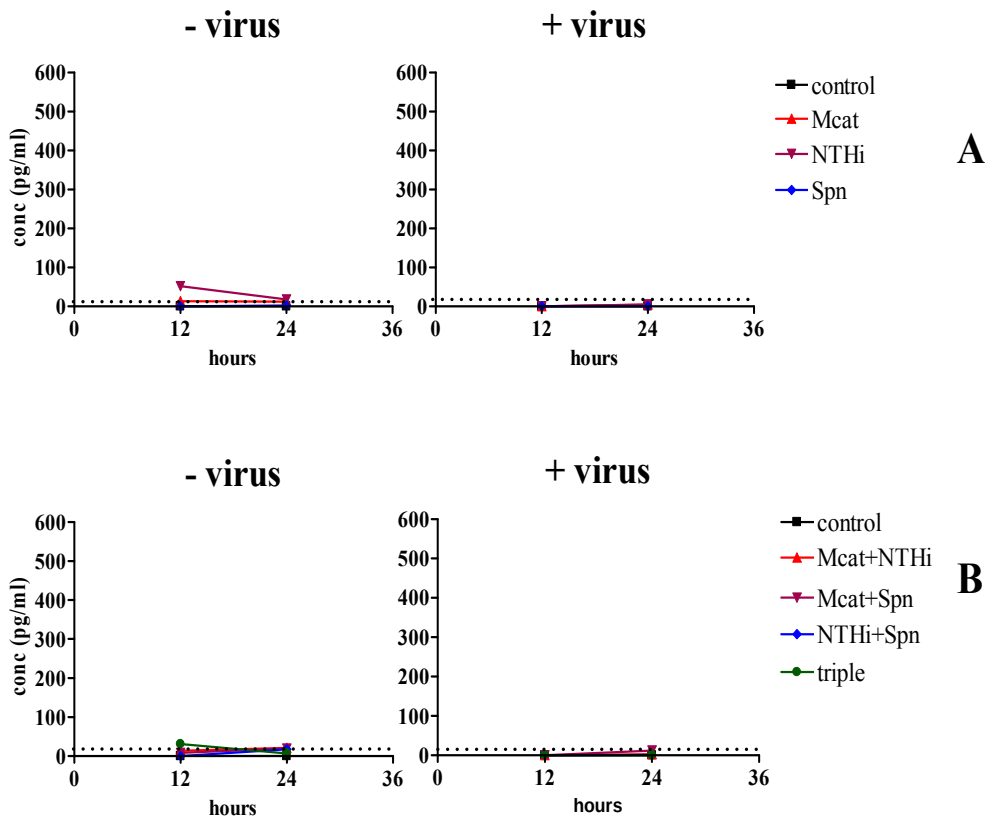
Levels of IL-12(p70) released from non-virus and virus infected A549 cells



Levels of IL-12(p70) released from non-virus and virus-infected A549 cells. The bars represent the median values of IL-12(p70) released at 12 hr and 24 hr from A549 cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-12(p70) set by the instrument.

