Acute Respiratory Infection in Pre-School Children

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master in Philosophy

by

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Abbreviations

%	Percentage
^{o}C	Degrees Celcius
µl	Microlitre
A&E	Accident & Emergency
AdV	Adenovirus
APC	Antigen presenting cell
ARI	Acute respiratory infection
ASF	Airway Surface Fluid
ATL	Animal tissue lysis
BAL	Bronchoalveolar lavage
B-cells	B-lymphocytes
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
Caspase	cysteine-aspartic protease
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CoV	Coronavirus
CpG	Cytosine-phosphate-guanine
Cpp	*Chlamydia pneumoniae*
DALY	Disability adjusted life year
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
EVW	Episodic viral wheeze
Flu	Influenza virus
Flu A	Influenza virus type A
Flu B	Influenza virus type B
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
hBoV	human Bocavirus
Hib	*Haemophilus influenzae* type B
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hMPV	human Metapneumovirus
hRSV	human respiratory syncytial virus
hRV	human rhinovirus
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IMIP	Instituto de Medicina Integral Professor Fernando Figueira
ITU	Intensive care unit

LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
MAC	Membrane attack complex
MASP	Mannan binding lectin-associated serine protease
MBL	Mannan binding lectin
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
Mpp	*Mycoplasma pneumoniae*
NaCl	Sodium Chloride
NF-κB	Nuclear factor-Kappa B
NK	Natural Killer
NPA	Nasopharyngeal aspirate
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV7	Heptavalent pneumococcal conjugate vaccine
pg	Picogram
PICU	Paediatric intensive care unit
PIV	Parainfluenza virus
PRR	Pattern recognition receptor
R$	Brazilian Reais
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SARS	Severe acute respiratory syndrome
SD	Standard deviation
S. pneumoniae	*Streptococcus pneumoniae*
TB	Tuberculosis
Th	T-helper
TLR	Toll-like receptors
TNF	Tumour necrosis factor
T-cells	Thymus-derived lymphocytes
UK	United Kingdom
URTI	Upper respiratory tract infection
USA	United States of America
US$	United States dollar
WHO	World Health Organisation

Abstract

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Katie Rose

Introduction: Acute respiratory infection (ARI) is a major cause of morbidity and mortality worldwide, being responsible for 3.5 million deaths annually. Despite this the precise aetiology of ARI is often unclear, and the pathogenesis remains poorly understood. The aims of this study were i) to investigate the viral and atypical bacterial causes of ARI in a clinically and demographically well defined population of children less than five years of age and ii) to investigate host immunological response to ARI caused by different pathogens and in different severities of disease.

Methods: Clinical and demographic data, along with nasopharyngeal aspirate samples were collected from 407 pre-school children who presented with ARI to the IMIP Children's Hospital, Recife, Brazil over a 12 month period. Multiplex PCR was used to detect 17 different respiratory pathogens.

The concentrations of nine cytokines were determined in a selection of samples from children with ARI caused by different pathogens and in differing severities of human respiratory syncytial virus (hRSV) disease.

Results: At least one pathogen was detected in 85.5% of samples. The pathogens detected most frequently were hRSV (in 37.3% of samples), adenovirus (AdV; 24.8%), human rhinovirus (hRV; 18.9%), human bocavirus (hBoV; 18.7%), human metapneumovirus (hMPV; 10.3%) and *Mycoplasma pneumoniae* (*Mpp*; 9.8%). Co-infection with multiple pathogens was detected in 39.6% of samples, with the pathogens most frequently detected in co-infection being AdV (in 52.8% of co-infections), hRSV (44.1%), hBoV (41.6%) and hRV (26.7%). Infection with *Mpp* or hRSV was associated with hospital admission and more severe disease.

Cytokine profiles were similar in ARI caused by different pathogens and in different severities of disease.

Conclusions: We have described high rates of pathogen detection and co-infection, with particularly high rates of *Mpp*, AdV and hBoV infection. Our results may reflect the type of samples collected, the number of pathogens tested for, and/or the population demographics. The high prevalence of *Mpp* is more commonly associated with ARI in older children, and our findings may have implications for antibiotic use in this age group.

Differing pathogens and differing severities of disease were not associated with particular patterns of cytokine production in the upper respiratory tract. This may reflect the type of sample or the particular cytokines investigated.

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1 Introduction

1.1 Acute Respiratory Infection

1.1.1 Definition

Acute respiratory infection (ARI) describes a multitude of signs and symptoms, essentially including all types of infection of the respiratory tract. It is characterised by cough or wheeze, with or without the presence of fever, acute rhinitis, otitis media and pharyngitis[1].

1.1.2 Burden

The World Health Organisation (WHO) estimates that respiratory infections account for 6% of the total global burden of disease – a higher percentage than diarrhoeal disease, cancer, human immunodeficiency virus (HIV) infection, ischaemic heart disease or malaria[2]. ARI is the third most common cause of death worldwide, and the most important in developing countries[3 4]. Worldwide, ARI is responsible for 3.5 million deaths each year, and just under two million of these are children under the age of five[5].

Pneumonia is the most common cause of death in children under the age of five, causing an estimated 19-21% of all deaths in this age group[6].

The WHO has calculated the burden of disease associated with ARI using disability adjusted life years (DALYs), which incorporate both loss of life in terms of deaths due to disease (mortality) and loss of 'healthy' life (i.e. disability) due to disease (morbidity), it was found that ARI caused the loss of 2,983 DALYs per 100,000 population in the world's poorest regions, in comparison to loss of 137 DALYs per 100,000 population in the world's richest regions[2].

In developed countries, ARI are the largest contributor to the total infectious disease burden. In the United States of America (USA) it has been estimated that ARI cause between 545,000 and 840,000 admissions in under 18 year olds each year, accounting for between 14 and 20% of all admissions in this age group[7]. Statistics show that for the majority of health problems, advancement in management or prevention is decreasing the burden of particular diseases. However, in ARI there has been very little change in the number of DALYs lost per 100,000 population between 1990 and 2002[2]. Firstly, this may be explained by the ageing population seen in so many developed countries. Along with young children, the elderly are more likely to develop an ARI in the first place, and more likely once they have an ARI to develop a severe infection requiring hospitalisation[8]. Secondly new respiratory pathogens, or strains of already known pathogens, are emerging frequently, which can be increasingly virulent causing widespread problems worldwide, or increasingly severe and more difficult to manage[2].

1.1.3 Epidemiology

Age groups

Acute respiratory infections affect all age groups, although the very young and the elderly are most likely to suffer severe disease and require hospital admission[8].

Seasonal Distribution

ARI occur throughout the year, however most studies find more infections occur in the autumn and winter months[9-11]. In paediatric studies, there is a strong peak incidence in the winter months, particularly in children under one year of age. This is due to the large number of infections caused by human respiratory syncytial virus (hRSV), the most common ARI causing pathogen in this age group. hRSV has a strong seasonality, peaking in the winter months in temperate climates, and in the wetter months in tropical climates[10], with very few infections being detected in the summer months. Peaks are seen in other pathogens,

however, none have such a distinct seasonality as hRSV infection, and patterns of particular pathogens tend to vary from year to year. Human metapneumovirus (hMPV) for example, has been found to peak in some years at the same time as hRSV infection[12], while other years it may peak immediately after the hRSV peak, or months later[13 14]. Other pathogens such as human rhinovirus (hRV) and adenovirus (AdV) are prevalent throughout the year[10 15].

1.1.4 Risk Factors

Risk factors for ARI can be split into clinical and demographic factors. Clinical risk factors include male sex; prematurity; low birth weight; co-existing cardiac or respiratory problems; immunodeficiency and birth during the first half of the hRSV season. Demographic risk factors include a lack of breastfeeding; multiple siblings/crowded living conditions; a low socioeconomic status and smokers in the household[16].

Breastfeeding

Many studies have found associations between breast-feeding and protection against ARI, specifically against ARI hospitalisation, and with a greater effect in developing countries[17-19]. Breast-feeding is beneficial due to colostrum containing hRSV-IgA and lactoferrin, both of which have antiviral properties, and it is also thought to promote lung maturation, having a particularly positive effect in the very young, and premature infants[16].

Overcrowding

Many studies have found associations between crowded living conditions and an increased risk of ARI. Increasing numbers of people sharing a bedroom with the child; increased numbers of children under the age of 12 in the household and living with school-age siblings, have all been shown to be independent risk factors[20-23].

Socio-economic status

Low socioeconomic status has itself been found to be an independent risk factor for bronchiolitis, with one study finding children in the most deprived area of one particular city in Sweden to be almost twice as likely to need hospitalisation for bronchiolitis than those in the rest of the city[24]. Explanations for low socioeconomic status leading to increased risk of ARI are probably due to previously mentioned risk factors, including an increased likelihood of over-crowding, and parental smoking, as several studies have found increased rates of smokers in lower socioeconomic groups[25-27].

Smoking

Both passive smoking and maternal smoking during pregnancy have been linked to an increased risk of the development of ARI, and increased risk of severe ARI requiring admission to hospital[28 29].

Air Pollution

Air pollution has been reported as a risk factor for ARI for many years, with data from the 1950's during the 'London fog' suggesting increased mortality due to respiratory causes during that period[30]. Indoor air pollutants such as the use of particular cooking fuels have also been shown to be risk factors of ARI in children. This is particularly relevant in developing countries, where biomass fuels are still commonly used, often in poorly ventilated settings, and may also be a contributing factor to the increased morbidity and mortality from ARI in developing countries[31 32].

HIV infection

Human immunodeficiency virus (HIV) infection is an important risk factor for ARI, with the biggest impact being in Sub-Saharan Africa.

1.1.5 Aetiology

Causative organisms are predominately bacterial or viral, with *Streptococcus pneumoniae* (*S. pneumoniae*) and *Haemophilus influenzae* type b (Hib) being the most common bacterial pathogens and hRSV, hRV, AdV, and parainfluenza virus (PIV) being the most commonly identified viruses[33].

1.1.5.1 'Typical' Bacterial Pathogens

Streptococcus pneumoniae

Streptococcus pneumoniae (S. pneumoniae) is the most common cause of community-acquired pneumonia worldwide[34]. It also causes meningitis and otitis media. *S. pneumoniae* is commonly carried in the nasopharynx of healthy individuals, with one particular study finding 26% of two month old infants, and 62% of two year olds to be carriers[35]. Problems arise when *S. pneumoniae* spreads from the nasopharynx to the lower respiratory tract causing pneumonia, or other sites such as the meninges, blood or middle ear, causing meningitis, bacteraemia or otitis media – these together are termed invasive pneumococcal disease[34]. Risk factors for carriage of *S. pneumoniae* include a young age, young siblings, day care attendance, asthma and, in adults, smoking[34]. Invasive pneumococcal disease is a huge problem, causing an estimated one million deaths in children under the age of five annually[34]. Human immunodeficiency virus (HIV) infection is an important risk factor for invasive pneumococcal disease, particularly in Sub-Saharan Africa. Those with HIV have a 10-100 times increased risk of developing invasive pneumococcal disease when compared to the general population[36].

Haemophilus influenzae type b

Haemophilus influenzae type b (Hib) is another leading cause of community-acquired pneumonia and meningitis, with the WHO estimating it caused 8.13 million serious illnesses

and 371,000 deaths in the year 2000[37]. Successful Hib vaccines have been available for a number of years, and have led to dramatically reduced levels of Hib disease. A study published in 2009 estimated that in 2006, 55% of children in the world were included in Hib vaccination programmes. However, it is in a number of less developed countries where the vaccine is not routinely given, where Hib is still a major problem. It is estimated that 61% of all Hib childhood deaths occur in just 10 countries in Asia or Africa[38].

1.1.5.2 'Atypical' Bacterial Pathogens

'Atypical' bacterial pathogens are so called due to their ability to cause atypical pneumonia – pneumonia with symptoms different from those caused by *S. pneumonia*, including a sub-acute onset, low-moderate grade fever, dry cough, prominent constitutional symptoms, absent or moderate leukocytosis and more extensive radiographic involvement than would be expected after physical examination[39]. The three most important atypical bacteria are *Mycoplasma pneumoniae* (*Mpp*), *Chlamydia pneumoniae* (*Cpp*), and *Legionella*[40].

Mycoplasma pneumoniae

Mycoplasma was first described in 1944[41], but was classified as *Mycoplasma pneumoniae* (*Mpp*) by Chanock et al in 1963[42]. It is estimated that *Mpp* may cause between 14 and 34% of lower respiratory tract infections in children, but it is generally accepted that it is more commonly associated with disease in older children and adolescents[43 44], and is thought to be the most common cause of community acquired pneumonia in the 5-20 age group[40]. Studies specifically investigating children under the age of five with ARI have found *Mpp* rates to be between 0 and 1.5%[10 45 46].

Chlamydia pneumoniae

Chlamydia pneumoniae (Cpp) is Gram negative, intracellular bacterium that, like other respiratory pathogens, may be responsible for a host of respiratory symptoms. *Cpp* has been found to be associated with between 6 and 20% of cases of community acquired

pneumonia[40]. Few studies have investigated the role of *Cpp* in children under the age of five, but those that have found rates of 0-1.3%[10 45].

Legionella

Legionella is a Gram negative bacterium that was first named after an outbreak at an American Legion convention in 1976[47], although after identification previous outbreaks without known aetiology were discovered to have been caused by species of *Legionella*[48]. *Legionella* is usually transmitted by direct inhalation from contaminated water supplies, usually man-made reservoirs, where the bacterium thrives[40]. It causes a wide variety of clinical manifestations, from mild respiratory disease, to Legionnaires disease, with severe pneumonia and multisystem involvement, with neurological, hepatic and haematological abnormalities. *Legionella* is thought to be associated with between 1 and 27% of community acquired pneumonias[40], however it is very rare in young children, with most cases occurring in the immunocompromised, or those with risk factors such as prematurity or bronchopulmonary dysplasia[48].

1.1.5.3 Viral infection

Human Respiratory Syncytial Virus

Human respiratory syncytial virus (hRSV) is a single-stranded RNA virus of the Paramyxoviridae family, from the pneumovirinae subgroup. It gets its name from its ability to cause the cell membranes of nearby cells to fuse and form syncytia[49]. It is the most common cause of ARI in infants and young children, with almost all children having come into contact with the virus by the age of two years. One large study found hRSV to be responsible for 20% of hospitalisations, 18% of emergency department visits and 15% of general practitioner visits in children under five with ARI in the United States[50]. It is also thought to be responsible for 50-90% of hospitalisations for bronchiolitis[51].

There are many other respiratory viruses which have been implicated in the pathogenesis of ARI. Influenza (flu), PIV, AdV, coronaviruses (CoV) and hRV have all been accepted as common aetiological agents, and have been found in varying prevalences in populations of children and adults with ARI.

Rhinoviruses

Rhinoviruses have been found to cause about two-thirds of common colds and asthma exacerbations in adults and older children[52]. Previous work has shown rhinoviruses have optimum replication temperatures of 33^{o}C – the temperature in the nasal passages, as opposed to the higher temperatures in the lower respiratory tract, another factor in support of rhinoviruses being associated with upper respiratory tract infections (URTI) and the common cold. However, further research has shown that although the optimum temperature for hRV replication is 33^{o}C, they are still able to replicate effectively at 37^{o}C, proving it is feasible that they are a cause of lower respiratory tract infection[53].

Kusel et al. in 2006 detected hRV in 48% of nasopharyngeal aspirates from children under the age of one year with ARI[10], however this study population comprised children who were all at high risk of atopy (i.e. had at least one parent with asthma, eczema or hayfever), so results may not apply to general populations. Other studies looking specifically at children with ARI found the prevalence of hRV to be between 3.6 and 24.7%[1 15 45 54].

Adenovirus

Adenovirus (AdV) infection can be classified according to over 50 different serotypes, which can be further divided into six main subgroups, each of which has some general clinical characteristics. AdV can cause a wide range of manifestations, with subgroups B1, C and E generally causing respiratory disease, subgroup F causing gastroenteritis, and subgroup D causing keratoconjunctivitis[55]. AdV infection of the respiratory tract can lead to a wide spectrum of disease from mild upper respiratory tract symptoms to severe pneumonia,

with one particular serotype (serotype 14) being associated with particularly severe disease and a high mortality rate[56].

Parainfluenza Viruses

Parainfluenza viruses (PIV) are common causes of ARI, especially in children, with studies estimating that most children will have evidence of infection with multiple serotypes by the age of 5[57].

Influenza Viruses

Influenza (flu) viruses are responsible for between 250,000 and 500,000 deaths each year. Influenza A (flu A) viruses commonly cause pandemics, with the most recent being the novel H1N1 pandemic (commonly referred to as 'Swine flu'), which was declared a public health emergency by the WHO on the 25th April 2009[58]. H1N1 influenza presented in a similar way to seasonal influenza, but had increased occurrences of vomiting and diarrhea, and an increased number of hospitalisations and deaths, with young people being particularly affected[58]. Influenza B (flu B) is also an important cause of morbidity and mortality, however it generally causes milder disease, and is responsible for 75% fewer hospital admissions than flu A[59].

Coronaviruses

Coronaviruses (CoV) are also a common cause of ARI, usually causing similar clinical manifestations to other previously mentioned viruses, with mild URTI being common. However, severe associated respiratory syndrome (SARS)-associated coronavirus was identified in 2002, which had particularly severe clinical manifestations, with some patients developing respiratory distress syndrome, requiring intensive care and ventilation[57].

Recently discovered viruses

The discovery of new respiratory pathogens is not uncommon. Recently discovered respiratory pathogens include human metapneumovirus (hMPV), first identified in 2001[60];

two new coronaviruses, NL-63 (CoV NL-63), discovered in the Netherlands in 2004, and HKU-1 (CoV-HKU-1), discovered in Hong Kong in 2005[61 62]; as well as human bocavirus (hBoV) discovered in Sweden in 2005[63].

Human metapneumovirus

Since discovery in 2001, hMPV has been shown to play a major role in ARI, being found in 1.5-14% of samples from children with ARI[1 33 45]. Some studies have found that hMPV is associated with more severe disease when found in co-infection with hRSV[64].

Human Bocavirus

Human bocavirus (hBoV) was discovered by random polymerase chain reaction (PCR) amplification of respiratory samples from children[63]. Since its discovery it has been described by several groups worldwide, with prevalences in those with ARI being found to be between 1.5 and 18%, with detection most frequent in young children[46 65]. However, proving causality of hBoV in ARI has not been fully achieved, and it is still uncertain as to whether or not the detection of hBoV is clinically significant[65-67].

1.1.5.4 Tuberculosis

Mycobacterium tuberculosis (TB) is an aerobic, acid-fast Gram positive bacillus which can infect all organs of the body, with pulmonary infection being most common[68]. Although vaccinations and treatment of TB have been successful, it is still a major global problem. Worldwide, 9 million new TB infections occurred in 2007, with 1.4 million of these being in those with HIV infection, most commonly in Africa (79%) or South-East Asia (11%)[69]. TB infection in children is usually due to close contact with an infected family member, and those with HIV infection or other form of immunocompromise are most at risk[68]. Infants are at the highest risk of developing disease after infection, as opposed to asymptomatic infection. Acute infections are most likely in the 3-12 months after initial infection, and may present with cough, fever and lymphadenopathy, as well as complications such as miliary TB where the bacterium spreads through the bloodstream, and causes disseminated disease throughout the lungs, as well as TB meningitis[68]. In developed countries the incidence of TB in children may be as low as 10 per 100,000 population. In contrast, in South Africa in 1993 the incidence rates were 273 per 100,000 in males under the age of one, and 205 per 100,000 in females under the age of one, and in a particular Cape Town community in 1991 the TB rate in children under the age of four was 3588 per 100,000[70].

1.1.5.5 Previous studies into aetiology

Many previous studies have investigated the aetiology of ARI, looking at various populations and a varying number of pathogens. **Table 1** outlines the findings of a selection of studies that have investigated the prevalence of a range of pathogens in ARI. The studies in the table have been included due to their populations being restricted to pre-school children.

As well as indicating the % prevalence of each pathogen, the table highlights co-infection rates described in the studies. Since the introduction of multiplex PCR and its ability to simultaneously detect multiple pathogens within one sample, many studies have included co-infection rates in their analysis, with findings ranging from 4% to 33%[10 15 33 45 46].

One notable study that is not included in the table is that by Brunstein et al. They investigated the prevalence of 21 pathogens in 1742 samples, from a population made up of 80% adults and 20% children. They detected at least one pathogen in 68% of samples, with co-infection in 26.8%[54]. However, one of the pathogens included was *S. pneumoniae*, detected in 20% of the population, and in a high proportion of co-infections. The authors acknowledged the point that they were not able to tell if the *S. pneumoniae* positive samples were examples of carriage of the bacterium, or if it was actually causing disease[54].

Table 1: Summary of a selection of studies investigating the epidemiology of ARI in pre-school children

Study	Bharaj et al[13] 2009	Kaplan et al[46]	Sung et al[45] 2009	Lambert et al[71] 2007	Souza et al[15] 2003	Canducci et al[33] 2008	Regamey et al[1] 2008	Kusel et al[10] 2006
Age Group (years)	**<5**	**<5**	**<5**	**<5**	**<2**	**<2**	**<1**	**<1**
Total +ve	**35.2**	**78**	**47**	**74**	**43**	**46.6**	**79**	**69**
Co-infection	**6.6**	**33**	**4**	**9.9**	**4.8**	**14**	**20**	**10.3**
AdV	**X**	**37**	**4.8**	**3.1**	**5.9**	**X**	**3**	**1.5**
hBoV	**X**	**18**	**X**	**X**	**X**	**2.2**	**5**	**X**
CoV	**X**	**1.2**	**3.8**	**1.5**	**X**	**8.7**	**18**	**5.5**
Cpp	**X**	**4.5**	**0**	**X**	**X**	**X**	**X**	**1.3**
Flu	**3.0**	**0.6**	**10.6**	**3.7**	**3.0**	**X**	**4**	**4.5**
hMPV	**3.7**	**2.5**	**1.5**	**3.7**	**X**	**14.3**	**13**	**1.8**
Mpp	**X**	**0**	**1.5**	**X**	**X**	**X**	**X**	**1.4**
PIV	**16.3**	**0**	**8.8**	**4.1**	**5.1**	**X**	**17**	**5.2**
hRSV	**20.3**	**43**	**8.4**	**6.6**	**1.8**	**28**	**16**	**10.9**
hRV	**X**	**11**	**3.6**	**n/a**	**24.7**	**X**	**23**	**48.5**

Note: All values shown are % prevalence. **X** indicates that the particular study did not test for that pathogen. (AdV, adenovirus; hBoV, human bocavirus; CoV, coronavirus; *Cpp*, *Chlamydia pneumoniae*; Flu, influenza virus; hMPV, human metapneumovirus; *Mpp*, *Mycoplasma pneumoniae*; PIV, parainfluenza virus; hRSV, human respiratory syncytial virus; hRV, human rhinovirus)

1.1.6 Clinical Manifestations of ARI

The term 'acute respiratory infection' encompasses all infections of the respiratory tract, from a mild upper respiratory tract infection (URTI), to severe bronchiolitis or pneumonia. Diagnoses are usually made on clinical grounds, which are subjective, being dependant on the clinician involved, with lots of overlaps between different clinical diagnoses.

1.1.6.1 Upper Respiratory Tract Infection

URTI are very common, and usually mild, with the common cold being the most common manifestation. Other manifestations classed as URTI include otitis media, croup, whooping cough and epiglottitis.

Otitis Media

Otitis media describes infection or inflammation of the middle ear, and is very common, affecting over 80% of children by the age of three years[72]. Symptoms of otitis media include ear-ache, which in a young child may present with them being irritable and tugging at one or both ears, fever, loss of balance and a degree of hearing loss. It is thought that up to one third of cases of otitis media are caused by *S. pneumoniae*[73].

Croup

Laryngotracheobronchitis, or croup, describes inflammation of the larynx and trachea. It causes hoarseness and a loud, harsh, barking cough. It can also cause stridor, commonly when the child becomes upset, but also in more severe disease. The most common cause of croup is PIV infection, but it can also be caused by other respiratory pathogens[74].

Whooping cough

Pertussis, or whooping cough, is so called due to the 'whooping' sound made during the inspiration of air against a closed glottis after a bout of coughing. It is caused by the Gram negative bacterium *Bordetella pertussis*. The main symptom is bouts of coughing, often

ending in vomiting. The illness is usually mild, although it can be severe in young children, and can last for a prolonged period of time[74].

Epiglottitis

Epiglottitis is a particularly serious manifestation of URTI. It describes inflammation of the supraglottic larynx, usually caused by *Haemophilus influenzae* type b. It is characterised by acute onset stridor, and can rapidly cause obstruction of the trachea, meaning prompt management is vital[74].

1.1.6.2 Bronchiolitis

Bronchiolitis predominately affects infants and young children, with the main clinical features being initial coryza, developing into cough, with the presence of wheezing and/or crackles and signs of respiratory distress, such as tachypnoea, hyperinflation and recession, commonly complicated by acute otitis media[75 76].

1.1.6.3 Pneumonia

Pneumonia describes inflammation of the parenchyma of the lung[77]. In children the signs and symptoms include fever, tachypnoea and respiratory distress, often with focal or diffuse crackles on auscultation. In young children pneumonia is commonly caused by both bacteria and viruses, with *S. pneumoniae* being the most common bacterial cause[78].

1.1.6.4 Episodic Viral Wheeze/Asthma

Wheeze can be defined as 'a continuous high-pitched sound with musical quality emitting from the chest during expiration', and is caused by limitation to the airflow during expiration[79]. Episodic viral wheeze (EVW) can be described as 'wheezing during discreet time periods, often in association with clinical evidence of a viral cold'[79]. Episodic viral

wheeze is most common in children under five, and usually disappears by the age of six, although it may continue[79]. The Global Initiative for Asthma (GINA) has described asthma as 'a syndrome with a highly variable clinical spectrum, characterised by airway inflammation'. However, the presence of inflammation is difficult to determine in very young children, so EVW is a more accurate description, until symptoms can be more accurately assessed when the child is older, and if necessary a diagnosis of asthma can be made[79].

1.1.7 Investigations

The diagnosis of ARI is a clinical one, based on history and physical examination findings. Specific diagnoses such as URTI, bronchiolitis, pneumonia, and episodic viral wheeze are used and do have an impact on management, with children diagnosed with pneumonia being more likely to be given antibiotics than children diagnosed with bronchiolitis, which is presumed to be viral.

There are many techniques available for the detection and identification of pathogens responsible for ARI. Studies have shown major advantages to viral identification[80]. Knowledge of the correct diagnosis helps determine the optimum management of the infection, decreasing morbidity and mortality. Studies have shown decreased antibiotic use and shorter hospital stays, with children with proven viral pathogens not being given unnecessary antibiotics[81]. Viral identification is also used to separate children infected with particular pathogens[76], allowing uninfected children, and particularly vulnerable children, such as those with congenital cardiac and respiratory problems, to be protected from exposure.

Sampling Techniques

The British Thoracic Society guidelines on community acquired pneumonia state that all children suspected of having bacterial pneumonia should have blood cultures taken, and all children under the age of 18 months should have a nasopharyngeal aspirate (NPA) taken for viral antigen detection with or without viral culture[78].

Obtaining a nasopharyngeal aspirate involves inserting a catheter between five and seven centimeters into the child's nostril, then pulling back on the catheter while applying gentle suction[82]. An alternative method involves taking a nasal swab, where a cotton swab is inserted around 1.5 centimeters into the nostril and rotated against the nasal cavity to obtain a sample[83]. Taking a nasal swab is quicker, less invasive and less uncomfortable for the child. However, several studies have found that the sensitivity of detection of respiratory viruses is higher in NPA samples when compared to nasal swabs[82-84].

The other problem with sampling using NPAs or nasal swab samples, is that in both cases, the sample is being taken from the upper respiratory tract, rather than the lower respiratory tract where the infection is causing the clinical manifestations of disease. Bronchoalveolar lavage (BAL) is a way of obtaining fluid from the lower respiratory tract, and although this would make it ideal for the detection of pathogens which are causing lower respiratory tract infection, it is invasive and requires sedation, so is not a practical option for the majority of those with ARI. It does, however, have a place in severely ill children who are already ventilated, and non-bronchoscopic BAL may then be a practical way of detecting pathogens in the lower respiratory tract[85].

Pathogen detection

Polymerase chain reaction (PCR) has been shown to be the gold standard in the rapid detection of respiratory viruses, and many advantages over other methods have been

described. More traditional methods include virus culture and antigen detection. However, virus culture can take between 2 and 14 days to obtain results[86], so in most cases would not be of much use in guiding the management of a patient. Antigen detection methods such as enzyme immunoassay are quicker than virus culture and results can be available in less than 24 hours. However, the sensitivity and specificity of PCR has been found to be greater than that of other methods[86 87]. PCR will be discussed in more detail in the methods section.

Chest Radiographs

In ARI decisions about antibiotic use are often based on radiographic findings, with signs of consolidation on chest x-ray being presumed to be caused by bacteria and thus treated with antibiotics. Studies have shown that the use of chest radiographs are associated with increased likelihood of antibiotics and longer stays in hospital. One study randomised children with ARI to receive or not receive a chest x-ray, and found that children who received a chest x-ray were more likely to be diagnosed with having bacterial pneumonia or lower respiratory tract infection and treated with antibiotics, although the median time to recovery was the same in both groups[88]. This study concluded that chest x-rays did not affect clinical outcome, and were not beneficial, in children over two months of age with acute lower respiratory infection[88].

Indicators of disease severity

Perhaps more important than labelling a condition, is the ability to recognise more severe disease, or the potential to develop more severe disease. Several studies have investigated clinical predictors of disease severity in children with suspected bronchiolitis, with the most consistent finding being that oxygen saturations derived from pulse oximetry are the single most useful predictor of severe disease[89 90]. Other independent predictors of severe disease were found to be young age and young gestational age, as previously mentioned, and high respiratory rate[90].

1.1.8 Management of the Clinical Manifestations of ARI

The majority of children with ARI will have a mild, self-limiting illness and do not require any treatment. However, ARI can cause very severe infections, especially in the very young and those with other co-morbidities, leading to many hospital admissions each year. The mainstay of treatment is supportive, and includes the use of supplemental fluids and oxygen.

1.1.8.1 Upper Respiratory Tract Infection

Upper respiratory tract infection (URTI) is the most common manifestation of ARI, covering everything from the common cold to otitis media, croup, whooping cough and epiglottitis. The common cold requires supportive treatment, making sure the child is kept well hydrated, and the use of anti-pyretics such as paracetamol for fever.

Otitis Media

The treatment of otitis media is debated. Untreated, bacterial otitis media can cause serious complications such as mastoiditis and chronic supperative otitis media leading to hearing loss. Because of the risk of these complications, and the fact that *S. pneumoniae* is responsible for around one third of cases of otitis media, antibiotics such as amoxicillin are often overprescribed, and this itself is causing problems by increasing bacterial resistance. Antibiotics may also cause adverse effects including diarrhea, vomiting, rashes and fungal infections[91]. Recent studies have investigated a 'watch and wait' strategy, in which children with less severe otitis media were given supportive treatment and followed up to assess for any worsening of symptoms, and then subsequently given antibiotics if necessary. The study found that 61% of children improved without antibiotics, and those that did need them were no more likely to have complications requiring surgery[91]. The study therefore

recommended that in children without any severe signs or symptoms, a 'watch and wait' strategy should be used, which will eliminate any adverse effects from antibiotic use, and it may help reduce antibiotic resistance[91].

Croup

The management of croup is supportive, ensuring the parents are aware of signs of deterioration such as increasing respiratory rate, fatigue, chest-wall indrawing and a softening stridor. Severe disease may require intubation under general anaesthesia to avoid airway obstruction[74].

Whooping Cough

The macrolide antibiotic erythromycin may be given to children in the early stages of *Bordetella pertussis* infection, although it is of little use once the paroxysmal cough has begun. It does, however, have a prophylactic use in contacts of someone with *Bordetella pertussis* infection. Treatment is otherwise supportive, with admission necessary in young children who are at risk from apnoea, and may require ventilation[74].

Epiglottitis

Epiglottitis can be life-threatening and rapid management is vital. The throat should not be examined until an experienced anaesthetist is present. The throat can then be examined, and if a swollen, inflamed epiglottis is visible, the child should be intubated immediately under general anaesthetic. *Haemophilus influenzae* type b (Hib) is the most common cause of epiglottitis and a third generation cephalosporin such as cefotaxime should be commenced immediately[74]. The incidence of epiglottitis had dramatically decreased since the introduction of the Hib vaccine into immunisation schedules[77].

1.1.8.2 Bronchiolitis

The mainstay of treatment for bronchiolitis is supportive. Oxygen should be given if saturations are below 93%, if the child has severe respiratory distress or if the child is cyanosed. Adequate hydration should be maintained, and if this is not possible through oral feeding nasogastric feeding or the use of intravenous fluids should be considered[92].

Bronchodilators are widely used in the treatment of ARI[76]. However, a Cochrane review investigating evidence for the use of bronchodilators in bronchiolitis found that although bronchodilator therapy led to a significant improvement in clinical scores, it recognised that the studies had included children with recurrent wheeze or asthma, which may have skewed the results[93]. This study also found no difference in rates of hospitalisation or oxygen saturations in those who had been given bronchodilators compared to controls[93]. A more recent systematic review found similar results, with studies finding improvements in short-term clinical measurements in children receiving bronchodilators, but no difference in the need for hospital admission or duration of hospital stay[94]. As with all pharmacological treatments, bronchodilator therapy is not without adverse effects, and these include increased heart rate and temporarily decreased oxygen saturations[95]. There is therefore little evidence supporting the use of bronchodilators for bronchiolitis, with only short term improvements in clinical measures being demonstrated.

Many studies have investigated the use of corticosteroids in bronchiolitis. Two large studies reviewed the evidence available, and both concluded that although corticosteroids may improve short term clinical scores, they did not improve the need for hospital admission or length of stay, and are not indicated in bronchiolitis[94 96].

1.1.8.3 Pneumonia

Pneumonia may be viral or bacterial, and while viral pneumonia requires only supportive treatment, the correct treatment of bacterial infection is important. The British Thoracic Society guidelines suggest bacterial pneumonia should be considered in children less than three years if there is fever over 38.5^{o}C, along with a respiratory rate greater than 50 breaths per minute and chest recession, and that bacterial pneumonia is unlikely if wheeze is present[78]. Antibiotics are given according to local or national guidelines, which should take into account patterns of particular pathogens and resistance in that area. Current British Thoracic Society guidelines suggest that the most appropriate treatment for suspected bacterial infection in children under five is with amoxicillin, and in those with severe pneumonia co-amoxiclav, cefuroxime and cefotaxime are appropriate[78]. Macrolide antibiotics are only advised as first line treatment in children over the age of five, in whom infection with *Mpp* is more common[78]. Overuse of antibiotics, especially in viral infection when they are completely unnecessary, can lead to increased antibiotic resistance of bacteria, which is an important clinical problem. There is significant penicillin resistance to *S. pneumoniae* worldwide with the main risk factor being previous antibiotic use, and a further risk factor for penicillin resistant invasive pneumococcal disease being recent respiratory tract infection[34].

1.1.8.4 Episodic Viral Wheeze/asthma

As well as supportive therapy such as fluids and oxygen when necessary, bronchodilators are the mainstay of treatment for acute episodes of wheeze. Inhaled, short-acting β2-agonists are the first line treatment, giving rapid bronchodilatory effects and relief of symptoms. Adverse effects such as headaches and palpitations are rare with inhaled therapy unless particularly high doses are given[79].

Corticosteroids are often used during acute episodes of wheeze in children due to their proven role in acute exacerbations of asthma in adults and older children[97]. However, a recent review article looking into the results of several trials has found conflicting evidence on the use of corticosteroids in this age group, and recommended that a trial of oral corticosteroids should be given only children admitted to hospital with severe wheeze[79].

1.1.9 Prevention

The burden of ARI is great on many levels, from its effects on individuals, to the large hospital costs from the high number of admissions that it causes each year. Due to the lack of effective treatment for ARI, with management being largely supportive, prevention may be key to decreasing the burden. Prevention of ARI can be viewed from two perspectives. Firstly, prevention can be attempted by addressing some of the risk factors mentioned in section 1.1.4. The biggest problem faced here is that many of the issues are already large health problems, which many countries are already attempting to address. Smoking cessation, for example, is the focus of many government schemes in both developed and developing countries. Although this may have a positive impact on the prevalence of ARI, the schemes are already large and costly and to further them specifically for the purpose of preventing ARI would not be feasible. Poverty is the other major risk factor that contributes to ARI. Again, this is a global problem for which there is not an easy solution.

1.1.9.1 Prophylaxis

Prophylaxis is the other major route to prevention of ARI. However, there are some considerable problems with the development of suitable vaccines for ARI. The first and biggest problem lies in the varying aetiology of ARI – many pathogens, both viral and bacterial, are responsible for ARI. It would not be feasible to attempt to develop

vaccinations, or vaccinate against a large number of pathogens that in most cases cause mild, self-limiting illnesses.

hRSV prophylaxis

It is not surprising that most efforts to develop a vaccine have been focused on hRSV infection, as it has been shown to cause 50-90% of hospitalisations for bronchiolitis and 20-50% of hospitalisations for winter pneumonia. The development of hRSV vaccination has been slowed by safety issues following the administration of a formalin-inactivated hRSV vaccine to 13 residents of a children's home in 1966[98]. An outbreak of hRSV infection at the centre approximately nine months following the vaccinations, demonstrated that the children who had received the vaccinations were not only not protected against hRSV, but developed more severe disease, with significantly higher rates of hospitalisation, higher rates of pneumonia, and longer duration of illness[98]. A breakthrough in immunisations against hRSV occurred with the development of Palivizumab, a humanised monoclonal antibody, which has been shown to significantly reduce hospitalisations in children at high risk of hRSV infection[99 100]. Palivizumab is given monthly as an intramuscular injection to high risk infants during the hRSV season. Although Palivizumab has been shown to be clinically effective at reducing hospitalisations in high-risk infants, the cost of vaccinating these infants is very high, and studies have found the use of palivizumab is not cost effective in all high risk children[101].

Pneumococcal conjugate vaccine

The heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in the year 2000, vaccinating against the seven most common serotypes of *S. pneumoniae*. In the USA all children under the age of two receive the vaccine, as well as children under five who are at an increased risk of invasive pneumococcal disease. The vaccine has been shown to reduce invasive pneumococcal disease, as well as nasopharyngeal carriage of these serotypes of

the bacteria in both children and adults[102-104]. Large studies have found reduced rates of meningitis and invasive pneumonia that have been sustained in the years since the vaccine was introduced[105]. Although the vaccine has been shown to be effective at preventing cases of invasive pneumococcal disease caused by the seven common serotypes, since the introduction of the vaccine, the presence of invasive disease caused by other non PCV7 serotypes has increased dramatically, with a higher percentage of these being resistant to penicillin as well[34]. Similarly, a large study in Massachusetts found that after introduction of the vaccine there was very little carriage of any of the PCV7 serotypes, but greatly increased carriage of the non-PCV7, often penicillin resistant serotypes[106]. Invasive pneumococcal disease therefore remains an important problem causing significant mortality worldwide. More recently, a 13-valent pnumococcal conjugate vaccine (PCV13) has been introduced into the vaccination schedules, replacing PCV7. It was introduced in the US in February 2010, shortly followed by the UK in April 2010, protecting against 13 strains of the bacteria, which are responsible for three-quarters of invasive disease in young children, although as with PCV7, the prevalence of non-PCV13 serotypes is likely to increase[107].

1.1.10 Sequelae of ARI

Although the immediate morbidity and mortality associated with ARI comes from the acute illness, there is a wealth of literature investigating associations between viral infection in infancy or childhood with respiratory problems later in life, such as recurrent wheeze and asthma, or with an increased likelihood of developing invasive pneumococcal disease.

1.1.10.1 Recurrent Wheeze

Several studies have investigated the development of wheezing and asthma in children who had a viral respiratory illness, often focusing on particular viruses or particularly severe disease. One large study by Jackson et al in 2008, found that rhinovirus infection causing wheezing in the first three years of life, but especially in the third year, was strongly associated with an increased risk of asthma by the age of six (odds ratio = 9.8)[108]. They also found increased asthma at age six in children who had had wheezy illness caused by hRSV infection in the first three years of life, although this association was not as strong (odds ratio = 2.6)[108]. A limitation to this study is that all study participants were at high risk of asthma development – in order to be eligible for inclusion into the study the child must have one parent with proven allergen sensitivity or diagnosed asthma[108]. Sigurs et al in 2009 reported asthma/recurrent wheezing, allergic rhinoconjunctivitis and sensitisation to common inhaled allergens were all significantly more frequent in a group of children who were hospitalised with hRSV bronchiolitis in infancy, when compared to matched controls[109]. Stein et al in 1999 found an association between hRSV LRTI before the age of three, and wheeze up until the age of 11. However, after age 11 the risk decreased and unlike the previously mentioned studies, was not significant by the age of 13[110].

Although all these studies show significant associations between ARI in infancy or childhood and the development of further respiratory morbidity, no causal relationship has been established. As mentioned in the study by Jackson et al. their results indicate that rhinovirus infection in infancy may be used as a *predictor* of asthma later in childhood[108]. It is not known whether these infections predispose to later illness and the development of recurrent wheeze or asthma, or whether these children may already be predisposed to

developing recurrent wheeze or asthma and, for the same reason, are at higher risk of having a viral respiratory illness.

1.1.10.2 Invasive pneumococcal disease

A study by Ampofo et al found that viral infection with hRSV, influenza or hMPV was associated with increased risk of admission with invasive pneumococcal disease in the weeks following the viral infection. The increased risk existed for four weeks after hRSV infection, and two weeks after influenza or hMPV infection[111]. In a large study of adults and children with invasive pneumococcal disease in New Zealand, Murdoch et al. found statistically significant correlations between incidence rates of invasive pneumococcal disease and detection rates of flu viruses, AdV and PIV3, and in the under 5's RSV[112].

Madhi et al found that a pneumococcal conjugate vaccine introduced in South Africa prevented nearly one third of ARI caused by influenza A, RSV, PIV 1-3 and AdV. This highlighted the importance of viral-bacterial co-infection, and its role in causing disease, as well as the role of pneumococcal vaccination in the prevention of viral ARI as well as those caused directly by Streptococcus pneumoniae [113].

1.1.10.3 Bronchiolitis obliterans

Bronchiolitis obliterans describes a pathological process in which inflammation and fibrous tissue deposition lead to the obstruction and obliteration of the terminal and respiratory bronchioles[85]. It is associated with a number of conditions, including toxic fume inhalation, graft-vs-host disease after transplants, drugs such as penicillamine, and post-infection following acute respiratory infection[114]. Specifically, particular types of AdV – 1, 3, 7 and 21, as well as influenza virus, *Mpp* and *Legionella* infection are associated with a risk of developing bronchiolitis obliterans[114]. The prognosis of bronchiolitis obliterans is variable,

with some patients having a rapidly declining lung function with a fatal outcome, while some patients develop chronic lung disease and others have actually improved over time, with gradual resorption of the fibrovascular tissue and improvement to almost normal airways and lung function[114].

1.1.10.4 Bronchiectasis

Bronchiectasis describes chronic dilation of the airways and thickening of the bronchial walls[115]. This in turn leads to destructive changes and abnormalities in the epithelium, with loss of cilia giving a decreased ability to clear secretions and mucus gland hyperplasia leading to increased sputum production[115 116]. Ultimately this causes an increased likelihood of chronic infection, chronic inflammation and airway obstruction[115]. There are a many causes of bronchiectasis, including post-infectious, congenital, immunodeficiencies, ciliary abnormalities, as well as foreign body aspiration, toxic gas inhalation and asthma[116]. Post-infectious causes relevant to this study of ARI include AdV, Mpp, *Bordetella pertussis*, TB and necrotizing bacterial pneumonia[116]. Clinical features of bronchiectasis include chronic cough, large amounts of sputum production and recurrent infections[116]. Management of bronchiectasis involves treating the underlying cause, using postural drainage to remove mucus, vaccinating against infectious agents where possible and prompt antibiotic treatment of new infections. Surgical interventions such as pneumonectomy are used for localised disease which is causing severe symptoms[116].

1.2 Immunological responses to ARI

The lung makes up the largest epithelial surface in the body, with each breath exposing the airways to new micro-organisms which may give rise to acute respiratory infection[117]. As well as the need for a rapid acting, efficient immune system to deal with these micro-organisms, the complex physiology of the respiratory system, with its ability to facilitate gas exchange, is a delicate, balanced environment, in which an inflammatory response, aimed at destroying a potentially harmful pathogen, may itself create problems and affect the normal functioning of the lung[118].

The immune response can be divided into the innate and the adaptive responses. The innate response comprises rapid, first line, non-specific mechanisms in the defence against infection by pathogens. The adaptive response is in contrast, a much more complex system, with specialised cells mounting specific responses against the particular pathogen involved in each 'attack', and producing an immunological 'memory' to enable the rapid elimination of that particular pathogen should it come into contact with the host immune system on a subsequent occasion. However, the adaptive immune system's specificity, although essential, can take three to five days to produce an adequate response to effectively deal with a pathogen, a time when the innate immune system plays a vital role in controlling the replication of the pathogen, and limiting its damaging effects[119].

1.2.1 Innate Response

As already mentioned, the innate immune response is the earliest line of defence against infection, and plays an important role in the initial response to a pathogen. However, the innate immune system also has another major role in the activation of the adaptive immune response, which will be discussed further at a later stage.

Innate immunity can be divided into three groups, the physical barriers to infection, the non-cellular response and the cellular response.

1.2.1.1 Physical Barriers to Infection

Physical barriers to infection are the very first line of defence any invading organisms will encounter, and include the cough reflex, the barrier function of respiratory epithelium and the muco-ciliary escalator. The respiratory epithelium is highly specialised, with numerous roles in the protection of the respiratory tract from invasion by pathogens, but firstly, it provides a physical barrier to foreign particles and pathogens. The epithelium also plays a pivotal role in the muco-ciliary escalator, with pseudostratified columnar epithelial cells, which form a large part of the epithelial surface of the conducting airways, being ciliated cells. The co-ordinated beating of the cilia on these cells provides a mechanism for clearing mucus and with it any trapped material, out of the airways, to the epiglottis, where it can be safely swallowed, clearing any potential pathogens away from the delicate respiratory surfaces[120].

Mucus has several roles including the lubrication of mucosal surfaces, and the immunological functions of trapping inhaled particles or organisms in order to clear them from the respiratory system. Mucus is made up of water, ions, proteins, lipids and 'mucins'[121]. Mucins are large gel-forming glycoproteins that give mucus its gel-like properties, and its ability to trap bacteria. Mucus, however, can also play a major role in pathological processes, with cystic fibrosis being a prime example, when the mucus is dehydrated, causing thick purulent sputum, providing an environment that traps pathogens, which, because of the increased viscosity, are not cleared by the muco-ciliary escalator, giving a high risk of infection. Pathogens such as bacteria and viruses themselves, along with the release of inflammatory mediators in response to infection have been shown

to influence and increase mucus production, leading to some of the clinical manifestations associated with respiratory illness.

For pathogens that get past the initial physical barriers of the respiratory tract, the next line of defence comprises soluble mediators of the immune system that can be found in the fluid lining the respiratory tract – the airway surface fluid (ASF)[117].

1.2.1.2 Pathogen recognition/detection

The ability to recognise a pathogen in order to initiate an immune response is the first, vital role of the immune system. The innate immune system uses a range of genetically pre-programmed receptors, which, instead of being able to recognise specific pathogens, like the adaptive immune system, are able to recognise structures or patterns common to many pathogens, which, importantly, are absent in self molecules. These structures are called 'pattern-associated molecular patterns' or PAMP's, and the receptors are called 'pattern recognition receptors' (PRR's)[119]. Examples of PAMP's are lipopolysaccharide (LPS), an endotoxin and component of the cell membrane of gram negative bacteria; forms of bacterial DNA – unmethylated cytosine-guanosine-rich areas – CpG sequences; and double stranded RNA[117 119].

Pattern Recognition Receptors

Pattern recognition receptors (PRR's) are proteins that are expressed on cells of the innate immune system, such as macrophages, dendritic cells and B-cells. PRR's can be divided into secreted, endocytic or signalling PRR's. Secreted PRR's act as opsonins, which find and bind to pathogens in order to alert other cells of the immune system to their presence, so an appropriate response may be mounted. Endocytic PRR's are found on the surface of phagocytes, mediating the delivery of pathogens to lysosomes, where they are destroyed.

Signalling receptors recognise PAMP's and activate pathways that lead to the transcription of many genes vital to an effective immune response[119].

Toll like receptors

Toll-like receptors (TLR's) are a family of signalling PRR's that upon recognising a particular PAMP, activate pathways that result in the transcription of inflammatory mediators. These mediators, such as inflammatory cytokines, not only play a major part in the initiation of the innate inflammatory response, but are vital in the initiation of the adaptive response[119]. At present, there are 10 known toll-like receptors in humans, and 12 in mice[122]. The TLR's can be divided according to whether they are found on cell surfaces (TLR1, TLR2, TLR4, TLR6 and TLR11), or in intracellular vesicles (TLR3, TLR7, TLR8 and TLR9), such as in lysosomes, where they recognise microbial nucleic acids[122]. Different TLR's have are activated by different PAMP ligands, and then activate specific pathways resulting in the production of specific inflammatory mediators. The general pathway used by the TLR's involves activation of nuclear factor κB (NF-κB) transcription factors using various pathways according to the specific TLR and ligand. The NF-κB is released and enters the nucleus, initiating the activation of a range of inflammatory genes[119]. The toll-like receptors also play a vital role in the activation of the adaptive immune system, with T-cells (whose functions will be discussed in section 1.2.2.2), needing both an antigen against which to mount a response, as well as the expression of CD80 or CD86 molecules on an antigen presenting cell, in order for the T-cell to be activated. The TLR's induce the expression of the CD80 and CD86 molecules[119]. This is important not only for activation of the adaptive immune response, but also to ensure that the adaptive immune response occurs only when necessary. The activation of TLR's, and the subsequent expression of CD80 or CD86 only when infection is present, is a vital mechanism in the prevention of an immune response against self molecules[119].

Several TLRs are involved in the recognition of pathogens which cause ARI. TLR-2 is known to recognise bacteria, including *Mpp*, as well as viruses, and is able to initiate different responses depending on the particular ligand it comes into contact with by forming heterodimers with other TLRs[123]. TLR-4 has also been shown to be important in the recognition of respiratory pathogens. As well as recognising bacteria, it has been found to be important in the recognition of hRSV, as well as being implicated in the pathogenesis of H5N1 avian influenza. It is thought that TLR4 recognises one of the molecules released from cells damaged by H5N1 (a damage-associated molecular pattern, DAMP), and mice deficient in TLR-4 were found to be resistant to the H5N1 induced lethality[123]. This illustrates one example of the immune response being damaging rather than protective.