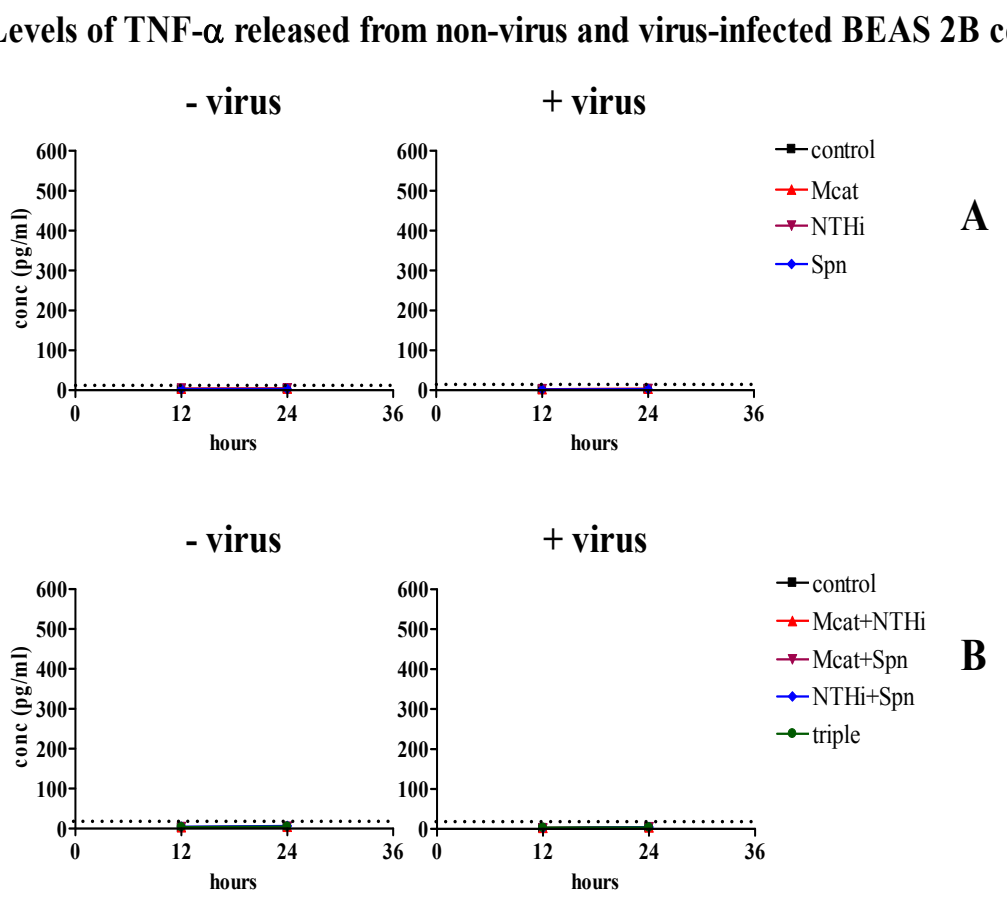
C.2 Cytokines released following infection of BEAS-2B cells

C.2.1 Levels of IFN-γ released from non-virus and virus-infected BEAS-2B cells



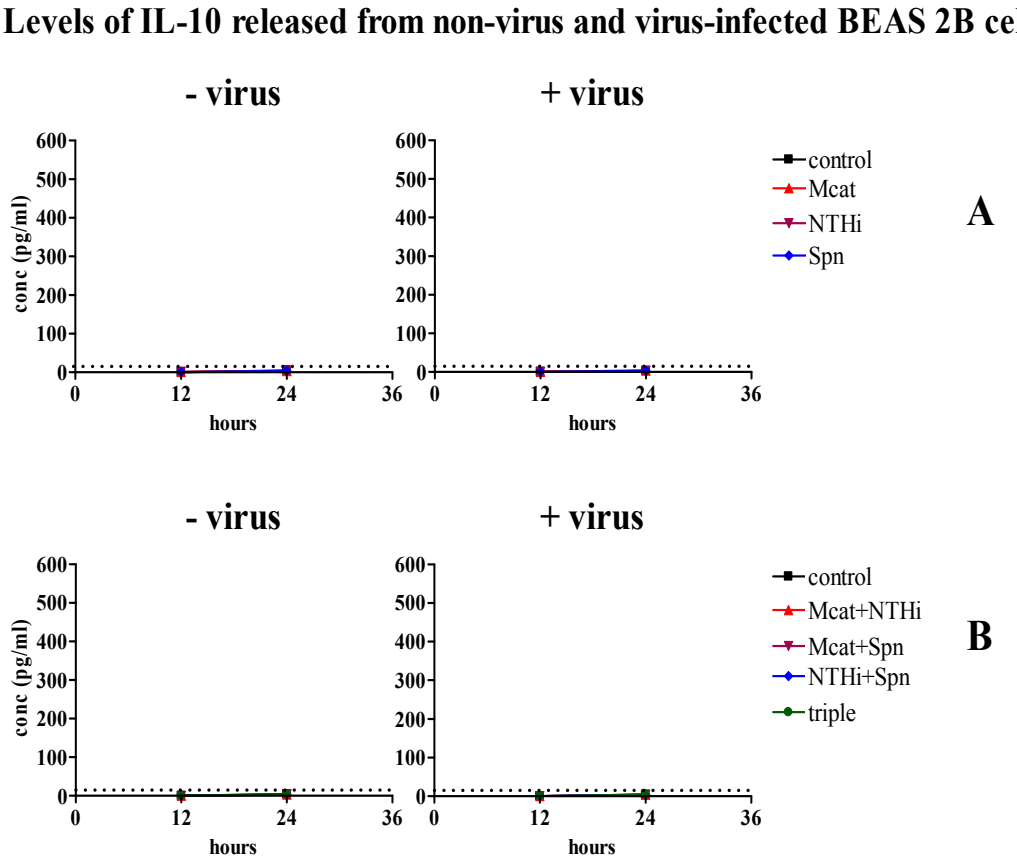
Levels of IFN-γ released from non-virus and virus-infected BEAS-2B cells. The bars represent the median values of IFN-γ released at 12 hr and 24 hr from BEAS-2B cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IFN-γ set by the instrument.