Collectins

Collectins are secreted pattern recognition receptors which bind to carbohydrates on pathogens including gram negative and gram positive bacteria, and some viruses, parasites and yeasts. Examples of collectins include mannan-binding lectin (MBL) and surfactant proteins A and D. The main functions of these are to alert and recruit other cells of the immune system, such as macrophages and lymphocytes, and to activate the 'lectin pathway' in which the activation of a series of proteases leads to the activation of the complement pathway – one of the effectors of the innate immune system[119]. The surfactant proteins are also particularly important in the regulation of inflammation in the lungs, inhibiting the activation of alveolar macrophages until they themselves come into contact with a pathogen[118].

Lysosome

Endocytic pattern recognition receptors are proteins attached to the surface of phagocytes which mediate the uptake and delivery of pathogens to lysosomes, which endocytose (engulf) the pathogens, and destroy them using acid hydrolases. Proteins from the

pathogen are then processed and displayed on the surface of the phagocyte by major histocompatability complex (MHC) molecules, in order to alert the adaptive immune system, which will be described later[119].

1.2.1.3 Soluble mediators of the innate immune system

Defensins

The defensins are small peptides found in the respiratory tract, as well as the gastro-intestinal and reproductive tracts. The α-defensins are produced by neutrophils, and the β-defensins are produced by epithelial cells[124]. They have broad antimicrobial activity against bacteria, fungi and some viruses[117]. The antimicrobial action of defensins relies upon attachment to the cell membrane, where it is thought they then form pores in the membrane[124]. They are also able to activate the alternative complement cascade, which will be discussed in more detail in section 1.2.1.4. The defensins have been shown to be up-regulated in response to epithelial cell exposure to pathogens, TLR agonists and inflammatory cytokines[125]. Murine studies have found that mice deficient in β-defensin had delayed clearance of *Haemophilus influenzae* from the lung after infection[126], and also that mouse β-defensin inhibits influenza A replication[127], both studies suggesting defensins may have an important role in the defence against ARI.

Lysozyme

Lysozyme is found in airway lavage fluid and sputum. They are enzymes which are made by glandular serous cells, surface epithelial cells and macrophages, and are highly effective at defending the airway against Gram-positive bacteria[117]. They are effective by catalysing hydrolysis of peptoglycan links in the bacterial cell wall. They are also implicated in neutrophil recruitment[117].

Lactoferrin

Lactoferrin is another broad-spectrum, anti-microbial protein found in high concentrations in lavage fluid[120], but is probably better known for its high concentration in colostrum and breast milk, where it provides important anti-microbial activity to infants. In the lungs it is produced by serous cells and neutrophils. Lactoferrin's antimicrobial activity is based on its ability to cause agglutination of bacteria, upon recognising them by their carbohydrate motifs[117], as well as its ability to remove iron from pathogens[125] .

Increased amounts of both lysozyme and lactoferrin have been detected in the lungs of mice after treatment with *Haemophilus influenzae*, as well as other infectious agents, implicating them in the immune response to ARI, although further details on specific modes of action against specific pathogens are not known[125].

Immunoglobulin A

Immunoglobulins are secreted by B-lymphocytes, and while these are usually associated with the adaptive immune response, immunoglobulin A (IgA) release has been shown to be stimulated by the release of stimulatory cytokines from epithelial cells. IgA is found in the secretions of the respiratory tract, as well as the gastro-intestinal tract, genito-urinary tract, breast milk and colostrum, and is important in the rapid response, especially to viruses[117].

Type I Interferon Response

The type I interferon (IFN) response is particularly important in the defence against viruses, with the name describing the ability to interfere with viral infection[128]. IFN-α and IFN-β are the most important IFN's in the response, and are released by numerous cells of the innate immune system, including epithelial cells, particularly in response to NF-κB, and by

macrophages, in response to viral infection[117]. Mice deficient in receptors for type I interferons have shown increased viral replication in the lung, although the infection was still cleared by other mechanisms, illustrating the complexity of the immune response with various different levels of defence[117].

1.2.1.4 Effectors of innate Immunity

The complement system

The complement system consists of a number of plasma proteins that may be activated by one of three pathways: the classical pathway, the alternative pathway, and the lectin pathway. Once activated these pathways lead to a number of steps which eventually all result in destruction of the pathogen with the formation of membrane attack complexes (MAC); or opsonisation of the pathogen for destruction by other parts of the immune system.

The classical pathway, named due to its earlier discovery than the other two pathways, is activated by certain antibody-antigen complexes, and is a major component of the adaptive immune system.

The adaptive and lectin pathways are both components of the innate immune system. The alternative pathway is activated by microbes themselves, without the need for antibodies. The activation takes place on the surface of the microbes, in the absence of regulatory proteins that prevent the activation on human cells. The complement protein C3b binds to the microbial cell surface, and plasma protein factor B is cleaved and forms the complex C3bBb – C3 convertase. C3 convertase leads to further cleavage of C3, amplifying the response and leading to more C3b, and the release of C3a. The C3b binds to the C3 convertase, forming the alternative pathway C5 convertase[129].

The lectin pathway was briefly mentioned earlier in the chapter. Lectins such as the mannan binding lectin (MBL), bind to polysaccharides on bacteria, as well as MBL-associated serine proteases (MASPs), including MASP-1, MASP-2 and MASP-3. The subsequent complexes activate the lectin pathway by initiating the cleavage of C4 to C4b and C2 to C2b, forming the complex C4bC2b – C3 convertase, which, as in the alternative pathway, cleaves C3 to C3b. The C3b this time forms a C4b2b3b complex – another (classical) form of C5 convertase[129].

Both these pathways, as well as the classical pathway, lead to the formation of C5 convertase. C5 convertase initiates the activation of further components of the complement cascade, including C6, C7, C8 and C9, which ultimately lead to the formation of the membrane attack complex (MAC). The MAC has the ability to form pores in the cell membrane, causing cell lysis[129].

Other functions of complement activation include opsonisation and stimulation of the inflammatory response. Opsonisation occurs when the pathogen becomes coated in C3b or C4b, both of which bind to receptors on neutrophils and macrophages, and initiate phagocytosis of the pathogen[128].

The C3a, C4a and C5a fragments released during the cleavage of C3, C4 and C5, respectively, have their own function in the stimulation of the immune response. C5 is the most potent, causing mast cell degranulation and stimulating neutrophil motility and adhesion to endothelial cells. C3b and C4b have similar effects but are less potent[128 130].

Phagocytes

Neutrophils and macrophages are important effectors in the innate immune response to infection. They are phagocytes, their role being to recognise, internalise and subsequently destroy pathogens or particulate matter that is potentially harmful[130]. They are also

important antigen presenting cells (APC's), having a vital role in the activation of the adaptive immune system.

Neutrophils

Neutrophils are the most numerous circulating leukocytes, and are constantly produced in the bone marrow. Production and release of neutrophils is stimulated by granulocyte colony-stimulating factor (G-CSF). Neutrophils circulate in the blood, and in the absence of infection they will undergo apoptosis and phagocytosis after a period of around six hours[130]. However, when infection is present, neutrophils migrate to the site of infection, in response to the release of mediators such as IL-8, particular complement proteins and chemokines. This is very true in the lung during hRSV infection, with neutrophils accounting for 93% of cells taken from the upper respiratory tract, and 76% in the lower respiratory tract[131]. The process of neutrophil recruitment can be divided into a number of steps and begins with adhesion of the neutrophils to the endothelial surface, followed by migration through the endothelial cells towards the site of infection. Once reaching the site of infection, the neutrophils are kept in place, fulfilling their function as important phagocytes[130].

At the site of infection, neutrophils engulf potentially harmful organisms and destroy them via a number of mechanisms, including the use of reactive oxygen species, antimicrobial proteins, and degradative enzymes[118]. Neutrophils also have important roles in the production of pro-inflammatory mediators. They produce pro-inflammatory chemokines and cytokines, such as IL-1 and TNF-α, which encourage further neutrophil recruitment; and other mediators such as chemerin which activates dendritic cells; B-lymphocyte stimulator, stimulating B-cells and IL-12 - an important activator of T-cells. So neutrophils do not only further stimulate innate response, but play an important role in the activation and manipulation of the adaptive immune response[130].

During infection there is rapid proliferation and migration of neutrophils to the affected tissue, in response to the release of a variety of mediators, one of the most important being IL-8. IL-8 is released by macrophages and epithelial cells in response to infection, with initial responses being incredibly rapid. Studies of respiratory epithelial cells have shown IL-8 responses initially peaking within two hours of infection with hRSV[132]. IL-8 release by macrophages and epithelial cells is stimulated by TNF-α and IL-1[133], both of which are produced by macrophages in the early immune response to pathogens. IL-8 causes a rapid accumulation and activation of neutrophils, with neutrophils being abundant and IL-8 concentrations being increased in respiratory samples from patients with ARI. Recent studies, however, have implicated IL-8 in the pathogenesis of ARI, particularly in hRSV disease. An association between severe hRSV bronchiolitis and a polymorphism near the IL-8 gene was detected, with those with the polymorphism having increased IL-8 levels, and increased severity of disease[134]. This will be discussed further in section 5.4.1.

Macrophages

Macrophages are also important phagocytes, but they have some major differences in their functions. Whereas neutrophil production is dramatically increased during infection, when they rapidly migrate to infected tissues, monocytes (as immature macrophages are known), circulate in the blood, and migrate into tissues, becoming macrophages, on a regular basis. Although macrophages are phagocytic, they have important roles in the production of cytokines and chemokines that modify neutrophil recruitment, adhesion and chemotaxis[130]. Studies into hRSV disease have shown macrophages to secrete a variety of cytokines, including IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α[132]. Surfactant proteins A and D are thought to be important in the regulation of alveolar macrophages, ensuring they are only activated in response to infection, avoiding unnecessary activation of the immune system and subsequent inflammation. The surfactant proteins bind to the alveolar macrophages when

no infection is present, and inhibit their activation. When a pathogen is detected, the surfactant proteins bind to the pathogen, no longer inhibiting the macrophage, which will then be activated and begin to produce its inflammatory mediators which will alert other aspects of the immune system[118]. Macrophages are also important antigen presenting cells (APC's), which will be discussed further in section 1.2.1.5.

Natural Killer Cells

Natural killer (NK) cells are important in the innate response to viral infection. They are derived from the same lineage as T-cells in the bone marrow, but do not need to mature in the thymus. NK cells recognise cells that have abnormal expression of human leukocyte antigen (HLA) class I tissue antigens[117]. These antigens are present on the surface of cells, and function to present peptides from pathogens, in order to alert the immune system. If a virus is present inside the cell, the expression of HLA molecules will be altered, and this will be recognised by the NK cell. HLA recognition is very important in the adaptive response to infection. However, some viruses, instead of altering the HLA molecules, are able to down-regulate them in order to avoid recognition by the adaptive immune system. In this respect, NK cells are very important, as they are able to recognise these cells with low HLA expression which are missed by other parts of the immune system[130].

NK cells are able to destroy infected cells in a number of ways. Firstly, they contain perforin, and upon contact with an infected cell the perforin is transferred to the NK cell's surface. Perforin forms pores in the infected cell membrane, leading to apoptosis. The second mechanism NK cells uses is granzyme, three proteolytic enzymes which enter the infected cell and activate caspases (cysteine-dependent aspartate-directed proteases), enzymes within the cell which initiate degradation of DNA and therefore induce apoptosis. The third mechanism used by NK cells is Fas ligand. Fas ligand is expressed on NK cells when they

come into contact with infected cells, and binds to Fas on the infected cell, and again induces apoptosis through activation of caspases[130].

As well as causing apoptosis, NK cells, like many of the other effectors of the innate immune system, secrete cytokines, specifically large amounts of interferon-gamma (IFN-γ), which is important in the activation and recruitment of cells of the adaptive immune system[130].

1.2.1.5 Antigen-Presenting Cells

Macrophages have already been discussed in terms of their role as phagocytes, and it was mentioned that a further role of theirs is as antigen presenting cells (APCs). APCs provide a vital link between the innate immune response and activation of the adaptive immune response. Antigens are presented with major histocompatibility complexes, which enable recognition by T-cells, and the subsequent development of a specific response to the antigen, as well as the development of immunological memory.

Dendritic Cells

Dendritic cells are specialised antigen presenting cells (APCs). They are produced in the bone marrow, and are named according to their shape, with long 'dendritic' processes extending from the cell. Dendritic cells are mobile, and travel to peripheral sites where they are more likely to come into contact with antigens. Upon encountering antigens, dendritic cells bind the antigen, and then travel back toward lymphoid tissue or organs. The dendritic cells present the antigen to T-cells which are specific for that MHC-antigen complex. The T-cell responses will be discussed in further detail in section 1.2.2.2. In the lung, the processes on intraepithelial dendritic cells extend into the airway lumen, and are able to pick up antigens that have been trapped by the mucociliary escalator[130].

1.2.2 Adaptive Response

The adaptive immune system has two main functions. Firstly, if the innate immune system isn't able to destroy pathogens immediately, an adaptive immune response develops, specific for that particular pathogen, enabling complete clearance of the invading organism. Secondly, the adaptive immune system is responsible for immunological memory, whereby the immune system is able to 'remember' a particular pathogen from a prior infection. When the same pathogen is subsequently encountered on a later occasion, immunological memory allows a very rapid, specific, response to be mounted against the pathogen[130].

The adaptive immune system can be divided into two parts – the humoral response, and the cell-mediated response.

1.2.2.1 Humoral Immunity

Humoral immunity describes the antibody response. Antibodies are proteins produced by B-lymphocytes (B-cells), which use various mechanisms to destroy pathogens. Antibodies are particularly useful in the defence against extracellular pathogens, as the antibodies are able to bind to the free pathogens, and support the destruction by other mechanisms[128].

B-cells are produced in the bone marrow, and like T-cells, the genes for the receptors are randomly formed by recombination of DNA segments during the maturation of the cells[128]. This means that a huge number of receptors are randomly generated, being able to recognise a diverse range of pathogens which they may come into contact with[119]. B-cells migrate to peripheral lymphoid tissues where they will meet antigens. A B-cell becomes activated when it recognises the specific antigen for which it has receptors. The antigen binds to IgM and IgD receptors on the B-cell, and depending on the antigen, may or may not require stimulation from a helper T-cell in order to be activated. Once activated the B-

cell undergoes clonal expansion, with production of the same antigen specific B-cells, with differentiation to antibody secreting plasma cells, and memory B-cells[128]. The proliferation and differentiation of B-cells is stimulated by a range of cytokines, produced by other parts of the immune system. IL-2, IL-4 and IL-21 produced by helper T-cells, as well as IL-6 produced by helper T-cells, macrophages and many other cells, are all important in B-cell proliferation, and also have roles in determining the differentiation. For example, IL-6 has been found to have a role in promoting the differentiation of B-cells into antibody secreting plasma cells[135], while IL-4 has been found to promote the development of memory B-cells[136]. Different immunoglobulins have different effects, and are released at different times in the response. IgM is released early in the immune response and is particularly good in the defence against bacteria. It has 10 potential binding sites, and is able to attach to and cause agglutination of bacteria, as well as activating the complement system and opsonising the bacteria for phagocytosis[130]. Viruses and bacteria promote the release of IFN-γ from T-helper cells, which then promotes the differentiation of B-cells into IgG secreting plasma cells. IgG also opsonises pathogens for phagocytosis by macrophages[128]. IgD has already been mentioned as a receptor on B-cells, where it has a role in the identification of antigens and the activation of the B-cells. IgA was mentioned in the previous chapter and is found on mucosal surfaces in the respiratory, gastrointestinal and reproductive tracts, as well as in saliva, breast milk and tears. It is thought to be one of the earlier defences against pathogens, preventing their movement across mucosal surfaces[130].

An example of antibody responses can be seen with hRSV infection. During initial hRSV infection IgM is the first detectable antibody, remaining detectable for 1-2 weeks. IgG then increases and declines after a couple of months. The role of antibodies in hRSV has, however, been debated. hRSV most commonly occurs in infants, when maternal hRSV antibodies are highest, leading to thoughts that the antibodies may have a role in the

pathogenesis of hRSV disease. Conversely, it has also been shown that infants with low placental hRSV antibodies are more likely to suffer severe hRSV disease, indicating a protective role[132].

1.2.2.2 Cell-mediated Immunity

Cell-mediated immunity is based on the response by T-lymphocytes (T-cells). Cell-mediated immunity, in contrast to humoral immunity which is only effective against extracellular pathogens, is effective against intracellular pathogens, which may escape other parts of the immune system, as well as pathogens within other immune cells, such as those that have been phagocytosed by macrophages as part of the innate immune response[128].

T-cell responses are dependent upon presentation of antigen by major histocompatibility complexes (MHCs). MHCs are groups of molecules that are able to recognise and present antigens. In humans the MHC is known as the human leukocyte antigen (HLA). The HLA genes are found on chromosome 6 and encode the HLA molecules. T-cells are only able to recognise foreign antigens when they are presented in a complex with a HLA molecule[130]. Two main classes of HLA molecules are important in the activation of T-cells – class I HLA molecules are found on almost all cell types within the body, where as class II HLA molecules are found on B-cells, macrophages and dendritic cells. Extracellular antigens are phagocytosed by macrophages, the proteins from the antigens are degraded and the resulting peptides are picked up by HLA class II molecules. The HLA-antigen complex is then displayed on the outside of the cell and is recognised by T-cell receptors on CD4+ T-cells[130]. Intracellular antigens such as proteins from viruses activate T-cells via a different route. Within the virus-infected cell, proteasome enzymes are able to degrade the viral proteins, providing peptides that are able to form complexes with HLA class I molecules. Again these complexes are then displayed on the cell surface, and recognised by T-cell receptors, this

time on CD8+ T-cells[130]. The 'CD' part of the T-cell is the 'cluster of differentiation' which describes the molecules on the surface of the T-cell. The CD molecules are essential for the stabilisation of the T-cell receptor and the HLA-antigen complex, as well as for signal transduction which is vital for activation of the T-cell, with the CD4 molecules being specific for the class II HLA and the CD8 being specific for the class I HLA[130].

T-helper cells

The CD4+ cells are also known as T-helper cells, and once activated may differentiate into either Th1 or Th2 cells, depending on the cytokines present at the time.

Th1 response

A Th1 response is stimulated by IL-12, which is released from macrophages after IFN-γ stimulation. Th1 cells then release Th1 mediators, including IL-2, IFN-γ and TNF-α. The Th1 mediators stimulate B-cells, macrophages, neutrophils and CD8+ cells (cytotoxic T-lymphocytes). The macrophages dramatically increase phagocytic activity and the destruction of bacteria and viruses, while the B-cells under Th1 stimulation increase the production of IgG, again opsonising the pathogens for phagocytosis[130].

Th2 response

Without the IL-12 stimulation, and the absence of innate cytokines such as IFN-γ, T-helper cells differentiate into Th2 cells, releasing a different array of mediators, particularly IL-4, IL-5 and IL-13. These mediators stimulate B-cells to produce IgE, which then goes on to activate eosinophils and mast cells, characterising a Th2 response, which would be characteristic in atopic asthma. IL-13 has a role in mucosal activation, causing mucus hypersecretion and increased contractility in the airways[137].

Cytotoxic T-cells

The CD8+ cells are also known as cytotoxic T-cells, as they are directly able to destroy pathogens. As class I MHCs are found on almost all cell types within the body, CD8+ cells do not immediately react to a class I MHC-antigen complex. CD8+ cells are activated either by a class I MHC-antigen complex presented by a professional APC, or by a class I MHC-antigen complex on a non-professional APC, along with a secondary signal in the form of cytokines released by a an activated CD4+ T-helper cell. Once activated cytoyoxic T-cells use the same mechanisms as NK cells to destroy pathogens. Perforin is used to form pores in the cell membrane causing cell lysis, and granzyme and fas ligand are used to induce apoptosis[130].

Th17 Response

More recently, a new T-cell lineage has been described – Th-17 cells. They are a group of CD4+ T-cells that produce so-called Th-17 cytokines including IL-17A, IL-17F, IL-26 and IL-22[138]. Th-17 cytokines were initially associated with autoimmune disease, but have more recently been implicated in the host immune response to infection. They are strong mediators of inflammation, and abnormalities in the regulation of this inflammation has been implicated in autoimmune disease[138]. IL-17A is produced by Th-17 cells, as well as CD8+ T-cells, NK-cells and neutrophils[137].

Differentiation of CD4+ T-cells into Th17 cells is thought to result from the effects of TGF-β, IL-1β, IL-6, IL-21 and IL-23. The production of IL-17 is dependent on IL-6 and IL-23, released from macrophages and dendritic cells. Regulatory mechanisms are in place with neutrophils that are apoptotic following pathogen destruction inhibiting IL-23 production by dendritic cells, and therefore suppressing the differentiation of CD4+ cells into Th17 cells.

IL-17A and IL-17F exert their pro-inflammatory actions via a number of mediators. They induce G-CSF and GM-CSF expression, increasing the production of neutrophils and monocytes. They also stimulate neutrophil mobilisation, recruitment and activation, as well as stimulating fibroblasts to release IL-6 and IL-8[137]. IL-22 is also strongly pro-inflammatory, working synergistically with IL-17A and IL-17F, but also enhancing the expression of antimicrobial peptides such as the defensins[138]. IL-17E and IL-25 are somewhat different, and have been associated with a Th-2 response, with IL-17E being involved in eosinophil recruitment and the expression of Th-2 cytokines, and IL-25 stimulating eosinophils and basophils[137].

Studies have found Th17 responses to be critical in the defence against extracellular bacteria, as well as having a role in the defence against intracellular pathogens[138]. One of the first pathogens investigated was *Klebsiella pneumoniae*, a Gram negative bacterium that is often associated with hospital acquired pneumonia. IL-17 deficient mice were infected with *Klebsiella pneumoniae*, and found to have deficient neutrophil responses, increased bacteraemia and increased mortality when compared to controls. The lack of IL-17 was found to drastically reduce levels of certain chemokines as well as G-CSF, which led to the deficient neutrophil response[138]. IL-17 has also been shown to be important in the defence against *Mpp*, via an IL-23 dependant pathway. Wu et al used a murine model and reported increased IL-23 as early as four hours after *Mpp* infection, along with a subsequent increase in IL-17 in BAL. Upon blocking IL-23, IL-17 protein and mRNA expression was significantly reduced, along with neutrophil recruitment and neutrophil activity, which led to decreased clearance of the infection[139].

Th17 cytokines, along with all other cytokines and chemokines, are vital mediators of inflammation, and have critical roles in the co-ordination of the innate and adaptive immune responses to infection.

1.3 Aims of this study

The main objectives of this study were to characterise the viral and atypical bacterial causes of ARI in a clinically and demographically well-defined group of children under the age of five, and to investigate any associations between pathogens and particular demographics or clinical manifestations such as clinical diagnosis or severity of disease. We also aimed to characterise cytokine profiles in children with varying severities of disease, and with disease caused by different pathogens, to investigate the following hypotheses: a) different severities of hRSV disease are characterised by different host immune responses; and b) host immune responses in infection vary depending on infecting pathogen.

In order to address these aims we performed an observational, cross-sectional study. Nasopharyngeal aspirates, along with clinical and demographic information, were collected from children under the age of five with signs and symptoms of ARI. The NPAs were analysed for a range of viral and atypical bacterial pathogens, and the results were used to select samples for cytokine analysis. The study has been organised into the following chapters:

- Demographic and clinical information
- PCR results
- Cytokine analysis

2 Methodology

This chapter outlines the general methods used throughout the study. To avoid repetition details of all reagents and equipment mentioned in this chapter can be found in appendices 1 and 2, respectively.

2.1 Study Design

The study was a prospective, observational, cross-sectional study carried out over a period of twelve months at the IMIP Children's hospital, Recife, Brazil. The study was approved by the ethics committee at IMIP and the National Research Ethics Office of Brazil.

2.2 Recruitment

Between April 2008 and March 2009, children under the age of five, presenting with acute respiratory infection (ARI) to the IMIP children's hospital, Recife, Brazil, were enrolled in the study. All children less than five years of age presenting with upper and/or lower respiratory tract manifestations of ARI of less than seven days duration were eligible for inclusion. Written parental consent to take part in the study was obtained for each child. A total of 407 children were enrolled in the study, with 68.6% being under 12 months of age.

2.3 Demographic and Clinical Information

Demographics

The study was conducted in the IMIP Children's Hospital, Recife, Brazil. Recife is the capital city of the State of Pernambuco, and is situated on the North East coast of Brazil (**Figure 1**). The metropolitan region of Recife has a population of approximately 3.7 million, with the city itself having approximately 1.5 million inhabitants, making it the fourth largest metropolitan region and ninth largest city in Brazil. Recife's tropical climate sees average

temperatures between 22^{o}C and 31^{o}C. The coolest temperatures and maximum rainfall are experienced within Brazil's winter months, between June and August.

Recife has the highest proportion of favela residents, and the third highest number overall in the country, after only Sao Paulo and Rio de Janeiro, with an estimated 800,000 residents living in the shanty towns[140]. The majority of favelas in Recife have been around since the 1970's when a large number of people moved from rural areas into the city in search of work. The favelas are illegal settlements on the edge of the city, with high crime rates and poor standards of living. Many people living in favelas do not finish basic education, and although college and university attendance is free in Brazil, entrance exams have to be passed, which are impossible if secondary education has not been attained. The living conditions are often crowded, with very basic electricity supplies, and a lack of clean water (**Figure 2**).

Upon enrolment into the study the parent of each child was asked to complete a questionnaire containing 95 questions detailing the child's birth history; past medical history; social history and family history, as detailed in Appendix 3.

Figure 1: Map detailing the location of Recife

Figure 2: Photograph of one of Recife's favelas

Clinical Information

Clinical details such as the child's admission weight, heart rate, respiratory rate, temperature, and the presence or absence of symptoms such as chest in-drawing, cyanosis, cough and wheeze were recorded. The discharge diagnosis, as described by the attending physician, was recorded along with the severity of disease. Diagnoses were made upon discharge from hospital by clinicians who were not otherwise involved in the study, according to standard clinical criteria. Bronchiolitis was diagnosed in children under 18 months of age, who presented with a history of upper respiratory symptoms preceding lower respiratory symptoms of wheeze, tachypnoea and signs of respiratory distress. Pneumonia was diagnosed in children with fever, tachypnoea and respiratory distress, along with focal or diffuse crackles or decreased vesicular sounds on auscultation. A diagnosis of episodic viral wheeze (EVW)/asthma was made in children with discreet episodes of wheeze, often in association with presumed viral upper respiratory tract infection. Upper respiratory tract infection was diagnosed in children with symptoms such as coryza, earache, sore throat and stridor. Radiographs were available to assist diagnosis in some cases but not all.

Severity of disease was categorised as follows: *very mild* – upper respiratory signs only; *mild* – lower respiratory tract signs but no need for hospital admission; *moderate* – lower respiratory tract signs, with the need for hospital admission requiring nasogastric or IV fluids, but no oxygen; and *severe* – lower respiratory tract signs and the need for hospital admission and oxygen or ventilation. All children admitted to hospital were followed daily, with length of stay, outcome of illness and any further complications being recorded. Appendix 3 summarises the points covered by the questionnaire.

2.4 Sample collection & processing

2.4.1 Nasopharyngeal Aspirate collection

Nasopharyngeal aspirate (NPA) samples were collected from recruited children in A&E, on the ward or on the paediatric intensive care unit (PICU), to obtain samples of secretions from the upper respiratory tract.

NPAs were taken according to a previously described, standardised protocol[141].

All NPAs were collected by one research assistant (**Figure 3**) to minimise measurement bias. According to the study's protocol, all samples were processed within two hours, with dilution in 3mls of normal saline (0.9% NaCl in H_2O). The cell counts were calculated and the samples were then centrifuged at 500g for 10 minutes at 4^{o}C. The samples were split into the cell pellet and supernatant. The cell pellet was resuspended in a solution of 2-mercaptoethanol and RNA lysis buffer. The supernatant was split into two aliquots. All samples were stored at -70^{o}C. The samples were all transported in dry ice to the UK for analysis.

2.4.2 RNA Extraction

DNA and RNA were co-extracted from samples using the QIA symphony Virus/Bacteria Mini Kit (Pathogen Complex 200 protocol), according to manufacturer's instructions, using 300µl of each sample, added to 100µl animal tissue lyssis (ATL) lysis buffer. The RNA samples were stored at -70^{o}C until further analysis.

A)

B)

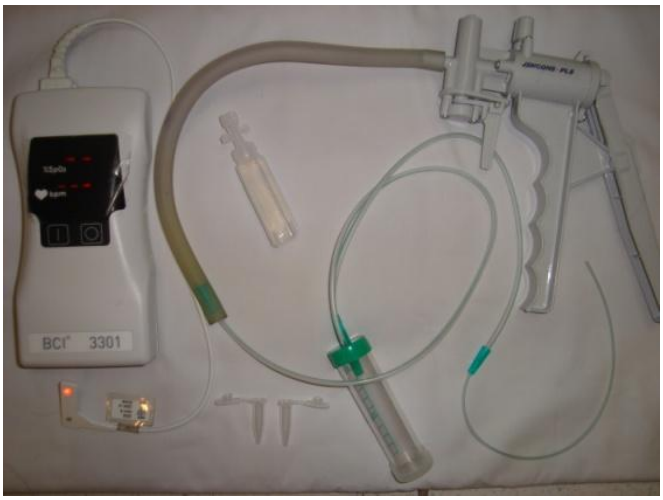


Figure 3: Nasopharyngeal aspirate collection

A) All NPAs were collected by one research assistant. B) NPA equipment

2.5 PCR Analysis

2.5.1 Introduction to PCR

The polymerase chain reaction (PCR) is a technique that allows the amplification of a specific region of DNA in vitro[142].

DNA consists of a double helix, made up of two strands of DNA running antiparallel to each other, with hydrogen bonds connecting complimentary bases – guanine (G) and cytosine (C), and adenine (A) and thymine (T)[142].

The first step in PCR is denaturation of the double helix. The DNA is heated to 94-95^{o}C in order to break the hydrogen bonds between the base pairs and separate the strands of DNA, providing two single stranded DNA templates for amplification. The temperature is then lowered according to the primers being used[143].

Primers are short sequences of DNA, usually around 20 nucleotides long. Primers complimentary to particular regions of the template sequence of DNA are added, to which they will anneal (form hydrogen bonds), giving a starting and end point for DNA synthesis. DNA polymerase will then catalyse DNA synthesis between the primers, adding the correct deoxynucleotides, complimentary to those on the template DNA to which they can anneal, subsequently forming a new double stranded structure, identical to the original[143]. The primers essentially define the region of DNA which is to be copied, and subsequently amplified.

Following this first replication of the initial DNA templates, two copies of the original DNA are produced, each containing one strand of original DNA (the template), and the new strand of DNA. The new double stranded molecules are once again heated to 94-95^{o}C, leading to denaturation, now providing four new templates for replication[143].

This cycle of denaturation and annealing is repeated between 20-40 times, each time doubling the number of copies of the DNA sequence, with the potential to provide millions of copies from a very small number of template DNA molecules[143].

The newly synthesised DNA can then be analysed, most commonly by gel electrophoresis, which will separate the DNA molecules in order of size. The newly synthesised DNA can then be compared to known DNA sequences, therefore allowing identification[143].

Multiplex PCR

Multiplex PCR uses multiple pairs of primers in the same reaction to amplify multiple sequences of DNA. This allows the detection and identification of multiple viruses within one reaction. Prior to the development of this, in order to identify more than one virus, each sample had to be prepared and PCR performed for each individual virus. Multiplex PCR has major advantages in terms of decreased cost and preparation time in comparison to original methods[144].

Real –Time PCR

Real-time PCR is another variant of PCR which includes an extra step in which the PCR products are analysed in the same reaction. Instead of gel electrophoresis to detect specific DNA, real-time PCR uses a specific DNA binding dye or primer which fluoresces when bound to the product, which is then detected[143]. The reaction can either be quantitative, in which the fluorescent signal is quantified, and can be compared throughout the cycle, or it can be qualitative, giving a 'positive' or 'negative' response to a sample[143]. Real-time PCR makes interpretation of results very simple, and eliminates the need for gel electrophoresis which can be time-consuming.

2.5.2 PCR Methods

Five separate multiplex PCR assays were used to detect 17 different respiratory pathogens. The first assay comprised of influenza A (flu A), influenza B (flu B), human metapneumovirus (hMPV) and human respiratory syncytial virus (hRSV), as well as primers and probes specific for novel H1N1 influenza. The second comprised of coronavirus (CoV) OC43, NL63, 229E and HKU 1. The third included parainfluenza virus (PIV) types 1, 2, 3 and 4 and hRV. The fourth included adenovirus (AdV), *Mycoplasma pneumoniae* (*Mpp)* and *Chlamydia pneumoniae* (*Cpp)*; and the fifth included human bocavirus (hBoV).

Multiplex reverse-transcription PCR was performed for the detection of RSV, hMPV, Flu A and B, PIV1-4 and hRV using the Invitrogen Superscript III Platinum One-Step Quantitative RT-PCR System as described previously[145]. Additional primer-probe sets were used for the detection of COVs (OC43, NL63, 229E and HKU1), also using the Invitrogen Superscript III Platinum One-Step Quantitative RT-PCR System as described by Gunson et al[146]. The RNA was denatured at 95^{o}C for one minute prior to mastermix addition on ice. 5µl nucleic acid was added to 20µl master mix. PCRs with DNA targets utilized 10µl nucleic acid to detect AdV*/Cpp/Mpp* and hBoV using the Roche LC480 Probes Master Kit and Qiagen Quantitect Probe PCR Kit respectively[147] [148]. Cycling conditions were as described previously[145], except for removal of the 50^{o}C hold for reverse transcription in the AdV/Cpp/Mpp and hBoV assays, and the 95^{o}C enzyme activation hold was extended to five minutes for the AdV/Cpp/Mpp assay, and to 15 minutes for the hBoV assay (Appendix 4). All primers and probes are listed in Appendix 5. All assays were performed in 96-well plates, in the LightCycler® 480 Real-Time PCR System.

2.6 Cytokine analysis

2.6.1 Protein Assay

Sample protein concentrations were measured to normalize cytokine concentrations by dilution and enable comparison across groups.

A protein assay was performed using the QuantiPro BCA Assay Kit to determine the protein concentration of each sample.

Protein concentrations were calculated from measured absorbances following interpolation to a bovine serum albumin (BSA) standard curve. The standard curve consisted of seven serial two-fold dilutions with a top standard of 30µg/ml. Samples were centrifuged at 8000RPM to pellet cellular debris. The samples were then diluted 1:14 with PBS, adding 20µl sample to 280µl PBS in each well. The reagents were made up by adding 75µl reagent A, 75µl reagent B and 3µl reagent C to each well. The plate was incubated for one hour at 60^{o}C before being read at 540nm on the ELx800 absorbance microplate reader.

2.6.2 Background – Bio-Plex technology

The Bio-plex system is a relatively new development in the detection of cytokines. The technology has developed from the enzyme-linked immunosorbent assay (ELISA), where the principle is based upon coating a plate with the antigen – the cytokine or biomarker in question – either directly or with the use of a capture antibody. The antigen is then bound by an added detection antibody. An enzyme is then added which will bind to the antigen-antibody complex, with levels of enzyme activity being determined by the addition of a colorimetric substrate solution. This colour change can then be quantified using a photometer[149].

Bio-plex technology uses similar principles, but the capture molecules are bound to polystyrene microbeads added to a microplate, instead of just the floor of the plate. The antigen, or substance containing the antigen, is then added, and binds to the capture molecules. A detection antibody, labeled with a fluorescent marker, is then added, which will bind to the antigen-antibody complex. The microplate is then read using the Bio-Plex array reader, which draws up the beads from each well in a single line, and each bead is then passed through two lasers which excite the beads and fluorescent markers, generating a fluorescent signal. The strength of the signal is determined by the amount of the captured antigen, allowing quantification. The biggest advantage of this technique is that many different colour coded beads can be added to the same assay, detecting multiple antigens simultaneously. This multiplex method has been developed to enable the detection of up to 100 different antigens at once. In this study we have used the Bio-Plex system along with an 8-plex kit, to which we added IL-17 beads in order to detect the following nine cytokines of interest: IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, TNF-α and IL-17.

2.6.3 Cytokine Analysis

Standard Preparation

One vial of premixed lyophilized standard was reconstituted with 500µl standard diluent. An eight point standard curve was constructed using 1:4 serial dilutions in standard diluent.

Sample preparation

Nasopharyngeal aspirate supernatant samples were centrifuged at 8000RPM for five minutes at 4^{o}C. A 1:6 dilution of each sample was used adding 25µl sample to 125µl human serum diluent.

Bead Preparation

Each set of coupled magnetic beads (8-plex kit containing IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ and GM-CSF; plus a separate IL-17 kit) were quick-spun, then resuspended before adding to 4600µl assay buffer.

Detection Antibody Preparation

Each detection antibody (10x) was vortexed for 10-15 seconds, and centrifuged for 30 seconds before being added to 2400µl detection antibody diluent to make a 1x dilution.

Streptavidin-PE Preparation

Streptavidin-PE (100X) was prepared by vortexing for 1-3 seconds, and centrifuging for 10-15 seconds, before adding to 5940µl assay buffer to make a 1x dilution.

Assay Procedure

The Bio-Plex Pro flat bottom plate was pre-wet with 100µl assay buffer per well. Excess buffer was removed from the plate using a vacuum manifold calibrated at 1-3”Hg. The prepared coupled beads were added to the plate, 50µl per well. The plate was washed twice by adding 100µl wash buffer, with excess liquid being removed using the vacuum manifold as before. Prepared standards and samples were added to the plate (50µl per well), before incubating for 30 seconds at 1100RPM, then for a further 30 minutes at 300RPM in the dark. After incubation the plate was washed x3 as before. Detection antibody was added to each well (25µl) before incubating as before. The plate was washed x3 before adding 25µl streptavidin-PE to each well, before incubating at 1100RPM for 30 seconds, followed by 10 minutes at 300RPM in the dark. The plate was washed x3 before adding 125µl assay buffer per well to resuspend the beads. The plate was shaken at 1100RPM for 30 seconds prior to reading using the Bio-Plex Array 200 system.

2.7 Statistical Analysis

All data was recorded on a database in Microsoft Excel 2007. All statistical analysis was performed on SPSS 18.0.1 statistical package (SPSS Inc, Chicago).

The Kolmogorov-Smirnov test

The Kolmogorov-Smirnov test was used to determine whether specific variables were normally distributed, in order to guide statistical testing. When a significant value was found, indicating that a variable was not normally distributed, non-parametric tests were employed for further testing.

Normally distributed data

Normally distributed data were expressed as mean with standard deviation (SD). In order to compare differences between two normally distributed groups, the students t-test was used.

Non-parametric data

Non-parametric data were expressed as median with the range. In order to investigate differences between two non-parametric groups, a Mann-Whitney U test was used. If there were multiple non-parametric groups for comparison, a Kruskal-Wallis test was used, and if differences were detected, a Mann-Whitney U test was performed to locate the groups within which these differences existed.

Categorical Variables

In order to compare differences between categorical variables, a Chi-square test was used.

Bonferroni Correction

When performing multiple comparisons, the likelihood of finding a significant difference between variables by chance (a type 1 error) is increased. A Bonferroni correction was used when performing multiple comparisons to decrease the likelihood of this.

Graphs

Bar graphs and box and whisker plots have been used to graphically display data. In box and whisker plots the median is marked as a line within the box, the edges of the box indicate the interquartile range, and the 'whiskers' indicate the overall range of the data.

Graphs were created using Microsoft Excel 2007 (chapter 3 and chapter 4), and SPSS 18.0.1 (chapter 5).

3 Demographic and Clinical Characteristics

3.1 Introduction

Although a number of studies have investigated the epidemiology of acute respiratory infection (ARI) in developing countries, none have detailed the demographic or clinical characteristics of the population studied, or addressed the impact these factors may have on results. It is important to analyse and document demographic and clinical information, not only because it enables findings of a study to be put into context with other studies, but to put the results themselves into context and allow them to be used in clinical practice. In the case of ARI this is particularly true, as demographic and clinical factors, such as overcrowding, smoking, prematurity and low birth weight, have all been shown to be risk factors for ARI. Knowledge of clinical information allows potential risk factors and causes to be linked to clinical conditions, and helps identify confounders which may affect the accuracy and reliability of results.

The aims of this chapter are:

1. To define the demographics of the study population.
2. To outline the background and presenting clinical information of the study population.

3.2 Methodology

Clinical and demographic information was obtained using a detailed questionnaire as described in section 2.3.

3.3 Results

A total of 407 children and infants under the age of five were enrolled into the study over a 12 month period from April 2008 to March 2009. **Table 2** summarises the demographic and clinical characteristics of the study population.

3.3.1 Demographics

The study population comprised of 171 girls (42.0%) and 236 boys (58.0%). The median age of study participant was 8.0 months (range 0-57), with 68.6% of participants being under 12 months old (**Figure 4**).

Table 2 : Summary of clinical and demographic characteristics of study population

Characteristic	Value
• Male (%)	236 (58.0)
• Female (%)	171 (42.0)
• Median age, months	8
• Age range, months	0-57
Socio-economic factors:	
• Median (range) primary household income	$R 415.00 (30-1800)
• Mean (SD) number of years parents studied:	
○ Mother	7.63 (3.065)
○ Father	7.54 (3.197)
• Number (%) of parents who attended higher education:	
○ Mother	3 (0.8)
○ Father	5 (1.3)
Living Conditions:	
• Median (range) number living in accommodation	4 (1-17)
• Median (range) number sleeping in same bedroom as child	3 (1-10)
• Number (%) of children exposed to smoking at home	174 (42.8)
Birth History:	
• Mean (SD) birth weight, grammes	3420.88 (1568.64)
• Gestation <37 weeks (%)	78 (19.2)
• Gestation <32 weeks (%)	9 (2.2)
• Number of children breastfed (%)	374 (91.9)
Past Medical History:	
• Co-morbidities (%)	19 (4.7)
• Asthma Diagnosis (%)	37 (9.1)
• Admission to hospital in last 30 days (%)	49 (12.0)
Presenting Features:	
• Cough (%)	399 (98)
• Wheeze (%)	322 (79.1)
• Coryza (%)	268 (65.8)
• Fever (%)	296 (72.7)
Severity	
• Very mild (%)	23 (5.7)
• Mild (%)	173 (42.5)
• Moderate (%)	169 (41.5)
• Severe (%)	42 (10.3)
Diagnosis	
• Bronchiolitis (%)	211 (51.8)
• Episodic Viral Wheeze/Asthma (%)	55 (13.5)
• Pneumonia (%)	109 (26.8)
• Upper respiratory tract infection (%)	23 (5.7)
• Other (%)	9 (2.2)

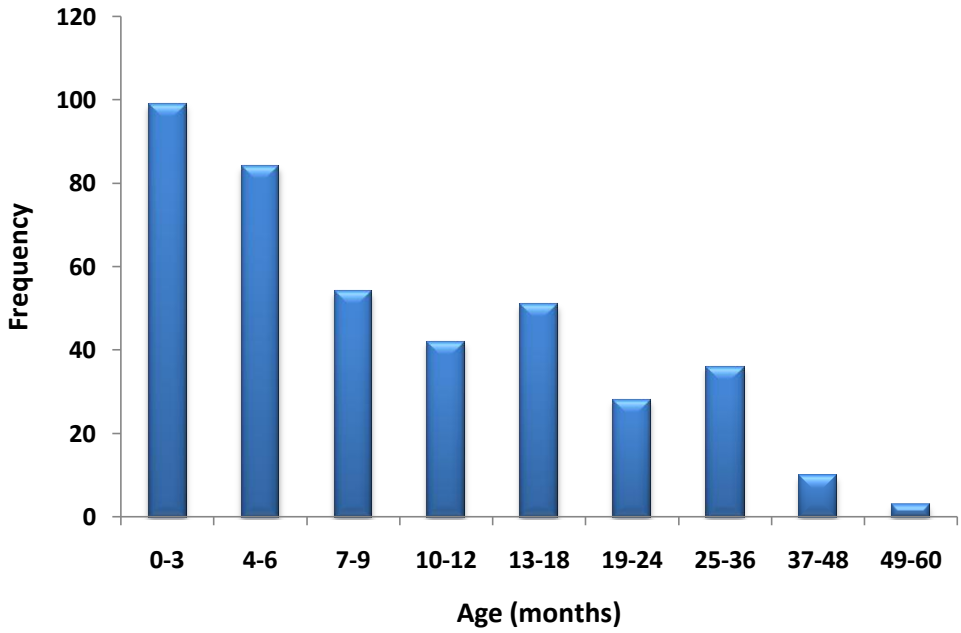


Figure 4: Distribution of age in study population.

Socio-economic Status

The questionnaire included several questions relating to the living conditions and socio-economic status of each participant and their family. The first indicator that was addressed was household income. The median (range) household monthly income for the study participants was found to be R$415.00 (30-1800) (Brazilian Reais). This equates to approximately US$235.99. Of the study participants, 85.9% had monthly incomes below R$800 (the overall mean Brazilian monthly income[150]). A total of 48 (11.8%) of participants lived in accommodation with no water supply, and 56 (13.8%) of participants lived in households that did not use any form of water purification. All participants were asked about the power supply to their home, and 404 (99.3%) study participants were found to have some form of electricity supply to their accommodation.

The questionnaire also documented the number of residents per household and in particular the number of people sharing a bedroom with the study participant. The median number of people living in the same household as the child was 4, with a range from one other person, to 17 others. The median (range) number of people sharing a bedroom with the study participant was 3 (0-10), with only 28 (6.9%) having their own room, and 105 (25.8%) having at least four in the same room. The median number of siblings of our study participants was 2, with a range from zero to 10.

One further parameter that may indicate socio-economic status is the number of years of education the parents of each participant received[151]. The mean (SD) number of years the mother studied for was found to be 7.63 (3.065), with 56.5% having studied for less than Brazil's 'mandatory' nine years. The mean (SD) number of years the father studied for was found to be similar - 7.54 (3.197) – with 55.5% having studied for less than nine years. Out of the whole study population, only 5 (1.3%) fathers and 3 (0.8%) mothers had attended

college or university (this data was deduced from the number having studied for more than 11 years).

Smoking

Out of the 407 study participants, 174 (42.8%) came from households in which at least one member of the household smoked.

Birth History

The questionnaire included questions on birth weight, gestation, breastfeeding and weaning. The median (range) birth weight for the study population was 3120.00g (970-3965g). Overall, 56 (13.8%) participants had a birth weight of less that 2500g, and 78 (19.2%) study participants were born prematurely (less than 37 weeks gestation), although only 9 (2.2%) were born at less than 32 weeks gestation. Out of the total 407 children enrolled onto the study, 374 (91.9%) had been, or were currently being, breastfed.

Vaccinations

The questionnaire included questions regarding whether or not the child had been vaccinated against tuberculosis (TB), diphtheria, *Haemophilus Influenzae* type b (Hib), *Streptococcus pneumoniae* (*S. pneumoniae*), and influenza (flu). Only four children (1.0%) had not had the BCG vaccine (against TB). However, 67 children (13.4%) had not received the diphtheria vaccine, and 73 children (14.6%) had not received the Hib vaccine. A total of 331 children (81.3%) had not been given the pneumococcal vaccine, while 334 (82.1%) had not had the influenza vaccine.

Past medical history and co-morbidities

Specific questions were asked regarding any co-morbidities the child may have, any recent hospital admissions or any previous respiratory symptoms, and specifically if the child had been diagnosed with asthma, or had ever had tuberculosis. Overall, 49 children (12.0%) had been admitted to hospital within the last 30 days. Out of the total 407 children, 37 (9.1%)

had previously been diagnosed with asthma by a doctor, and no study participants had ever been diagnosed with TB. A total of 52 patients (12.8%) had been admitted to hospital with pneumonia in the past, 238 children (58.5%) had previously had an episode of wheezing, and 21 (5.2%) had been diagnosed with allergic rhinitis by a doctor.

Out of the 407 study participants, 19 (4.7%) had a co-morbidity. The most common type of co-morbidity was heart disease in 7 patients, followed by neurological (4 patients) and genetic (3 patients) abnormalities (**Table 3**).

Table 3: Table detailing the presence of co-morbidities in study population

Co-morbidity Type	Frequency
Heart disease	7
Chronic Lung disease	1
Genetic abnormality	3
Neurological	4
Genetic/Immunodeficiency	1
Diaphragmatic eventration	1
Cardiac/neurological	1
Cow's milk protein allergy	1

3.3.2 Clinical Information

Presenting features

The most common symptoms present on admission were cough in 399 children (98%), wheezing in 322 (79.1%), coryza in 268 (65.8%) and fever in 296 (72.7%). Chest indrawing was observed in 48 children (11.8%), while 22 (5.4%) had oxygen saturations below 90% on admission. Episodes of apnoea were reported in 19 children (4.7%), and one child out of the 407 was found to be cyanotic on clinical examination (0.2%).

Severity

The severity of disease of each patient, as described in section 2.3, was recorded (**Figure 5**). The most common severity observed was *mild* disease (173 participants; 42.5%), followed by *moderate* (169 participants; 41.5%), then *severe* (42 participants; 10.3%), and finally *very mild* (23 participants; 5.7%). The low numbers of children with *very mild* disease reflects the fact that recruitment took place in a hospital, meaning that children with ARI attending general practitioners or nurse led clinics, or not consulting at all, were not included in the study. Just over half (212, 52.1%) the study participants were admitted to hospital with the ARI, while the other 195 were seen and discharged on the same day. Of the 407 children enrolled in the study, only two required admission to the intensive care unit. There were three deaths (0.7%) in the study population during the study period.

Clinical Manifestations

The diagnosis of each child was recorded upon discharge from hospital (**Figure 6**). Bronchiolitis, pneumonia, episodic viral wheeze (EVW)/asthma and upper respiratory tract infection (URTI) made up 97.8% of the diagnoses. Bronchiolitis was the most common, affecting 211 children (51.8%), followed by pneumonia, affecting 109 children (26.8%), then EVW/asthma, affecting 55 children (13.5%) and finally URTI, affecting 23 children (5.7%).

The other 2.2% (9 participants) were diagnosed with other conditions, including laryngitis, laryngotracheobronchitis and pneumonia with pleural effusion.

Over three-quarters of children diagnosed with pneumonia required admission to hospital (84/109; 77.1%), compared to 49.8% of those with bronchiolitis (105/211) and 23.6% of those with EVW/asthma (13/55).

Demographic effects on severity

The following demographic parameters were investigated with regard to severity of disease: household income; breastfeeding; birth weight; parental smoking and number of siblings. Following statistical analysis using a chi-square test, the only variable found to have a significant relationship with severity was birth weight. Children in the study with a birth weight under 2500g were found to be more likely to have moderate or severe disease than mild disease ($p=0.009$ and $p=0.005$, respectively). No other variables were found to significantly affect severity of disease.