C.2.2 Levels of TNF-α released from non-virus and virus-infected BEAS-2B cells



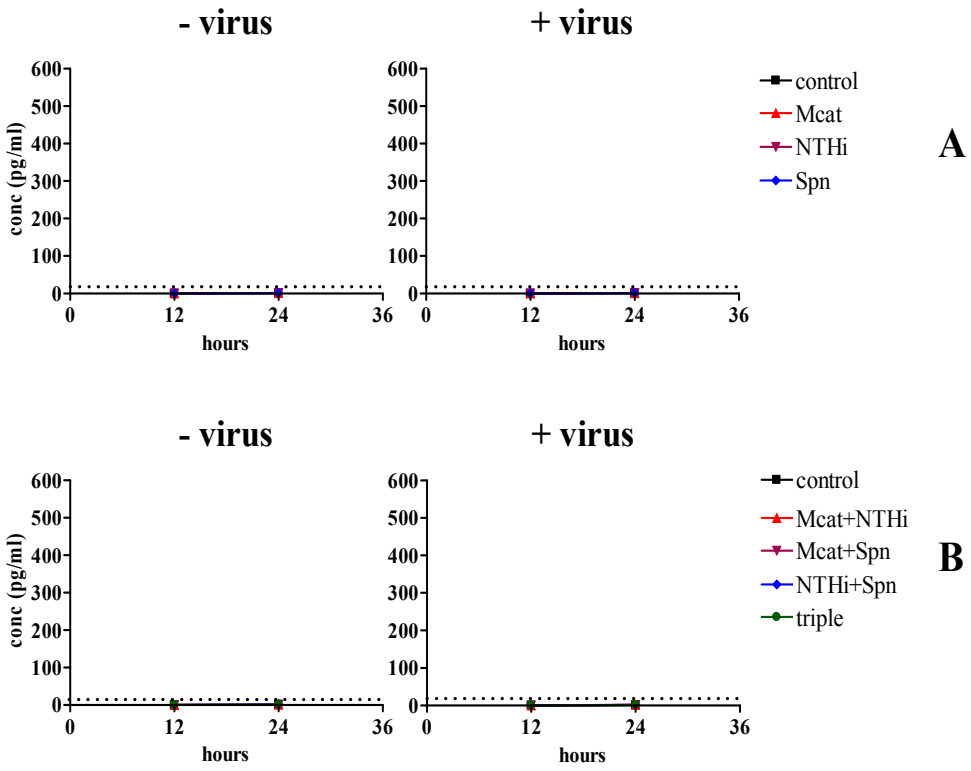
Levels of TNF-α released from non-virus and virus-infected BEAS-2B cells. The bars represent the median values of TNF-α released at 12 hr and 24 hr from BEAS-2B cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for TNF-α set by the instrument.