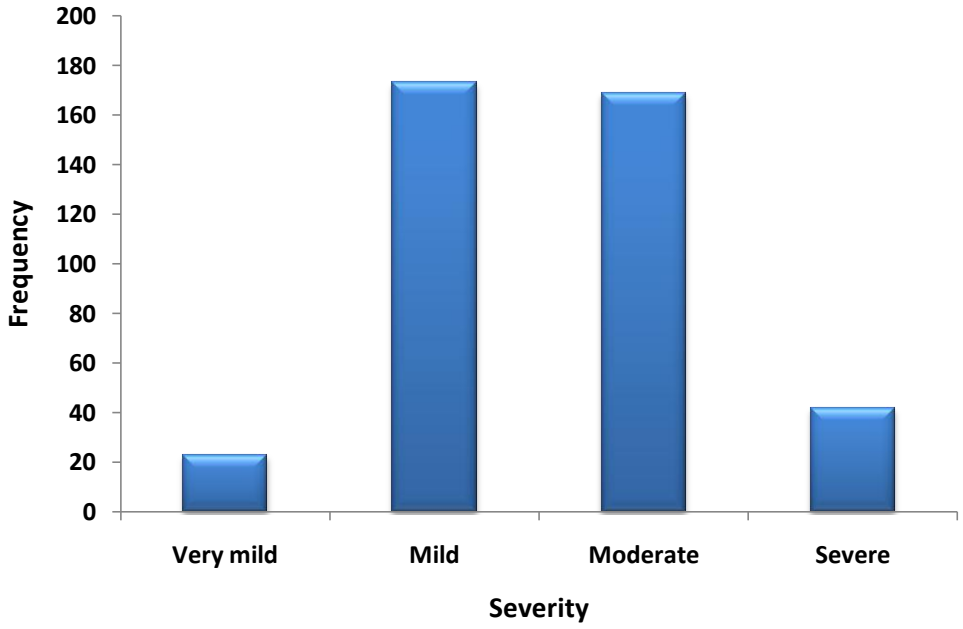


Figure 5: Distribution of severity of disease in study population.

Very mild – upper respiratory tract signs and symptoms only; *mild* – lower respiratory tract signs and symptoms but not requiring hospital admission; *moderate* – lower respiratory tract signs and symptoms requiring hospital admission; *severe* – lower respiratory tract signs and symptoms requiring hospital admission and oxygen or ventilation.

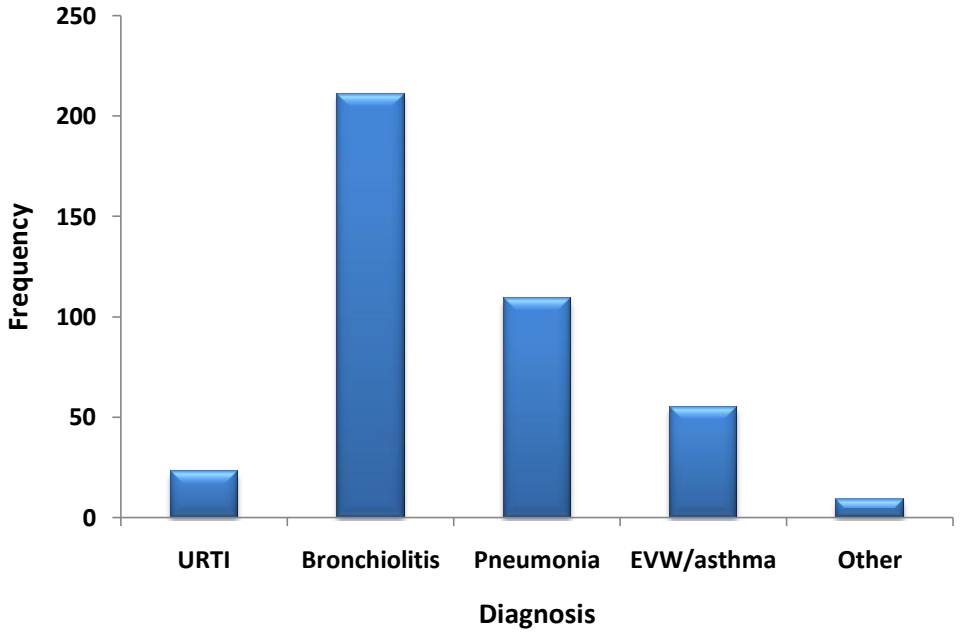


Figure 6: Clinical diagnoses recorded in study population.

3.4 Discussion

This chapter describes detailed demographic and clinical characteristics of the study population. Several factors suggest that this study population has an overall lower socio-economic status than that of Brazil as a whole.

The median monthly household income was found to be R$415 (US$235.99). This is in comparison to Brazil's overall mean monthly household income of R$805 (US$451)[150]. The number of people per household, (median 4, with up to 17 in one case), and the number of people sharing a bedroom with the study participant (25.8% had at least 4 in the bedroom), show a degree of overcrowding, or at least cramped living conditions. Poor living conditions, and especially overcrowding, are factors that will affect the spread of disease, and more specifically respiratory infections[16 20 22 23]. The low socioeconomic status of the study population, when compared to the whole population, can be explained, at least in part, by the structure of the healthcare system in Brazil. Brazil has a large private healthcare sector (52.1% of total health expenditure[152]). Such a large private healthcare sector will explain the discrepancies in mean income and living conditions of our study participants, with all recruitment to this study being from the IMIP Children's Hospital, a government run hospital providing free healthcare for those that need it.

Low parental literacy levels have been reported as a risk factor for ARI in children[151]. Pre-school education in Brazil for children under the age of 6 is optional. The only compulsory education or 'Fundamental Education' as it is known in Brazil, is given between the ages of 6 and 14, consisting of nine 'years' of learning. Secondary education is free to all, but is optional, and the 'Fundamental Education' must have been completed in order for someone to attend. Secondary education lasts for three years, and is mandatory for anyone wanting to pursue Higher Education. Higher Education is free at public universities,

although a competitive entrance exam must be passed in order to secure a place. The fact that over 50% of our study population's parents attended education for less than the mandatory nine years, with only 35.7% having attending secondary education, and a very small minority attending college or university (0.8-1.3%), highlights the fact that this population is not representative of the whole of Brazil, but represents a population with a lower socio-economic status, many of whom probably come from the poorest families in the city, living in favelas with poor living conditions.

An association between passive smoking and respiratory illness in children has been long established, with studies showing an increased risk of severe respiratory illness[28 29]. In our study population, 42.8% of children came from homes in which at least one person was a smoker. This rate of smoking is higher than the Brazilian national average, where in the year 2000, 35.4% of males and 26.9% of females were smokers, with both figures falling (males from 38.2% and females from 29.3% in 1995)[153]. This is also in contrast to the United States in which 23.9% of males, and 18.1% of females are smokers. We may speculate that the high percentage of our study population coming from homes in which there are smokers may be due to our study population having a lower socio-economic status than the overall Brazilian population. Many studies have shown that smoking rates are higher in lower socio-economic groups[25-27]. Secondly, several studies have found relationships between exposure to cigarette smoke and both increased likelihood of ARI in young children, and increased severity and likelihood of admission for ARI[28 29], meaning our high proportion of children from homes with smokers could explained because the exposure to smoke is putting them at a higher risk of admission. Finally, overcrowding will increase the likelihood of a child living in a household with a smoking adult.

The study population contained 56 children (13.8%) who had a birth weight under 2500g, and 19 children (4.7%) who had a co-morbidity. These statistics are lower than would be expected, as prematurity, along with cardiac and respiratory problems are all risk factors specifically for hRSV disease and are all associated with increased severity[154]. It is encouraging that 91.9% of the study population had been or were still currently being breastfed, as it has been found that infants age 0-5 months who have not been breastfed have a five-fold increased risk of death from pneumonia compared to those who had been[155]. These factors indicates that our study population is a generally otherwise healthy population, with a low co-morbidity rate and high levels of breastfeeding.

No children within the study population had ever had TB, which reflects the high uptake of the BCG vaccination. It is more difficult to assess the other vaccinations, because unlike the BCG (which the Brazilian Ministry of Health recommend is given in one dose at birth), diphtheria and *Haemophilus influenzae* type b (Hib) vaccines should be given at 2, 4 and 6 months[156]. As 39.1% of our study population is under 6 months of age, it is difficult to assess whether or not they should have completed the vaccinations, and with 13.4% and 14.6% having not received the diphtheria and Hib vaccines, respectively, this could be correct due to the patients' age, giving an overall high uptake of the vaccines. At the time of data collection the pneumococcal conjugate vaccine, and the influenza vaccine were not routinely recommended by the Brazilian Ministry of health, which explains why 81.3% and 82.1%, respectively, had not received the vaccines[156].

Bronchiolitis is the most common diagnosis in the study population. This reflects the age range of the population studied, with bronchiolitis being the most common cause of hospital admission in children under the age of one in winter months. There were relatively few children in the study population with URTI (23, 5.7%), reflecting the fact that although

URTI is very common, in the majority of cases it manifests as the common cold, which is not usually severe enough to warrant attendance at the emergency department, or hospital admission.

The classification of severity of disease in the study population has shown a spectrum from very mild disease with upper respiratory tract signs and symptoms only, to severe disease with children needing oxygen, and in some cases mechanical ventilation. Although this wide range of severities has been used, a limitation of this severity classification lies within the study design. As it is a cross-sectional study, it only investigates one point in time. Children with very mild or mild disease, who weren't admitted to hospital, were classified according to severity at the point of attendance at the emergency department. As children would have attended at different time points in the course of their illness, the severity may have changed following discharge. For example a child who initially only had upper respiratory tract signs and symptoms may have developed lower respiratory tract signs and symptoms once they had left hospital, which would have been changed their severity from very mild to mild, but as they had already left hospital they would have been included in the study as a 'very mild' patient.

4 PCR Results

4.1 Introduction

The correct identification of the causative agent in acute respiratory infection (ARI) is important as it may help determine management. A previous study of children with viral respiratory tract infections found that rapid knowledge of a diagnosis led to a decrease in duration of antibiotic use by 3.8 days per patient, and a decrease in length of admission by 0.9 days per patient, significantly reducing the costs of hospital care by up to 26%[81]. Also important is the collection of epidemiological data to analyse patterns and implement management plans for infection control.

The aim of this chapter is to characterise the viral and atypical bacterial causes of ARI in children under five years of age.

4.2 Methods

Nasopharyngeal aspirate (NPA) samples were analysed for 17 viral and atypical bacterial pathogens (human respiratory syncytial virus (hRSV), human rhinovirus (hRV), human metapneumovirus (hMPV), adenovirus (AdV), human bocavirus (hBoV), influenza virus A and B (flu), parainfluenza virus 1-4 (PIV), coronaviruses OC43, 229E, NL63 and HKU1 (CoV), *Mycoplasma pneumoniae* (*Mpp*) and *Chlamydia pneumoniae* (*Cpp*))using multiplex PCR as detailed in section 2.5.2.

Results were analysed using SPSS 18.0.1 as detailed in section 2.7.

4.3 Results

Multiplex PCR was performed on NPAs from each of the 407 children enrolled in the study, in order to detect the 17 different respiratory pathogens described above. Of the 407 samples, 348 (85.5%) were found to be positive for at least one viral or atypical bacterial pathogen. The most commonly detected pathogens were hRSV (detected in 152 (37.3%) samples), AdV (101, 24.8%), hRV (77, 18.9%), hBoV (76, 18.7%), hMPV (42, 10.3%) and *Mpp* (40, 9.8%). PIV was detected in 34 (8.4%) samples, CoV in 13 (3.2%), Flu in 11 (2.7%) and *Cpp* in 5 (1.2%) (**Figure 7**). The most common type of PIV detected was PIV3, found in 20 (4.9%) samples; PIV4 was detected in nine samples; PIV1 in four samples and PIV2 in one sample. The most common CoV detected was CoV-OC43, found in 7 (1.7%) samples; CoV-NL63 was detected in four samples; CoV-HKU1 in two samples and CoV-229E in one sample.

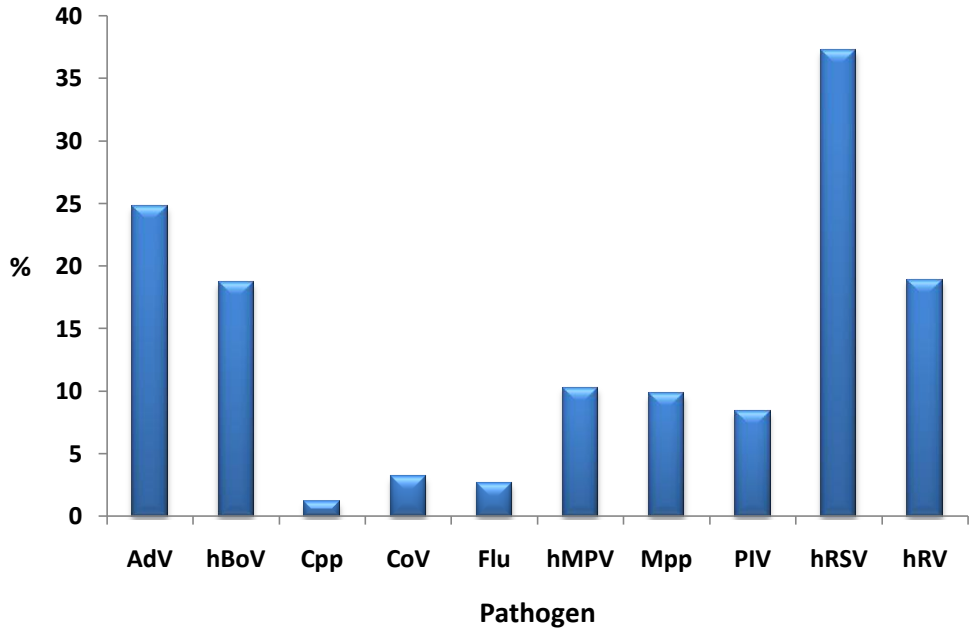


Figure 7: Pathogen prevalence

Pathogen prevalence (%) in nasopharyngeal aspirates from children less than five years of age with acute respiratory infection. Cumulative prevalence is greater than 100% due to the presence of co-infection. (AdV, adenovirus; hBoV, human bocavirus; *Cpp*, *Chlamydia pneumoniae*; CoV, coronaviruses; Flu, influenza viruses; hMPV, human metapneumovirus; *Mpp*, *Mycoplasma pneumoniae*; PIV, parainfluenza viruses; hRSV, human respiratory syncytial virus; hRV, human rhinovirus)

4.3.1 Co-infection

Co-infection with two or more pathogens was detected in 161 samples (39.6%). Two pathogens were detected in 124 samples (30.5%); three in 33 samples (8.1%); four in three samples (0.7%); and one sample was found to contain five pathogens (**Figure 8**).

Of the 187 single infections, the most common pathogens were hRSV, detected in 81 samples (43.3%), followed by hRV, detected in 34 samples (18.2%), followed by hMPV, detected in 11 samples (5.9%). AdV and hBoV, which were overall the second and third most commonly detected pathogens, were found as single infections in only 16 (8.6%), and nine (4.8%) samples, respectively (**Figure 9**).

AdV was found to be the most common pathogen involved in co-infection, being detected in 85 (52.8%) of the 161 samples. This was followed by hRSV, detected in 71 (44.1%) samples; and hBoV, detected in 67 (41.6%) samples (**Figure 10**).

The most common combinations of co-infection were dual infections with hRSV and hBoV (26 samples; 16.1% of co-infections), hRSV and AdV (18 samples; 11.2% of co-infections), and hRV and AdV (12 samples; 7.5% of co-infections). Other combinations observed in over 5 cases were hMPV and AdV, hMPV and hBoV, hMPV and *Mpp*, hRV and hBoV, PIV and AdV, and hRSV and hRV (**Figure 11**). The most common triple infection was found to be with AdV, PIV and hBoV (detected in 4 cases).

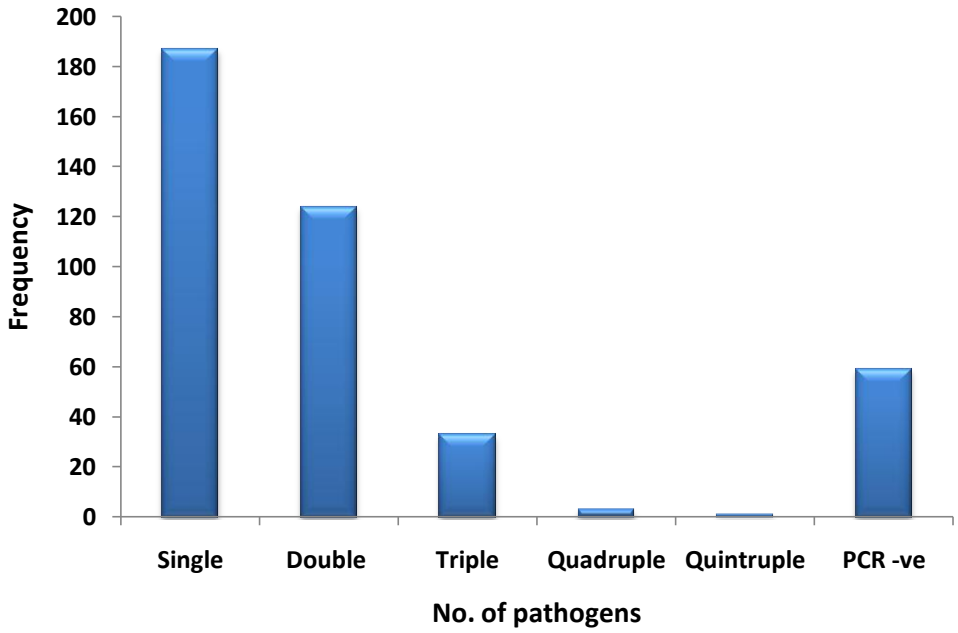


Figure 8: Number of pathogens detected per sample

Number of pathogens detected by multiplex PCR in each nasopharyngeal aspirate sample from children less than five years of age with acute respiratory infection.

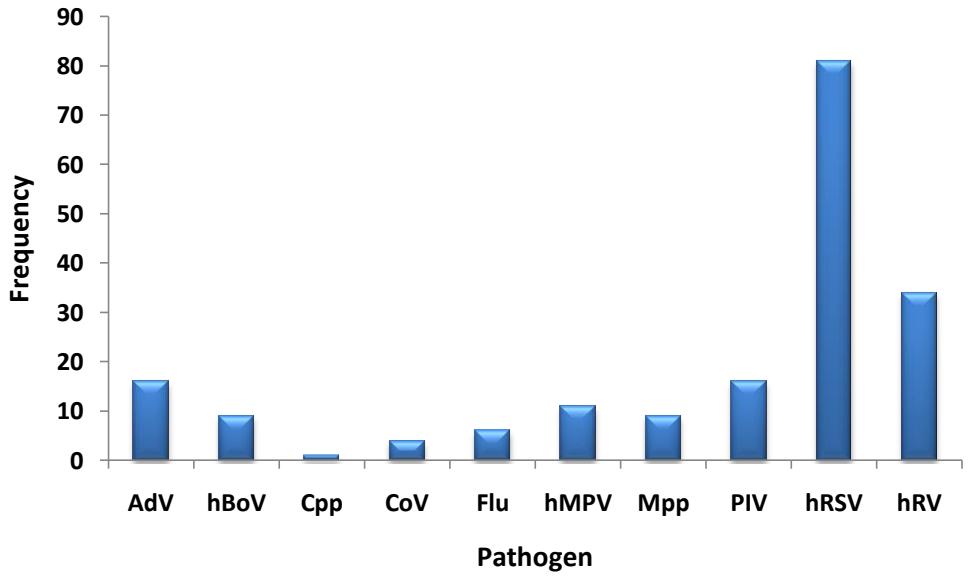


Figure 9: Pathogen frequency in single infection

Pathogen frequency in nasopharyngeal aspirates found to contain only single infection, from children less than five years of age with acute respiratory infection. (AdV, adenovirus; hBoV, human bocavirus; *Cpp*, *Chlamydia pneumoniae*; CoV, coronaviruses; flu, influenza viruses; hMPV, human metapneumovirus; *Mpp*, *Mycoplasma pneumoniae*; PIV, parainfluenza viruses; hRSV, human respiratory syncytial virus; hRV, human rhinovirus)

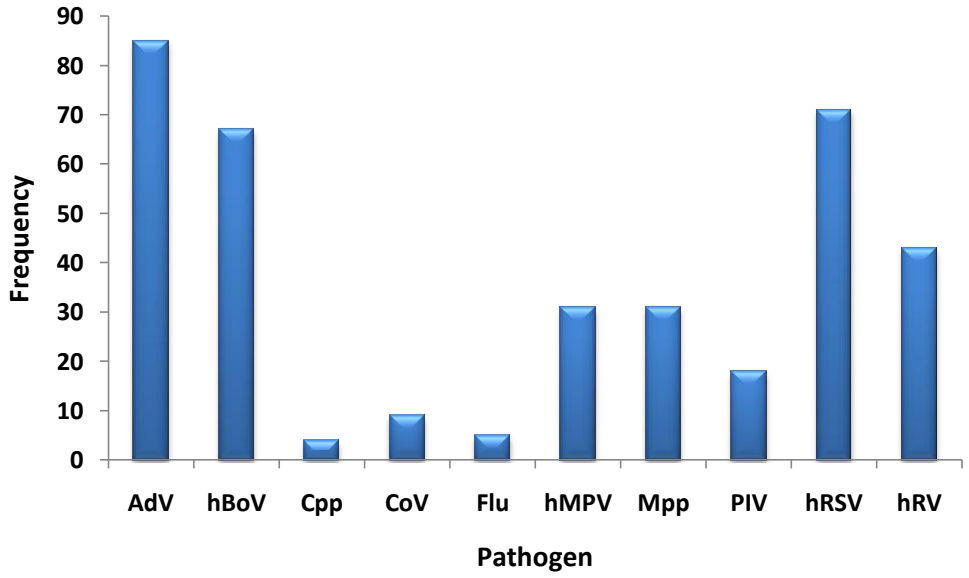


Figure 10: Pathogen frequency in co-infection

Pathogen frequency in nasopharyngeal aspirates found to contain co-infection with multiple pathogens, from children less than five years of age with acute respiratory infection. (AdV, adenovirus; hBoV, human bocavirus; *Cpp*, *Chlamydia pneumoniae*; CoV, coronaviruses; flu, influenza viruses; hMPV, human metapneumovirus; *Mpp*, *Mycoplasma pneumoniae*; PIV, parainfluenza viruses; hRSV, human respiratory syncytial virus; hRV, human rhinovirus)

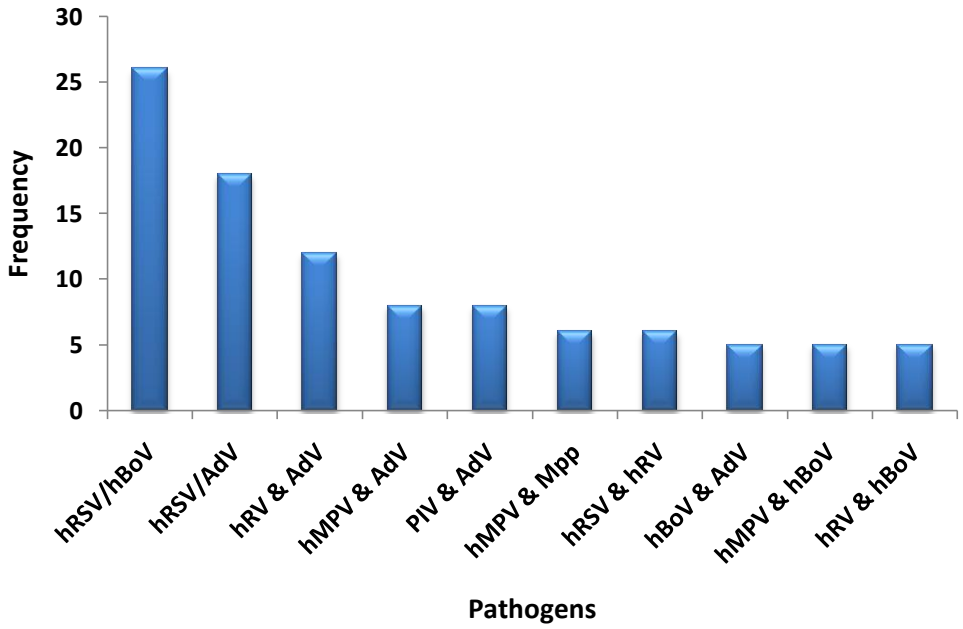


Figure 11: Most frequent co-infections

Combinations of pathogens most frequently detected in nasopharyngeal aspirate samples found to contain co-infection with multiple pathogens. (AdV, adenovirus; hBoV, human bocavirus; hMPV, human metapneumovirus; *Mpp*, *Mycoplasma pneumoniae*; PIV, parainfluenza viruses; hRSV, human respiratory syncytial virus; hRV, human rhinovirus)

The sample which was found to contain five pathogens contained hRSV, hMPV, hRV, hBoV and *Mpp*. On further investigation this child was found to be a one month old female, born at 39 weeks gestation with a normal birth weight (3400g), with no known co-morbidities and no previous hospital admissions. The child died in hospital from this episode, diagnosed as severe pneumonia. Her diagnosis was confirmed by chest x-ray findings.

Two other infants in the study population died in hospital during their admission for ARI. The first was a five month old boy who had no known co-morbidities, a normal birth weight and birth history, and was diagnosed with severe pneumonia. PCR results found the child to have hRV infection. The other child who died during the study period was a seven month old boy who had neurological co-morbidities. He was admitted with severe pneumonia, and died after seven days in hospital. All pathogens tested for were negative.

4.3.2 Pathogen detection and disease severity

When the relationship between respiratory pathogen and disease severity was studied, hRSV and *Mpp* infection were the only pathogens associated with particular disease severities. Children found to have *Mpp* infection were more likely to be admitted with moderate (12.4%) or severe (16.7%) disease, than discharged with mild (6.4%) disease, when compared to those without *Mpp* infection (p=0.04; p=0.039, respectively). Children with hRSV infection were more likely to be admitted with moderate disease than mild disease, when compared to children without hRSV infection (43.2% vs 31.8%; p=0.019). Otherwise, the prevalence of pathogens within the disease severity groups was similar, and there was no significant difference in disease severity groups in patients from whom no pathogen was detected, or in whom co-infection was found.

Children with hRSV infection were significantly more likely to need admission than those without hRSV infection (59.2% vs 47.8%; p=0.017). Similarly, those with *Mpp* infection were also significantly more likely to require admission than those without (70.0% vs 53.6% p=0.017). Conversely, those with PIV infection were less likely to need admission than those without (35.3% vs 53.6%; p=0.031).

4.3.3 Pathogen detection and clinical condition

When the relationship between respiratory pathogens and clinical condition was studied, only *Mpp* infection and hBoV infection were associated with particular clinical conditions. *Mpp* infection was more commonly associated with pneumonia than bronchiolitis (13.8% of cases of pneumonia vs 7.1% of cases of bronchiolitis; p=0.04), when compared to children without *Mpp* infection. HBoV was more commonly associated with EVW/asthma than bronchiolitis (29.1% of EVW/asthma vs 14.7% of bronchiolitis; p=0.013). No other relationships between pathogen and particular clinical conditions were found. The proportion of patients from whom no pathogen was detected or in whom co-infection was found, was not significantly different in any of the four clinical condition categories.

4.3.4 Seasonal Distribution

The peak incidence of ARI was found to be between April and September, with 70% of infections presenting during these autumn and winter months (**Figure 12**). hRSV showed a clear peak in between April and July, with no cases observed between October and February, clearly defining the hRSV season in Brazil. hMPV was prevalent throughout the year but had a clear peak after the hRSV peak, in September and October. *Mpp* was found to have a strong peak in August, with 80.0% of positives being found between July and October. AdV, hBoV and hRV were prevalent throughout the year. PIV, CoV, *Cpp* and flu all

had relatively few positive samples, meaning it is not possible to comment on a seasonal distribution. However, influenza was only detected between March and May, with one case also in July.

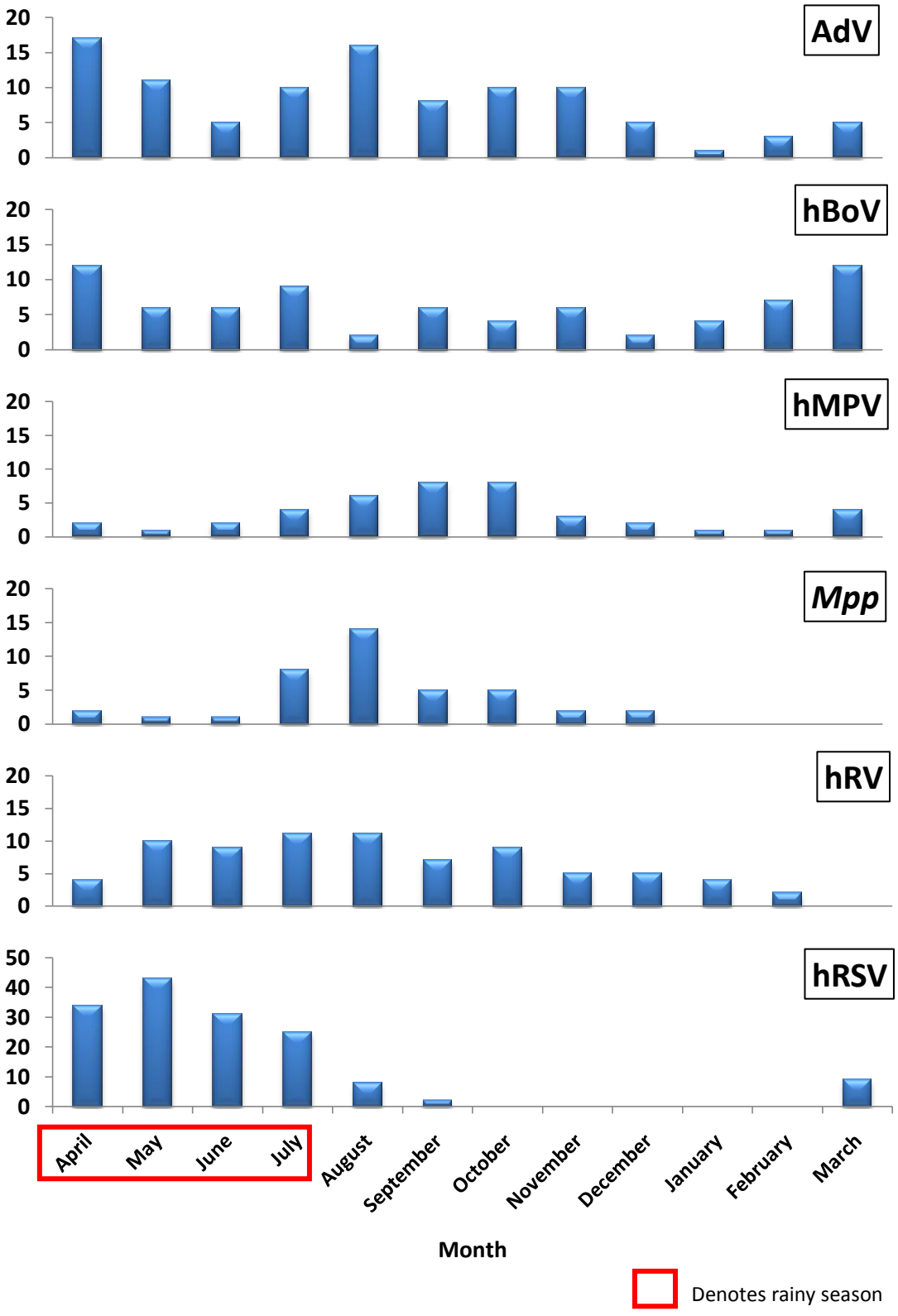


Figure 12: Seasonal distribution

Seasonal distribution of the most frequently detected pathogens in nasopharyngeal aspirates from children less than five years of age with acute respiratory infection. (AdV, adenovirus; hBoV, human bocavirus; hMPV, human metapneumovirus; *Mpp*, *Mycoplasma pneumoniae*; hRV, human rhinovirus; hRSV, human respiratory syncytial virus).

4.3.5 Relationships with age

The median (range) age of study participant was 8 (0-57) months. The median (range) age of study participant in the hMPV positive, hRSV positive and hRV groups were all lower than that of the population median (hMPV 5.5 months [1-36]; hRSV 6.0 [0-57]; hRV 6.0 [0-56]). The groups with the highest median age were found to be *Cpp* (11 months [4-15]), and CoV (13 months [1-57]). However, care needs to be taken when analysing this data as there is overlap between groups due to the high levels of co-infection in the study population.

4.3.6 PCR Negative Samples

Of the 59 children whose samples were found to be negative for all 17 pathogens, six (10.2%) were found to be very mild infections, 26 (44.1%) mild, 21 (35.6%) moderate and six (10.2%) severe. The diagnoses of these children were found to be bronchiolitis in 28 (47.5%), pneumonia in 16 (27.1%), episodic wheeze in 7 (11.9%), URTI in 6 (10.2%) and laryngotracheobronchitis and whooping cough each in one case.

4.4 Discussion

In this chapter we have used multiplex PCR to simultaneously detect 17 different respiratory pathogens in NPAs from a population of children and infants under the age of five, presenting with acute respiratory infection.

The overall positive rate, finding 85.5% of samples positive for one or more pathogens, was particularly high when compared to similar study populations. Regamey et al. found 79% of samples taken from children age 0 to 1 year, presenting with their first ARI, positive for one or more pathogen, when testing for 16 different respiratory viruses[1]. Other studies have found varying overall positive rates, ranging from 35.2% to 74%, although all were testing for varying numbers of viruses, ranging from 7 to 21[1 10 13 15 33 45 54].

The prevalence of hRSV, AdV and hBoV are all very high in the study population. hRSV has been found to be more prevalent in other studies, but these were looking specifically at bronchiolitis or children under the age of one[157]. Many other studies have found the prevalence of AdV to be below 10%[1 10 45 54 148 158]. Fewer studies have investigated the prevalence of hBoV, due to its more recent discovery in 2005, but the prevalence has been found to be between 2.2 and 18%[33 46 157 158].

The co-infection rate of 39.5% is higher than previously reported. Other studies have reported co-infection rates to be between 4 and 33% when using multiplex PCR to test for respiratory pathogens[1 13 45 46 54].

There are a number of possible reasons for the high detection and co-infection rates. This study looked for 17 respiratory pathogens, which is more than most similar studies[1 10 13 33]. All samples were NPAs collected by one research assistant, using a standard, reproducible protocol. Also, the demographics of the study population, as described in chapter 3, suggest that most children came from low income families, living in crowded environments, where living conditions may well facilitate the spread of pathogens.

AdV and hBoV were found to be particularly prevalent in our study population, and were found to be the first and third most common pathogens to be involved in co-infection. Previous studies have found both much lower prevalences of AdV and hBoV, along with much lower prevalences of co-infection[1 33 45]. The high rates may be due to prolonged nasopharyngeal shedding of the viruses post-infection. AdV has been detected in faeces months after infection[159], although it is unknown whether a similar situation occurs in the nasopharynx. A recent study detected hBoV in nasopharyngeal aspirates from three children, at least two months following their acute infection[67].

One study that has found prevalence rates of particular viruses to be very high, similar to those described here, is that by Kaplan et al. who studied a similar population of children under five years of age in Jordan. They described the prevalences of AdV and hBoV to be 37% and 18%, respectively[46]. Interestingly, they are also the only other study to have found similar co-infection rates (33%). However, they found the prevalence of hRV (11%) and hMPV (2.5%) to be much lower than those described here, and they found no cases of *Mpp* or PIV[46].

Seasonal Distribution

The seasonal distribution graphs show the hRSV season to be between March and August – Brazil's rainy season. Although hRSV is known to have a strong seasonal distribution, peaking in winter months, Recife has a tropical climate, with little variation in temperature throughout the year. It is somewhat surprising, then, that there is such a strong seasonal distribution, with no hRSV detected between October and February.

The *Mpp* results were particularly interesting, with a clear peak in the winter month of August, which was not expected. The prevalence of *Mpp* in the study population was also interesting, being higher than expected. *Mpp* is typically thought to affect older children and young adults[148], and is not thought to be one of the more common causes of pneumonia in pre-school children. These results may have implications for treatment of these children in this setting, as *Mpp* infections are treated with macrolide antibiotics such as erythromycin. This is in contrast to the supportive treatment of the viral pathogens or the antibiotic treatment of *S. pneumoniae* or *Haemophilus influenzae*, bacterial pathogens more commonly associated with pneumonia in this age group.

The three children included in the study population who died during their admission for ARI are all very interesting cases. The first child was one month old, previously healthy with a normal birth history and no previous admissions. The fact that she was admitted with severe pneumonia and then found to have five different respiratory pathogens in her NPA is surprising for a child of such a young age. She may have been very unlucky, coming into contact with five infections in her four weeks of life, which may reflect living conditions. Another possibility is that she had some form of underlying problem such as a congenital cardiac defect, or some form of immunocompromise, which predisposed her to ARI. Unfortunately, as the PCR results were only discovered during the study, after the child's death, it was not possible to confirm this. The second child who died during the study period was a five month old boy, again with no co-morbidities and an unremarkable past medical and birth history. This child was only discovered to have hRV in his sample. Human rhinoviruses are the most common respiratory tract viruses in older children and adults, and are thought to cause about two-thirds of common colds and asthma exacerbations[10]. They are most well known to cause upper respiratory illness, and are not specifically associated with severe illness. This child's death could be due to a number of factors. Although the majority of ARI are caused by viruses, some are caused by bacteria, and although we tested for 17 different respiratory pathogens, we only tested for two bacteria. This child's particularly severe illness could be because of a dual infection with hRV and another bacterial pathogen, or another viral pathogen which was not tested for.

5 Cytokine Analysis

5.1 Introduction

To further elucidate the pathogenesis of acute respiratory infection (ARI), and the complicated interplay between the pathogen and the host immune response, several studies have investigated cytokine profiles in the respiratory tract during ARI. The ideal way to investigate cytokine profiles would be to compare cytokine responses in healthy controls, to those with ARI. However, as previously mentioned, control subjects are difficult to recruit to this type of study and the nasopharynx is dry and difficult to sample in people without respiratory symptoms.

There is much conflicting evidence in the literature on the links between particular cytokines and chemokines and severity of disease in ARI. For example, one study reported an association between low levels of IL-6, IL-8, IL-10, IFN-γ and MIP-1β and a longer duration of supplemental oxygen therapy in children with RSV bronchiolitis[160]. However, strong associations between IL-8 and increased severity of disease have also been shown[161].

Many studies have looked at only one or two cytokines, or looked specifically at children or infants with bronchiolitis alone[161-163]. More evidence is therefore needed on which to draw conclusions on the relationship between the inflammatory response and disease severity in ARI.

Few studies have investigated the relationship between inflammatory response in the airways in relation to the particular pathogen involved in ARI. McNamara et al in 2007 found no difference in the concentration of a variety of cytokines and chemokines in bronchoalveolar lavage (BAL) samples from children with severe bronchiolitis caused by hRSV, compared to those with hRSV/hMPV co-infection[162]. Byeon et al in 2009 investigated

IL-4, IL-5 and IFN-γ levels in children with hRSV, influenza (flu), parainfluenza virus (PIV) and adenovirus (AdV) infection[163]. This study found significantly lower levels of IL-4 and IL-5 in the flu group when compared to the other three groups, in which they found a pattern of increased IL-4 and IL-5 levels, and decreased IFN-γ levels[163]. Again, these studies all investigated different aspects of the immune response, and where there was overlap between studies, results were not consistent.

The aims of this part of the study were to characterise the inflammatory response in nasopharyngeal aspirate (NPA) samples from previously healthy children under the age of five with acute respiratory infection (ARI). The following hypotheses were investigated:

1. Different severities of hRSV disease are characterised by different host immune responses
2. Host immune responses in infection vary depending on infecting pathogen.

5.2 Methods

5.2.1 Patient Samples

To maximise the number of samples containing single infection, samples from the following year's recruitment were also included.

hRSV Severity Groups

Initially the aim was to select equal numbers of samples containing only hRSV infection from each of the four severity groups: *very mild, mild, moderate and severe,* for cytokine analysis. However, even though hRSV was found to be the most prevalent pathogen in the study population, the exceptionally high prevalence of co-infection meant that the number of samples with hRSV infection alone was 81, with only six being very mild infections, and

only seven being severe infections. A total of 47 samples were selected for analysis, including six very mild, nineteen mild, fifteen moderate and seven severe.

Various Pathogen Groups

A second experiment was used to determine cytokine profiles in samples infected with different pathogens in order to test the hypothesis 'host immune responses in infection vary depending on infecting pathogen'. Samples containing the following pathogens were selected for cytokine analysis on the basis of numbers of single infections by that particular pathogen: human metapneumovirus (hMPV), human rhinovirus (hRV), *Mycoplasma pneumoniae* (*Mpp)*, adenovirus (AdV), and human bocavirus (hBoV). hRSV samples were not tested as data for these samples would already be available from the hRSV severity experiment. Samples with coronavirus (CoV), parainfluenza virus (PIV), influenza (flu) virus and *Chlamydia pneumoniae* (*Cpp*) were not selected for study due to the small sample size of single infection in each of the groups. Due to the high level of co-infection, only nine *Mpp* samples were available for analysis; all other groups contained ten samples.

5.2.2 Cytokine Analysis

Cytokine profiles of the NPA samples were determined using a human cytokine 8-plex bead immunoassay kit, along with a singleplex IL-17 kit as detailed in section 2.6.

A protein assay was performed to determine the protein concentration of each sample. Cytokine concentrations were then adjusted according to protein content and expressed as pg/ml/mg protein.

5.2.3 Statistics

The relationship between cytokine concentrations and different disease severities or pathogens was studied using a Kruskall Wallis test. Where differences were detected

between the groups, a Mann Whitney U test was used to find which groups were statistically different, with a p-value <0.05 being considered statistically significant. Statistical analysis was performed using SPSS 18.0.1 statistical package (SPSS Inc, Chicago).

5.3 Results

A total of 103 samples were analysed for the presence of nine different cytokines. IL-6, IL-8, IL-10 and GM-CSF had detectable concentrations in over 90% of samples, IL-17, IL-4 and TNF-α had detectable concentrations in 65-75% of samples, and IFN-γ and IL-2 had detectable concentrations in 50.1% and 33.7%, respectively.

5.3.1 hRSV severity samples

A total of 47 NPA samples containing single hRSV infection were analysed for the presence of nine different cytokines. One sample (*moderate* severity) was excluded due to a low protein concentration that was below the detection range of the standard curve, bringing the total number of samples for analysis to 46.

Demographics

The median (range) age of the study participants in this group was 4 (0-20) months. The age of participants in each of the severity groups were not significantly different. Overall 52.2% (24/46) were males. The diagnoses included URTI (15.5%); bronchiolitis (58.7%) and pneumonia (26.1%).

Cytokine Concentrations

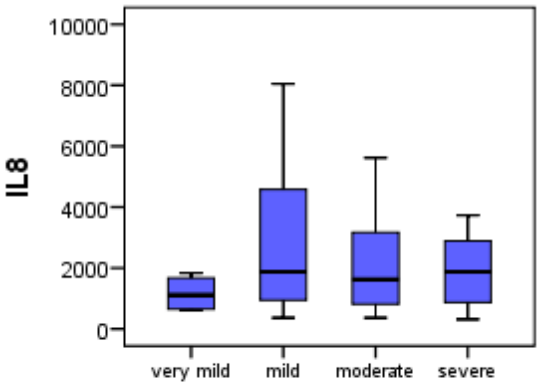
Figure 13 demonstrates the concentrations of IL-4, IL-6, IL-8, IL-10, GM-CSF, INF-γ and TNF-α found in nasopharyngeal aspirate samples from children with single hRSV infection, with

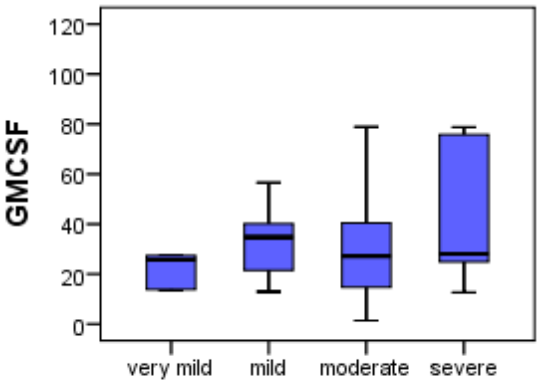
very mild, mild, moderate and *severe* disease. All results are shown as medians and inter-quartile ranges.

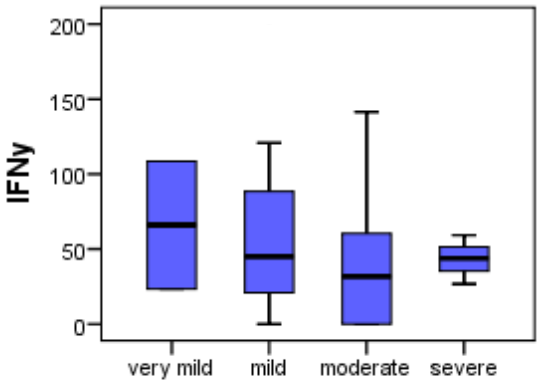
The results have been grouped according to function of the specific cytokines. The first shown are the innate, pro-inflammatory cytokines, IL-8, GM-CSF, IFN-γ and TNF-α. Next is IL-4, an adaptive, Th2 cytokine. Finally there are IL-6, IL-17 and IL-10, which have been grouped together due to their regulatory functions, with IL-6 included here due to its effects on IL-17 production.

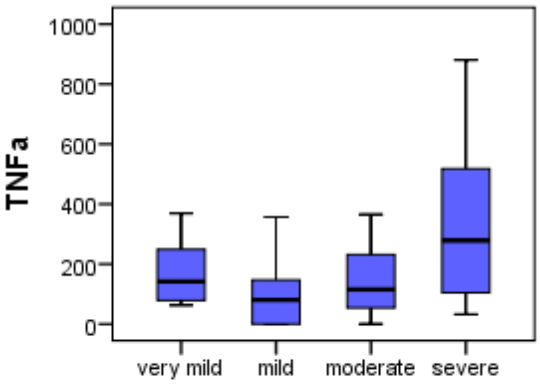
From the graphs you can see the wide variation in the range of results both within and between groups. After statistical analysis using a Kruskal-Wallis test, there was found to be no significant difference between cytokine concentrations according to severity of hRSV infection.

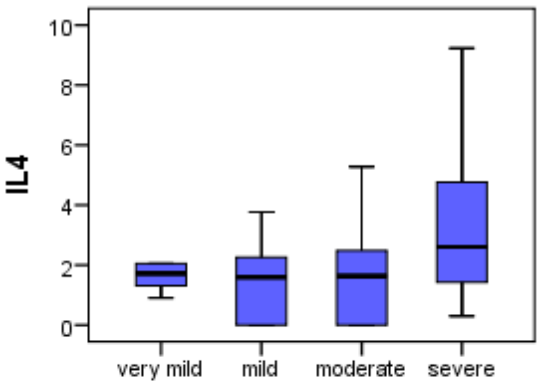
Although statistical analysis revealed no significant differences between groups, some patterns can be seen, particularly in the IL-4 and IL-10 graphs, with increasing IL-4 and IL-10 with increasing severity. Levels of GM-CSF also appear to increase with increasing severity, while levels of IFN-γ appear to be lower in the moderate and severe groups.

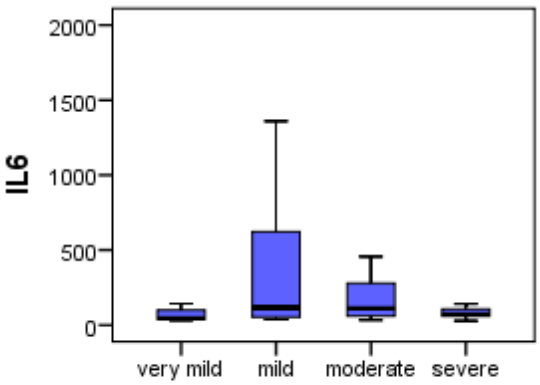












NB: All values shown are pg/ml/mg protein

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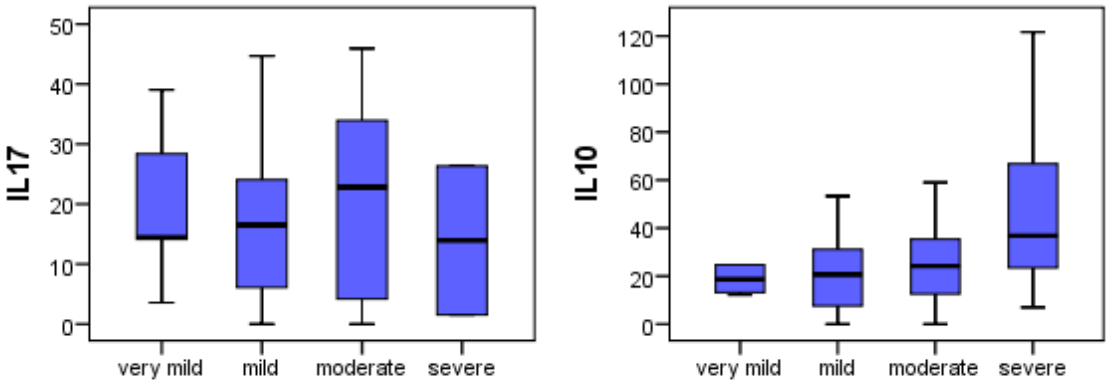


Figure 13: Cytokine concentrations in varying severities of single hRSV infection.

Concentrations of IL-8, IFN-γ, TNF-α, GM-CSF, IL-4, IL-6, IL-10 and IL-17 were measured in NPA samples from patients who had *very mild* (n=6), *mild* (n=19), *moderate* (n=15) and *severe* (n=7) hRSV infection. Samples were diluted 1:6 and all results were adjusted according to protein concentration. All values shown are pg/ml/mg protein. Outliers have been omitted from the graphs.

5.3.2 Cytokine Responses to Various Pathogens

A total of 49 samples which had tested positive for a single pathogen (hMPV, hRV, *Mpp*, AdV or hBoV) were analysed for the presence of nine different cytokines. Two samples (both *Mpp*) were excluded from the analysis due to low protein concentrations. The total number of samples included was therefore 47, including hMPV (n=10), hRV (n=10), *Mpp* (n=7), AdV (n=10) and hBoV (n=10). The previous hRSV results (n=46) were also included, bringing the total number of samples for analysis to 93.

Demographics

Of the 93 samples analysed, 54 (58.1%) were from patients who were male. The median (range) age was 6 (0-48) months. Samples in the hRSV group were from significantly younger children than either the AdV group (p=0.002) and the hBoV group (p=0.004). The ages across all other groups were similar. The most common diagnoses were bronchiolitis (52.7%); pneumonia (32.3%); URTI (9.7%) and EVW/asthma (5.4%). A total of six (6.5%) cases were *very mild*; 33 (35.5%) *mild*, 41 (44.1%) *moderate*, and 13 (14.0%) *severe*.