C.2.3 Levels of IL-10 released from non-virus and virus-infected BEAS-2B cells



Levels of IL-10 released from non-virus and virus-infected BEAS-2B cells. The bars represent the median values of IL-10 released at 12 hr and 24 hr from BEAS-2B cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-10 set by the instrument.

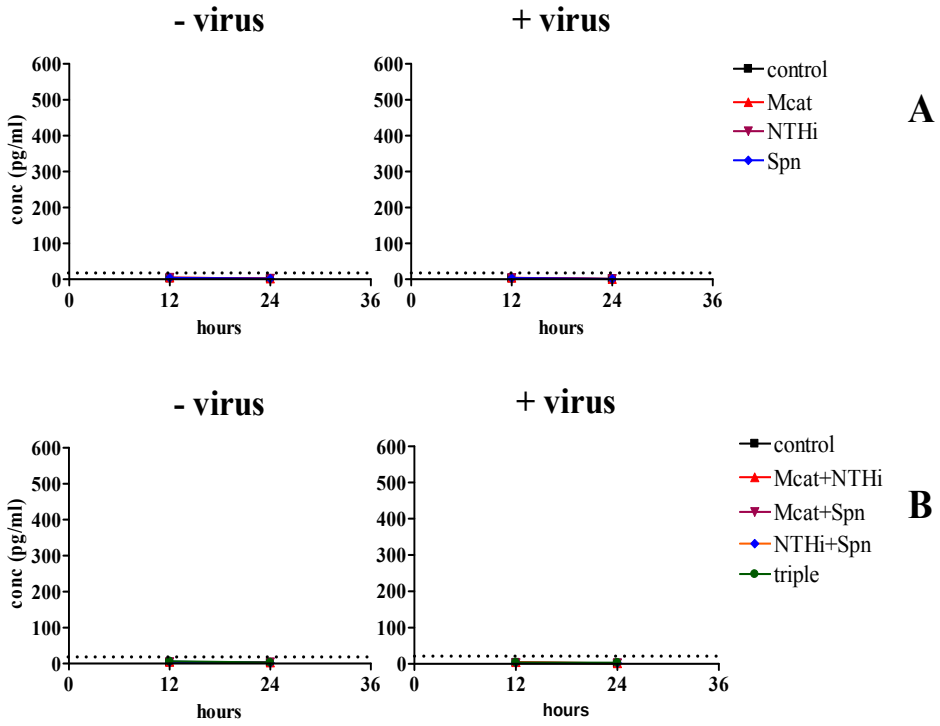
C.2.4 Levels of IL-1β released from non-virus and virus-infected BEAS-2B cells



Levels of IL-1β released from non-virus and virus-infected BEAS-2B cells. The bars represent the median values of IL-1β released at 12 hr and 24 hr from BEAS-2B cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-1β set by the instrument.

C.2.5 Levels of IL-12(p70) released from non-virus and virus-infected BEAS-2B cells

Levels of IL-12(p70) released from non-virus and virus-infected BEAS 2B cells



Levels of IL-12(p70) released from non-virus and virus-infected BEAS-2B cells. The bars represent the median values of IL-12(p70) released at 12 hr and 24 hr from BEAS-2B cells when infected with A] Single bacterium [*M. catarrhalis* (Mcat), NTHi or *S. pneumoniae* (Spn); B] Double and triple bacterial combination (Mcat+NTHi; Mcat+Spn; NTHi+Spn or Mcat+NTHi+Spn). The control samples represent the relevant untreated or virus only-treated cells. The dashed line represents the assays limit of detection for IL-12(p70) set by the instrument.