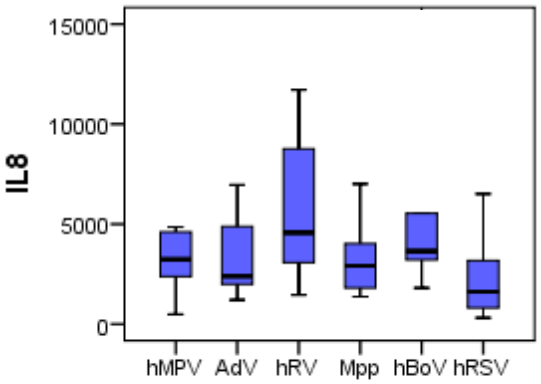
Cytokine Concentrations

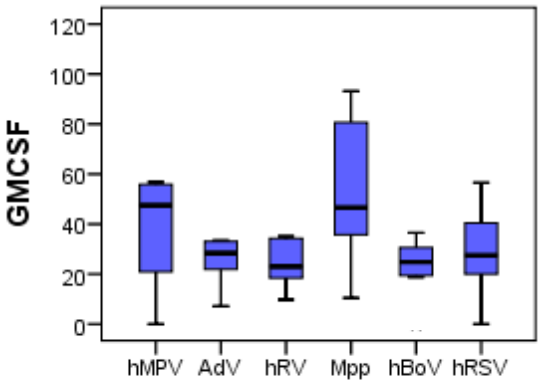
Figure 14 demonstrates the concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, GM-CSF, IFN-γ and TNF-α, according to the presence of pathogen: hMPV, AdV, *Mpp*, hBoV and hRSV. Again, large ranges both within and between groups can be observed.

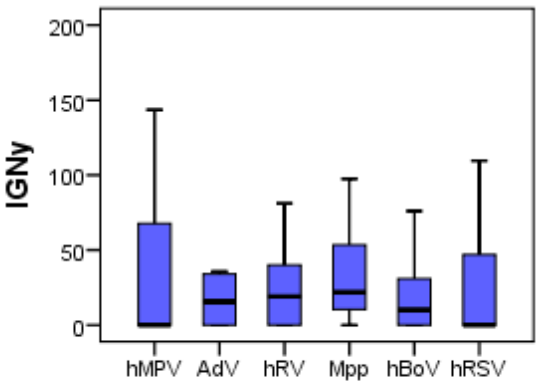
After statistical analysis, Kruskall-Wallis testing revealed differences between cytokine concentrations according to pathogen in IL-2 (p=0.039); IL-8 (p=0.001) and IL-17 (p=0.020). A Mann-Whitney U test was performed to detect where the differences were. After Bonferroni correction it was found that samples containing hRSV were associated with

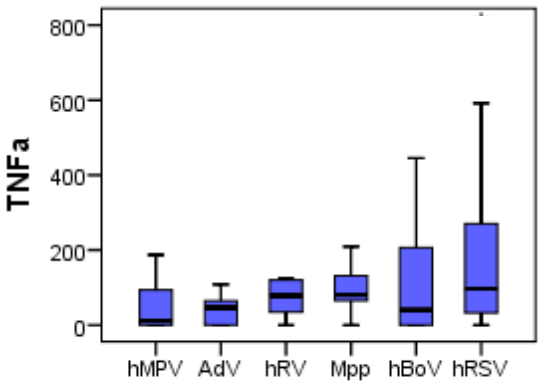
significantly lower concentrations of IL-8 than hRV (p=0.001). There were no other significant differences in cytokine concentration according to pathogen.

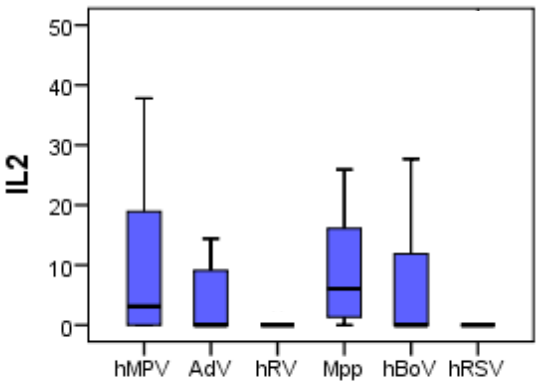
The available data was also analysed according to some of the demographic factors. It was found that there was no significant difference in cytokine concentrations according to clinical diagnosis or gender. When investigating cytokine concentrations according to age, infants under three months of age were found to have significantly lower concentrations of IL-2 (p=0.041), and significantly higher levels of IL-4 (p=0.013) and IL-10 (p=0.030) than those older than three months of age. No differences in cytokine concentrations were found according to severity of disease, which is similar to the previous findings when looking at hRSV infection alone.

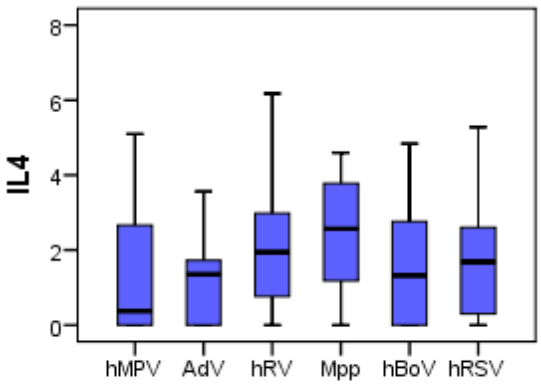












NB: All values shown are pg/ml/mg protein

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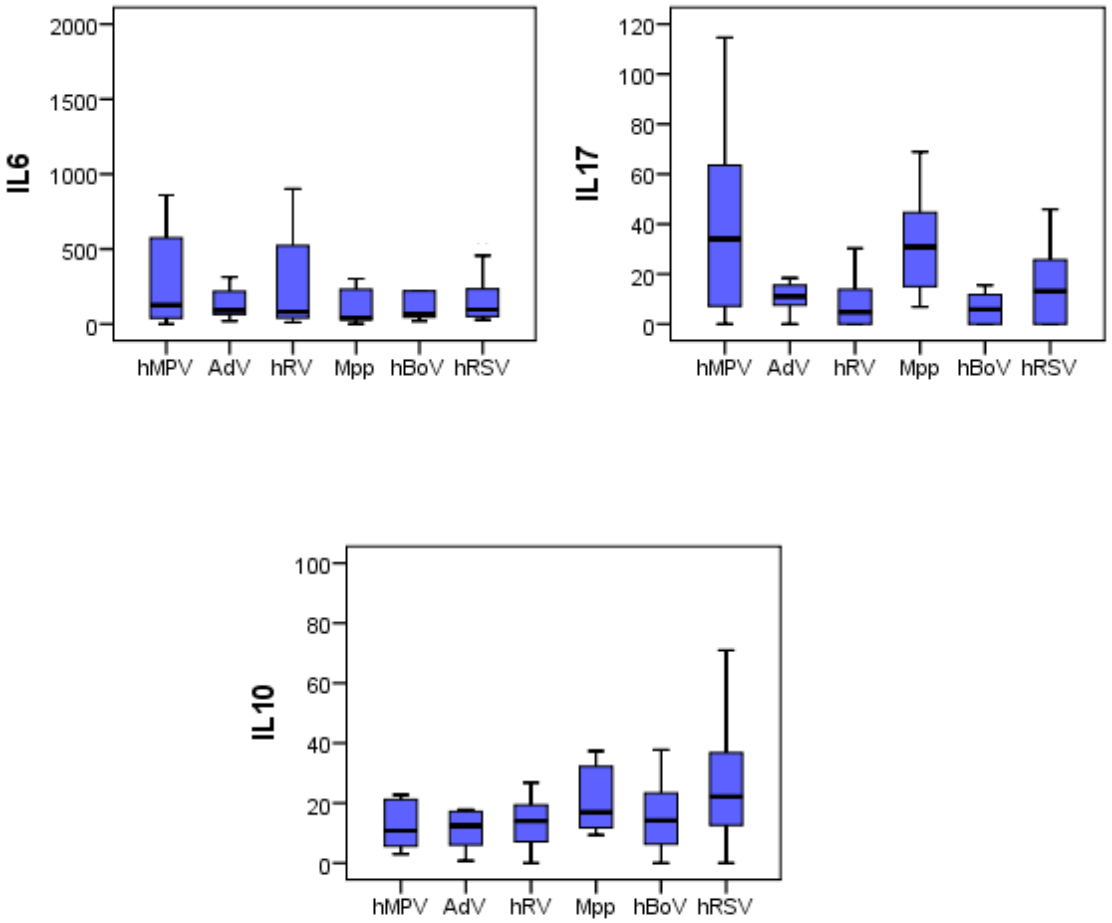


Figure 14: Cytokine concentrations according to pathogen.

All values shown are pg/mL/mg protein. Concentrations of IL-8, IFN-γ, TNF-α, GM-CSF, IL-2, IL-4, IL-6, IL-10 and IL-17 were measured in NPA samples from patients who had hMPV (n=10), AdV (n=10), hRV (n=10) *Mpp* (n=7), hBoV (n=10) and hRSV (n=46) infection. Samples were diluted 1:6, and all results were adjusted according to protein concentration. All values shown are pg/ml/mg protein. Outliers have been omitted from the graphs. (hMPV, human metapneumovirus; AdV, adenovirus; hRV, human rhinovirus; *Mpp*, *Mycoplasma pneumoniae*; hBoV, human bocavirus; hRSV, human respiratory syncytial virus).

5.3.3 Cytokine responses to co-infection

Very recent work which was presented at the American Thoracic Society conference in May 2010, investigated the role of IL-17 in the immune response to bacteria. Using a mouse model they found bacterial infection to be associated with a strong IL-17 response, while viral co-infection inhibited bacterial induced IL-17 production through interferon mediated inhibition of IL-23[164].

In light of this recent work, and the exceptionally high rate of co-infection observed in our study, cytokine responses and specifically IL-17 responses were investigated in single bacterial infection and viral/bacterial co-infection.

Therefore, as the final part of this study, 10 samples containing co-infection with a bacterium (*Mpp*) and a virus (hRV (n=3), AdV (n=4), hBoV (n=3)) were also tested for the same nine cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, TNF-α), using the same protocol and protein analysis as described previously. These results were then compared to those of atypical bacterial infection alone, to determine whether viral co-infection had an effect on the IL-17 response.

Demographics

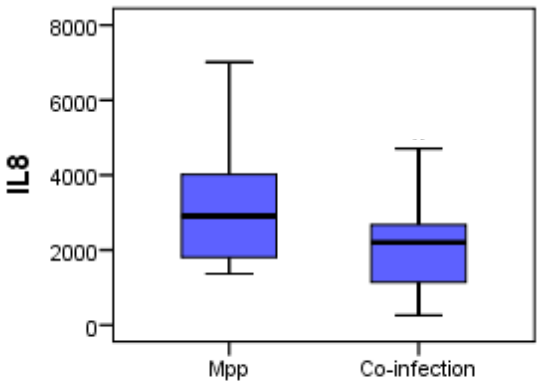
The median (range) age in this co-infection group was 10 (3-48) months, which was not significantly different from that of the *Mpp* group. Six samples were from male patients, and four from females. Three samples were classed as *mild*, four as *moderate*, and three as *severe*. The diagnoses were bronchiolitis (n=6), pneumonia (n=2) and episodic viral wheeze/asthma (n=2).

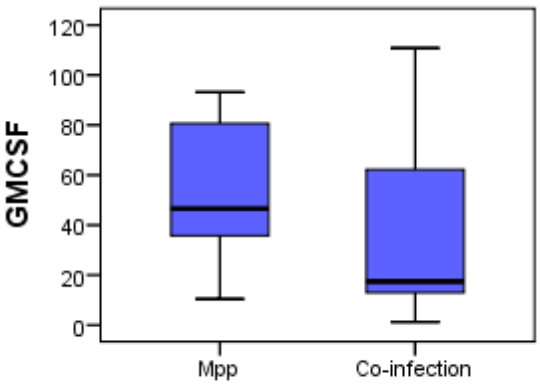
Cytokine concentrations

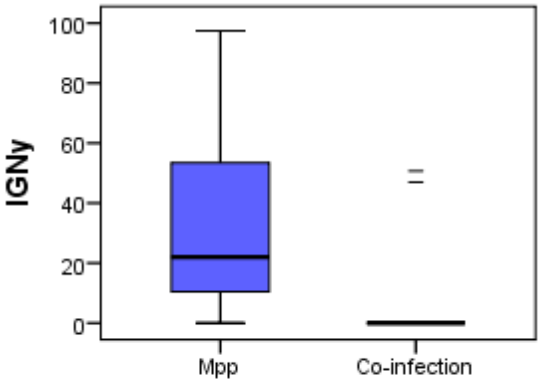
Due to the small sample size of each group, the co-infection results were grouped together for analysis, and compared to results from *Mpp* samples alone.

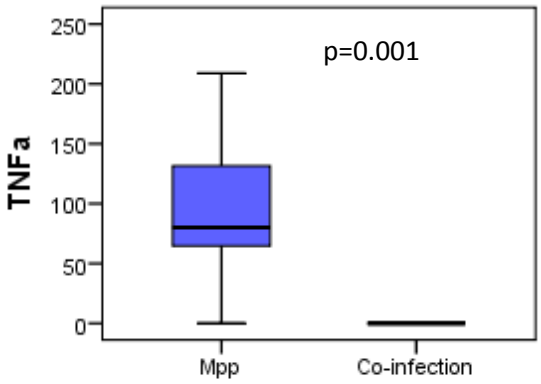
All samples had adequate protein concentrations.

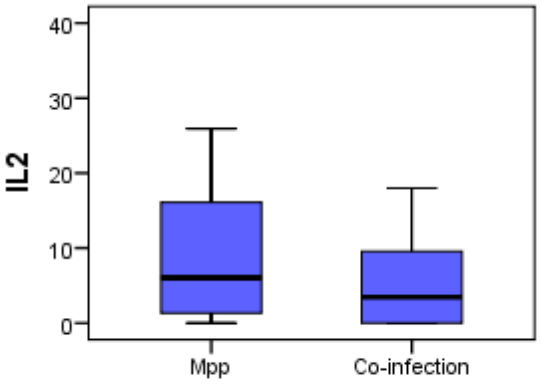
Figure 15 demonstrates the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, GM-CSF, IFN-γ and TNF-α, in samples with bacteria/virus co-infection, when compared to bacterial infection alone. After statistical analysis using a Mann-Whitney U test, several significant differences were detected. The bacteria/virus co-infection group was found to be associated with significantly lower levels of TNF-α, IL-4, IL-10 and IL-17, when compared to *Mpp* infection alone.

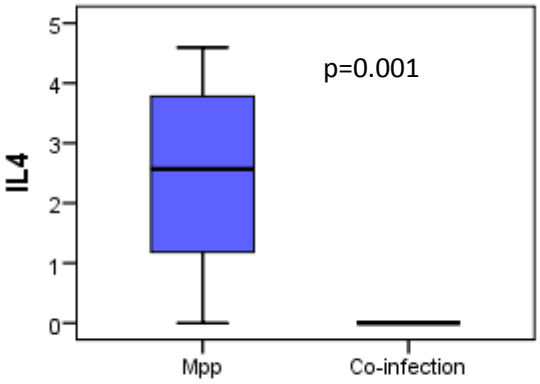












NB: All values shown are pg/ml/mg protein

Continued overleaf

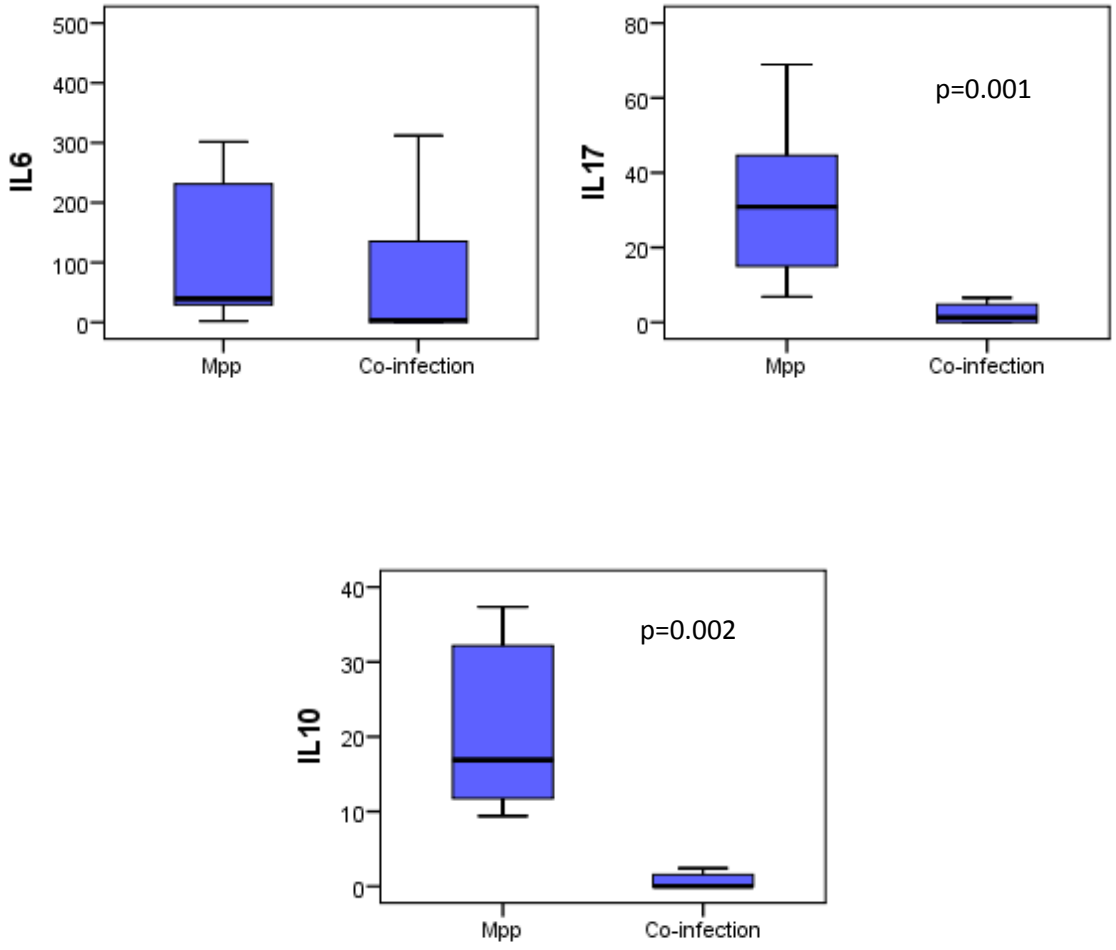


Figure 15: Cytokine concentrations according to single infection with Mpp, or co-infection with Mpp/virus.

Concentrations of IL-8, IFN-γ, TNF-α, GM-CSF, IL-2, IL-4, IL-6, IL-10 and IL-17 were measured in NPA samples from patients who had single *Mpp* infection (n=7) and *Mpp*/virus co-infection (n=10). Samples were diluted 1:6, and all results were adjusted according to protein concentration. All values shown are pg/ml/mg protein. - indicates outliers. All other outliers have been excluded from the graphs as they did not influence significance. (*Mpp*, *Mycoplasma pneumoniae*)

5.4 Discussion

In this chapter we have described host immunological responses in acute respiratory infection (ARI), investigating the concentrations of nine different cytokines in nasopharyngeal aspirate (NPA) samples from children with ARI with varying severities of human respiratory syncytial virus (hRSV) infection, varying pathogens, and bacterial/viral co-infection. We did not observe any differences in cytokine concentrations associated with differing severities or pathogens.

Throughout this part of the study, results were adjusted according to protein concentration of the sample. This was done to adjust for any variability in sample quality. Conversely, adjusting for protein may have affected the results by disguising true differences in cytokine concentration. For example, a more severe sample may have had a higher protein concentration because of the response to the particular pathogen.

5.4.1 Cytokine response to varying severities of hRSV infection

Similar cytokine concentrations were observed across different severity groups possibly due to the small sample size in each severity group. The very mild and severe groups were particularly small (n=6 and n=7, respectively), due to limitations in the number of these samples that were available for testing because of the high level of co-infection. This may have been important, as if a difference in response were to exist, it would be expected that it may be in one of these two groups, at either end of the severity spectrum.

In addition, although all samples were hRSV positive, with no known co-infections, the age range varied throughout the group, and the diagnoses were varied between bronchiolitis, pneumonia, upper respiratory tract infection, and episodic viral wheeze/asthma. Previous studies have tended to focus on one particular group, such as hRSV bronchiolitis[161], which

may have affected findings. Also, co-infection with either common bacterial infection such as *S. pneumoniae*, or with as yet undiscovered viruses, cannot be excluded. Interferon anti-viral responses were not fully investigated in this study. It is possible that differences in severity may be attributable to insufficient amounts of these cytokines, such as IFN-α and IFN-β, being produced.

Furthermore, the samples investigated were nasopharyngeal aspirates, which may not be representative of what is happening in the lower respiratory tract, which is where the infections manifest and cause clinical problems. However, one particular study compared IL-2 levels in NPA and BAL samples in children with hRSV bronchiolitis, finding no significant difference between the groups[165], indicating that NPAs do provide a good insight of what is going on in the lower respiratory tract.

Although no significant differences in cytokine concentration were found with regard to varying severities of disease, patterns could be observed when looking at some of the graphs. It appeared that IL-4 and IL-10 levels increased with increasing severity. IL-4 is a Th-2 cytokine, and has been found to be increased in murine models following stimulation with the RSV G protein[132]. IL-10 is a regulatory cytokine, with effects on the production of pro-inflammatory cytokines. Increased IL-10 levels in increased severity of disease may lead to thinking that the inhibition of pro-inflammatory cytokines may be an important factor in preventing the removal of pathogens and their harmful effects, consequently causing more severe disease. IFN-γ levels appeared to decrease with increasing severity of disease. This would fit with increasing IL-10 levels, with IFN-γ being one of many pro-inflammatory cytokines which would be inhibited by IL-10's regulatory effects. However, levels of GM-CSF, another pro-inflammatory cytokine, appeared to increase with increasing severity. This would not be expected with increasing levels of IL-10 – the opposite would be expected, as

with the IFN-γ levels. These were just patterns observed from the graphs, and the study would need to be furthered with a larger sample size to look further at these patterns, and identify whether or not they became significant trends.

Bennett et al in 2007 reported an inverse relationship between levels of IL-6, IL-8, IL-10, IFN-γ and macrophage inflammatory protein-1β (MIP-1β) and the need for oxygen therapy in nasal wash samples from children with bronchiolitis[160]. The study also found that levels of IL-6, IL-8, GM-CSF, IFN-γ, TNF-α, IL-1β, GM-CSF and MIP-1β were all increased in children with hRSV bronchiolitis, compared to those with non-hRSV bronchiolitis, although the severity of disease did not differ between the two groups. When investigating children with hRSV bronchiolitis alone, this group found that cytokine/chemokine levels were not significantly different in relation to the need for hospitalisation[160].

Although Bennett et al investigated a wide range of cytokines/chemokines, and had a fairly large sample size, there were several limitations to this study. Firstly, although the presence of other viruses were investigated in the samples, the effects of other viruses on inflammatory responses were not mentioned, and these may have had a confounding effect not only on the results from the 'non-hRSV' group, but also on the hRSV group with co-infection. Also, no bacterial pathogens were tested for, which may also have had confounding effects.

In contrast other studies have reported IL-8 to be closely correlated with severity of hRSV disease[134 161]. Smyth et al in 2002 found a strong association between increased IL-8 mRNA in NPAs and severe hRSV bronchiolitis[161]. This also agreed with the findings by Hull et al in 2000, who found an association between a single nucleotide polymorphism (SNP) near the IL-8 transcription site, causing increased IL-8 production, and increased severity of hRSV bronchiolitis[134]. Another study looking specifically at cytokine profiles from children with

AdV infection found no increase in IL-8 concentrations in nasal wash samples from children with AdV infection, when compared to healthy controls[166]. However, this same study did find an increase in IL-8 levels in patients admitted to hospital who were found to have AdV infection, compared to those who had AdV infection but were not admitted[166]. One other study investigated IFN-γ levels in infants with hRSV lower respiratory tract infection (LRTI), finding significantly decreased levels in infants with severe disease who required ventilation[167].

This, along with other studies that have looked specifically at one cytokine or one particular clinical condition, has demonstrated that there is an array of conflicting evidence, and lack of consensus as to whether individual cytokines have a role in the severity of disease. Previous studies[163] have illustrated theories that cytokine levels vary depending on virus and viral load, while other studies[160 161 168] have postulated that it is the inflammatory response, or lack of it, that may be causing increased disease severity. Our results do not show an association between cytokine concentrations and disease severity in single hRSV infection, and further study with a bigger sample size is needed to attempt to further understand the complex mechanisms and pathogenesis of this virus in the lungs.

5.4.2 Cytokine responses to various pathogens

A total of 46 samples containing hMPV, hRV, AdV, *Mpp*, hBoV and hRSV infection were tested for various different cytokines. After correcting for multiple comparisons the only significant difference was found between the concentration of IL-8 in hRSV compared to hRV. However, there are many other confounding factors that were not taken into account, including age. The samples in the hRSV group were from significantly younger children than two of the other groups. We are not able to determine whether, in this case, the difference in cytokine concentrations is due to the child's age, the hRSV infection, or another confounder that we have not investigated. Although age was not significantly different in any of the other groups, factors such as prematurity, breastfeeding and living conditions were not investigated or adjusted for. These are all factors that could affect immune responses and therefore may have an effect on cytokine concentrations.

The cytokines measured are all characteristic of a broad immune response to infection in the lung, and our results reflect a general response. This is not surprising when looking at the innate cytokines, where the aim of the innate response is to alert the adaptive immune system to the infection and to use non-specific mechanisms to destroy the pathogens, or at least keep the level of infection under control.

Previous studies that have investigated cytokine responses to different pathogens have found conflicting results. McNamara et al in 2002 investigated cytokine concentrations in bronchoalveolar lavage (BAL) samples from infants ventilated for bronchiolitis, with regard to the presence of single hRSV infection, or hRSV/hMPV co-infection. In this study clinical characteristics were similar between groups. No differences were detected in cytokine (IL-4, IL-9, IL-10, IL-13, TNF-α and IFNγ) or chemokine concentrations between the single infection or co-infection groups[162]. Byeon et al in 2009 investigated the response to IL-4, IL-

5 and IFN-γ in NPAs from children with LRTI caused by various pathogens. They found that hRSV, PIV and AdV infections caused a Th2 skewed response, with increased production of IL-4 and IL-5 and decreased IFN-γ, in comparison to influenza virus infection which was associated with significantly lower levels of IL-4 and IL-5[163].

Again the literature demonstrates many conflicting results, with a lack of large studies into this area.

5.4.3 Cytokine responses to co-infection

The final part of this study investigated cytokine concentrations of 10 samples with bacteria/virus co-infection. The bacteria/virus co-infection group was associated with significantly lower levels of several cytokines.

The IL-17 response is interesting, as recent work described by Alcorn et al. described the inhibitory effects of influenza A on IL-17 and IL-22 production and the subsequent increased inflammation and decreased clearance of bacterial infection with *Staphylococcus aureus*[164]. We have observed significantly lower levels of IL-17 in the bacteria/virus group, when compared to single bacterial infection with *Mpp*. However, the IL-17 responses to bacteria/virus co-infection are similar to those with virus alone, which leads to the question: is the *Mpp* in these infections active virus? The high sensitivity of PCR means that there is a possibility that we are picking up remnants of an older *Mpp* infection, and in fact what we are seeing in these results is caused by viral infection alone.

The low IFN-γ response in the co-infection group is interesting. As an innate cytokine involved in the defence against both viral and bacterial pathogens it would be expected to demonstrate a larger response in this group. However, we have not investigated IFN-α or IFN-β – the type I interferons which are important in the innate response to viral infection.

It may be the case that these type I interferons, or another cytokine or mediator we have not investigated, may be having the predominant effects.

Studies investigating the link between viral infection and increased susceptibility to bacterial infection have proposed various theories on the likely mechanisms. One particular study using a murine model found influenza virus to inhibit clearance of Streptococcus pneumoniae by decreasing tracheal mucociliary velocity – reduced clearance of mucus provides an ideal environment for pathogens[169]. Wang et al found that rhinovirus infection led to increased adhesion of Staphylococcus Aureus, Streptococcus pneumoniae and Haemophilus influenzae in primary human nasal epithelial cells, which was caused by increased production of fibronectin and other adhesion molecule receptors[170].

These mechanisms, as well as those proposed by Alcorn et al, should all be considered when interpreting the high levels of co-infection in this study. Further study, particularly on mechanisms involving co-infections with adenovirus and bocavirus, which have been shown in particularly high levels in this study, would be of interest.

6 Discussion

In this thesis we have described detailed demographic and clinical characteristics of children under the age of five in Recife, Brazil, with acute respiratory infection (ARI). We have investigated the causes of ARI by using multiplex polymerase chain reaction (PCR) to detect 17 different viral and atypical bacterial respiratory pathogens in nasopharyngeal aspirates (NPAs) from these children. In the final chapter we investigated cytokine profiles in NPAs from a selection of these children, looking at samples with differing severities, different pathogens and co-infection.

Using a questionnaire to ascertain details on the demographics of the study population we found several factors that suggest the population studied had a lower socio-economic status than in Brazil as a whole, with indicators such as family income, level of schooling and overcrowding. PCR results demonstrated a high level of PCR positive samples, high co-infection rates, and particularly high levels of adenovirus (AdV), human bocavirus (hBoV) and *Mycoplasma pneumoniae* (*Mpp*) infection. *Mpp* infection and human respiratory syncytial virus (hRSV) infection were associated with increased likelihood of admission to hospital. No other pathogens or co-infection were associated with severity.

Cytokine analysis of a selection of samples revealed similar cytokine concentrations across varying severities of hRSV infection, and with various pathogens.

6.1 Strengths of the study

This study has many strengths. The sample size of our study was large, including 407 participants enrolled over a 12 month period. Most similar studies have investigated study populations with a smaller sample size[1 10 13 15]. Those studies that have been larger have

often looked at all ages, including adults and children, rather than looking specifically at one age group[54 148].

All NPAs taken were collected by one research assistant, using a systematic and reproducible method, ensuring all samples were of as high quality as possible.

The questionnaire on demographic and clinical information was very thorough, and included a large number of questions covering a range of demographic and clinical topics. Each study participant was taken through the questionnaire with a research assistant, so that any queries could be answered, and so all questions were answered fully.

We investigated the presence of 17 different common viral and atypical bacterial respiratory pathogens. Previous studies using multiplex PCR have investigated between seven and 21 different pathogens. However, the studies investigating 20 and 21 pathogens, found positive rates of 47% and 68%, respectively, both figures lower than our 85.5%[45 54].

Our study included children with a whole spectrum of ARI disease severity, from very mild upper respiratory tract infections (URTI), which would include children with runny noses, to severe cases of bronchiolitis and pneumonia, requiring oxygen and in some cases ventilation and intensive care admission. Many other studies have focused on one particular group, whether it be inpatients or outpatients, or specific groups such as those at high risk of atopy[1 33 45 71].

6.2 Limitations

6.2.1 Demographics and clinical information

The questionnaire on demographic and clinical information was very thorough, with a vast number of topics being covered. However, upon analysing the results and comparing to

data in the literature, it was identified that although questions were asked related to the presence of smokers in the household, no specific question was asked about maternal smoking during pregnancy. Therefore associations between maternal smoking during pregnancy and severity of ARI could not be made from this study, as reported in the literature[171].

The accuracy of clinical diagnosis of each child's condition made by the attending physician upon discharge was one limitation of this study. Differentiating between bronchiolitis and pneumonia is particularly difficult in infants and young children. Information like this is always going to be a subjective assessment, and although it could have been made slightly more accurate using radiographs to investigate consolidation which may indicate pneumonia, studies have shown this to be a poor way of differentiating between pneumonia and bronchiolitis, with overlap between the signs shown in each condition[88]. However, despite being a limiting factor, it is representative of what happens in real clinical situations.

The nature of the study design - a prospective, cross-sectional study – gives a snap-shot of this particular population of children at this moment in time. Only those who were brought to hospital were included in the study, and although this has included a wide spectrum of conditions and severities, as demonstrated by the inclusion of very mild and severe cases, there will be a large proportion who did not attend hospital, or may have sought medical care elsewhere. This means that the results can only be applied to a hospital based population, and may not be representative of the population as a whole.

6.2.2 PCR Results

This study has successfully detected pathogens in 85.5% of NPAs, a higher percentage than has been previously reported in a similar population[13 45 46 71]. However this still leaves 14.5% of samples in which no pathogen was detected. A possible explanation for this is that this disease was caused by 'typical' bacteria. However, performing multiplex PCR looking for *S. pneumoniae* on NPA samples such as the ones collected in this study would have yielded inaccurate results, with previous studies finding over 60% of two year olds being asymptomatic carriers of the bacterium in their nasopharynx[35]. There would therefore be no way of determining if a positive result actually indicated that *S. pneumoniae* was responsible for the ARI. The collection of blood samples from each child, particularly to determine the full blood count (FBC), with differential white cell count, as well as the C-reactive protein (CRP), would have provided an insight into systemic response to infection, and may have indicated the likelihood of a bacterial infection. Unfortunately collecting blood samples was not included in the initial study design, and only a very small number of children in the study had blood taken as part of normal clinical protocols.

A further explanation of PCR negative samples within our study population would be the likelihood that there are as yet undiscovered respiratory pathogens, which may cause disease. Several new viruses have been discovered in recent years[60 62 63], and it is likely that more will be discovered in the future. The sensitivity and specificity of the PCR assays used may be another explanation for some of the PCR negative samples. Even though PCR has been shown to be the most sensitive and specific way to rapidly identify and detect respiratory pathogens, especially in comparison to more traditional methods, no method is going to be 100% sensitive and specific[172].

Tuberculosis (TB) was not looked for as part of this study protocol. The incidence of TB in Brazil was estimated by the World Health Organisation to be 45 per 100,000 population in 2009[173]. This is a further limitation to the study, as although the uptake of the BCG vaccine was very high, it is not 100% effective – it is estimated that it reduces the risk of TB by 50%[174]. With a high incidence of TB in the country, the possibility of TB in the study population cannot be discounted.

As mentioned in chapter 4, due to the sensitivity of PCR, and its ability to amplify DNA from both active pathogens, and remnants of old pathogens, we are unable to confidently say that the pathogens we have detected are the cause of the child's admission to the accident and emergency (A&E) department. A study using control samples to detect pathogens in asymptomatic children would help determine the likelihood of these pathogens being active, by giving base-line prevalence levels in the general population.

The only known study to date that has investigated control samples in a similar situation is that by van der Zalm et al in 2009[175]. They carried out a prospective, longitudinal study, following 18 healthy children, aged between zero and seven years, with the parents taking biweekly nasopharyngeal and oropharyngeal swabs both when the children were asymptomatic or when they had respiratory symptoms. The study used PCR to look for the presence of 13 pathogens. They found 56% of samples taken from symptomatic children to be positive for at least one virus, but they also found 40% of samples from asymptomatic children to be positive[175]. The most common viruses in asymptomatic children were found to be hRV (detected in 22%); coronaviruses (8%) and *Cpp* (5%). They also detected co-infection with multiple pathogens in 3% of samples[175]. AdV was not detected in any of the asymptomatic samples. These results are very interesting, with no other studies with similar data available. However, there are some limitations to this study. Firstly, they only

studied 18 children, and from these a total of 65 asymptomatic samples. Some samples will have come from the same children and the same latent pathogens may have been picked up multiple times from the same child. Also, the study only looked at a six month period, covering a only winter and beginning of spring seasons. A larger study investigating a control group over a longer period of time would be very interesting.

The statistics performed on the data only detected differences in severity due to the presence of *Mpp* infection and hRSV infection; and only detected differences in clinical diagnosis due to the presence of *Mpp* infection and hBoV infection. However, the sample size of the 'very mild' and 'severe' groups was much smaller than that of the 'mild' and 'moderate' groups, meaning the likelihood of a type 2 error, due to small sample size in those particular groups is high, and there may well be a difference that is not detectable at that particular power. The same can be said about the URTI clinical diagnosis group, again, with a sample size of just 23, is much smaller than the other diagnosis groups, and may be too small to detect a statistically significant difference.

6.2.3 Cytokine analysis

The main problem encountered with the cytokine analysis was the sample size in each group. Due to the very large numbers of samples containing co-infection, finding enough samples with one particular pathogen, and one particular pathogen with a particular severity for each group was very difficult. This, along with the heterogeneity of the study population, looking at such a variety of severities and clinical conditions, meant that comparisons between groups was very difficult, and was probably one of the reasons we found such a large level of variation of the results both between and within groups. However, as mentioned in section 5.4.1 and 5.4.2 other studies have found conflicting results, with some being similar to ours finding no association between cytokine

concentrations and severities of disease, and the ones that did find an association were ones looking at more specific groups such as a specific severity, age group or clinical condition[161-163].

6.3 Conclusions

In this study population of pre-school children who presented with ARI, we have found very high rates of pathogen detection and co-infection compared to previous studies. We also found particularly high rates of hBoV, AdV and *Mpp* infection, none of which have been described to the same extent in a similar study population.

Infection with *Mpp* or hRSV was associated with more severe disease. No other pathogens, co-infection, or inability to detect a pathogen were associated with particular disease severities.

Mpp detection peaked at the end of the rainy season, and was more likely to be associated with pneumonia. The high prevalence of *Mpp* infection has not previously been described in this age group, and may have implications for antibiotic use in this particular setting.

The high rates of pathogen detection may reflect the types of sample collected, the number of pathogens tested for, or the population demographic.

Cytokine profiles were found to be similar with varying severities of hRSV infection and with varying pathogens. This may be because the cytokines we investigated are all implicated in the broad response to respiratory infection. When taken with the results from previous chapters, in which most pathogens were not related with particular clinical manifestations or severities, it is not so surprising that particular pathogens did not elicit specific, discriminatory responses, just like they did not have specific clinical manifestations.

Differences in concentrations of some cytokines were found when comparing *Mpp* infection to bacteria/virus co-infection. However, samples sizes for all of the cytokine experiments were restricted due to the high level of co-infection in the study population, and further study is needed to enable more accurate conclusions to be drawn.

6.4 Further work

Completing this study has highlighted many avenues of work which could be followed to either expand the work that has already been completed, or to develop new studies.

Although we tested for 17 respiratory pathogens, we did not test for the common bacterial pathogens *Streptococcus pneumoniae* (*S. pneumoniae)* and *Haemophilus influenza* type b (Hib) or the less common *Legionella* or *Bordetella pertussis*. *S. pneumoniae* and Hib were discussed in detail earlier, and it would not be feasible to test for these due to the high levels of asymptomatic carriage in the nasopharynx. However, it would be possible to test for *Legionella* and *Bordetella pertussis*, which may add to the results of the study.

Another area of study which would add to the body of literature on ARI, would be to undertake a large study which included control samples. The only similar study that uses control samples is that by van der Zalm et al which was described, along with its limitations, in section 6.2.2[175]. A large prospective study, investigating the prevalence of pathogens in samples from children with no respiratory symptoms would be very useful, and may put some of our results into context in terms of the likelihood of the particular pathogens being causative in each case.

As previously mentioned, our cytokine work was restricted due to the high level of co-infection in the study population, meaning sample sizes for each group of single infections were small. In order to continue this work, more samples, with more single infections,

would be needed to increase sample size within each group. We also only looked at a limited number of cytokines, out of a whole range of known inflammatory mediators. It may be that the cytokines we investigated are all implicated in a broad response to respiratory infection, and to find a response that discriminates between pathogens, more specific mediators may need further investigation.

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Appendix 1: Reagents

Chemicals/Reagents	Company
Sample Collection/Processing	
2-mercaptoethanol	Sigma-Aldrich, Sao Paulo, Brazil
Normal Saline • **0.9% NaCl in H_2O**	Baxter, Sao Paulo, Brazil
RNA lysis buffer	Qiagen, Crawley, UK
RNA Extraction & PCR	
Qiasymphony Mini Kit Pathogen Complex 200 protocol	Qiagen, Crawley, UK
ALT Lysis Buffer	Qiagen, Crawley, UK
Invitrogen Superscript III Platinum One-Step Quantitative RT-PCR System	Invitrogen, Paisley, UK
Qiagen Quantitect Master Mix	Qiagen, Crawley, UK
Roche Probe Master Mix	Roche, West Sussex, UK
Protein Assay	
QuantiPro BCA Assay Kit: • **Reagent A** • **Reagent B** • **Reagent C**	Sigma-Aldrich, Dorset, UK
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Dorset, UK

Chemicals/Reagents	Company
Phosphate Buffered Saline • **One sachet was added to 1L distilled water. Each litre contained 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 at pH 7.2-7.4**	Sigma-Aldrich, Dorset, UK
Cytokine Analysis	
Human Cytokine 8-plex Assay, 1x 96 well: • **Coupled magnetic beads** • **Pre-mixed, lyophilized standard** • **Standard diluent** • **Detection antibody (10x)** • **Streptavidin-PE (100x)** • **Detection antibody diluents** • **Assay buffer** • **Wash buffer**	Bio-Rad Laboratories Inc., Hemel Hempstead, UK
Bio-Plex Human Cytokine Assay IL-17: • **Coupled magnetic beads** • **Detection antibody (10x)**	Bio-Rad Laboratories Inc., Hemel Hempstead, UK
Bio-Plex Human Serum Diluent	Bio-Rad Laboratories Inc., Hemel Hempstead, UK

Appendix 2: Equipment

Equipment	Company
Bio-Plex™ Pro Array 200 System	Bio-Rad Laboratories Inc., Hemel Hempstead, UK
Centrifuge – CR3i Multifunction	Thermo Scientific, Basingstoke, UK
Light Cycler 480 RT-PCR System	Roche, West Sussex, UK
Pipettes, single channel: 2-20µl, 20-200µl, 200-1000µl	Gilson Inc., WI, USA
Pipettes, multi-channel: 50-300µl	Thermo Scientific, Basingstoke, UK
Plate Reader ELx800 absorbance microplate reader	BioTek, UK
Plate Shaker – Titremax 1000	Heidolph, Essex, UK
QIASymphony	Qiagen, Crawley, UK
Vacuum Manifold	Bio-Rad Laboratories Inc., Hemel Hempstead, UK

Appendix 3: Questionnaire Details

Demographic Information	Clinical Information

• Housing:
  ○ Type
  ○ Size
  ○ Power supply
  ○ Water supply
  ○ Cooking fuel
  ○ Number of occupants
  ○ Number sharing a bedroom with the child
  ○ Smokers in the house
• Age
• Sex
• Weight
• Height
	• Family History:
	  ○ Parent/Guardian's age
	  ○ Parent/Guardian's education
	  ○ Parent/Guardian's occupation
	  ○ Number of siblings
	  ○ Household income
	• Birth History:
	  ○ Gestation
	  ○ Birth weight
	• Past medical history:
	  ○ Any previous admissions
	  ○ Allergies
	  ○ Any previous illnesses
	  ○ Any previous respiratory symptoms
	• Co-morbidities
	• Vaccinations
	• Breastfeeding
	• Need for admission
	• Discharge date/length of admission
	• Discharge status
	• Presenting signs & symptoms:
	  ○ Coryza
	  ○ Cough
	  ○ Difficulty breathing
	  ○ Wheeze
	  ○ Apnoea
	  ○ Cyanosis
	  ○ Grunting
	  ○ Fever
	  ○ Ear ache
	  ○ Feeding difficulties
	  ○ Lethargy
	  ○ Heart rate
	  ○ Respiratory rate
	  ○ Oxygen saturations
	  ○ Chest indrawing
	  ○ Temperature

Appendix 4: PCR Cycling Conditions

	Temperature (°C)	Duration	No. of cycles
Flu, hMPV, hRSV; CoV's; PIV's, hRV	50	20 minutes	1
	95	2 minutes	1
	95	10 seconds	50
	58	45 seconds	
	72	1 second	
	40	30 seconds	1
AdV, *Mpp*, *Cpp*	95	5 minutes	1
	95	10 seconds	50
	95	10 seconds	
	58	45 seconds	
	72	1 second	
hBoV	95	15 minutes	1
	95	10 seconds	50
	58	45 seconds	
	72	1 second	

Appendix 5: Primer Table

Assay	Oligonucleotide	Sequence (5' to 3')	Concentration (µM)	Citation
Flu 6-plex	hMPV 383 Fwd	ACAAAGARGCAAGAAAAACAATGG	0.4	[145]
	hMPV 451 Rev	GGTGTGTCTGGTGCTGArGG	0.4	
	hMPV 424A	(FAM)–TCATCAGGyAATATy**C**CA**C**AAAATCAGAG –(BHQ1)	0.2	
	hMPV 424B	(FAM)–TCATCAGGTAACATC**C**CA**C**AAAACCAGAG –(BHQ1)	0.1	
	hRSV Fwd	GCAAATATGGAAACATACGTGAACA	0.4	
	hRSV Rev	GCACCCATATTGTWAGTGATGCA	0.4	
	hRSV LC610	(LC610)–CTTCACGAAGGCTCCACATACACAGCWG –(BHQ2)	0.1	
	Flu B Fwd	AAATACGGTGGATTAAATAAAAGCAA	0.4	
	Flu B Rev	CCAGCAATAGCTCCGAAGAAA	0.4	
	Flu B Cyan 500	(Cyan500)–CACCCATATTGGGCAATTTCCTATGGC –(BHQ1)	0.1	
	Flu A Fwd	AAGACCAATCCTGTCACCTCTGA	0.4	
	Flu A Rev	CAAAGCGTCTACGCTGCAGTCC	0.4	
	Flu A VIC	(VIC)– TTTGTGTTCACGCTCACCGT –(MGB-NFQ)	0.1	
	H1 Fwd	ATTGCCGGTTTCATTGAAGG	0.4	
	H1 Rev	ATGGCATTYTGTGTGCTYTT	0.4	
	Swine H1 LC640	(LC640)–ATGAGCAGGGGTCAGGATATGCAGCCGACC –(BHQ2)	0.1	
	Human H1 LC670	(LC670)–ATGAGCAAGGATCTGGCTATGCTGCAGATC –(BHQ2)	0.1	
PIV 6-plex	PIV1 HN525 Fwd	GATTTCTGGAGATGTCCCGTAGG	0.4	[145]
	PIV1 HN722 Rev	TGACTTCCCTATATCTGCACATCC	0.4	
	PIV1 HN556	(FAM)– TACTGAGCAACAACCC –(MGB-NFQ)	0.16	
	PIV2 Fwd	CCATTTACCTAAGTGATGGAA	0.4	
	PIV2 Rev	CGTGGCATAATCTTCTTTT	0.4	
	PIV2 LC640	(LC640)–AATCGCAAAAGCTGTTCAGTCAC –(BHQ2)	0.16	
	PIV3 NP300 Fwd	CTTTCAGACAAGATGGAACAGTGC	0.4	
	PIV3 NP800 Rev	AGTTACCAAGCTCTGTTGAGACC	0.4	

Assay	Oligonucleotide	Sequence (5' to 3')	Concentration (μM)	Citation
	PIV NP766	(LC610)– CCAATCTGATCCACTGTGTCACCG CTCA –(BHQ2)	0.16	
	PIV4 NP271 Fwd	CAGGCCACATCAATGCAGAATC	0.4	
	PIV4 NP407 Rev	ATGTCATCCCAGCCAGATCTTG	0.4	
	PIV4 NP298	(LC670)– ATGATTGCTGCCAGAGCCCCAGAT GC –(BHQ2)	0.16	
	hRV Fwd	TGG ACA GGG TGT GAA GAG C	0.4	
	hRV Rev	CAA AGT AGT CGG TCC CAT CC	0.4	
	hRV HEX	(VIC)– TCC TCC GGC CCC TGA ATG –(BHQ1)	0.16	
	MS2 F1	TGG CAC TAC CCC TCT CCG TAT TCA CG	0.2	
	MS2 R1	GTA CGG GCG ACC CCA CGA TGA C	0.2	
	MS2 Cyan 500	(Cyan500)– CACATCGATAGATCAAGGTGCCTA CAAGC–(BHQ1)	0.08	
CoV 4-plex	OC43 Fwd	CGATGAGGCTATTCCGACTAGGT	0.4	[146]
	OC43 Rev	CCTTCCTAGCCTTCAATATAGTAAC C	0.4	
	OC43	(Cyan 500) TCCGCCTGGCACGGTACTCCCT (BHQ1)	0.16	
	NL63 Fwd	ACGTACTTCTATTATGAAGCATGAT ATTAA	0.4	
	NL63 Rev	AGCAGATCTAATGTTATACTTAAAA CTACG	0.4	
	NL63	(FAM) ATTGCCAAGGCTCCTAAACGTACA GGTGTT (BHQ1)	0.16	
	229E Fwd	CAGTCAAATGGGCTGATGCA	0.4	
	229E Rev	AAAGGGCTATAAAGAGAATAAGGT ATCT	0.4	
	229E	(HEX) CCCTGACGACCACGTTGTGGTTCA (BHQ1)	0.16	
	HKU1 Fwd	TTACTTCCACACTTTTCATCTCTCT G	0.4	
	HKU1 Rev	CGGAAGCAGCAACGAAATTC	0.4	
	HKU1	(LC640) CGCCCACTTGAAGCCGAGACCG (BHQ2)	0.16	
CMA 3-plex	Cpp Fwd	CAAGGGCTATAAAGGCGTTGCT	0.2	[148]
	Cpp Rev	ATGGTCGCAGACTTTGTTCCA	0.2	
	Cpp	(LC670) TCCCCTTGCCAACAGACGCTGG	0.1	

Assay	Oligonucleotide	Sequence (5' to 3')	Concentration (µM)	Citation
		(BHQ2)		
	Mpp Fwd	GGAATCCCAATGCACAAGAACA	0.4	
	Mpp Rev	GCTTTGGTCAACACATCAACCTT	0.4	
	Mpp	(LC610) GCCTTGAAGGCTGGGTTTGCGCTA (BHQ2)	0.1	
	Adv Fwd	GCCACGGTGGGGTTTCTAAACTT	0.4	[147]
	Adv Rev	GCCCCAGTGGTCTTACATGCACATC	0.4	
	Adv	(FAM) TGCACCAGACCCGGGCTCAGGTACTCCGA (BHQ1)	0.2	
HBoV 2-plex	hBoV NS Fwd	CTTGGGCGGGACAGAATGC	0.4	
	hBoV NS Rev	AACAGAATTGCCACCAACAACC	0.4	
	hBoV NS	(FAM) TCAAGCATAGAGACAGT (MGB)	0.2	
	hBoV NP Fwd	GCTCGGGCTCATATCATCAGG	0.4	
	hBoV NP Rev	CTCCCTCGTCTTCATCACTTGG	0.4	
	hBoV NP	(VIC) AATCAGCCACCTATC (MGB)	0.2	