The viral and atypical bacterial causes of acute respiratory infection in children in Recife, Brazil

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy (MPhil) by

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Abstract

Introduction:
Acute respiratory infections (ARI) are the commonest cause of mortality in children <5 years worldwide with viral pathogens being important. The aims of MPhil year were threefold:
1) To describe the epidemiology of viral pathogens in ARI of children <5 presenting to hospital in Brazil during the emergence of novel H1N1 pandemic
2) To characterise clinical and epidemiological features of ARI in the novel group of rhinoviruses (hRV), hRV-C
3) To investigate the interaction between interleukin (IL)-17 and IL-13 in RSV infection of airway epithelial cells.

Methods:
1) Multiplex PCR was utilised to test for viral (including H1N1) and atypical bacterial respiratory pathogens in nasopharyngeal aspirates from children <5 presenting with ARI to IMIP Children's Hospital in Recife, Brazil.
2) In samples in which hRV was detected, cDNA was amplified and sequenced to compare to traditional and novel hRV strains.
3) RSV-infected and non-infected bronchial epithelial cell (BEAS-2B) cultures were simulated with IL-17, IL-13 and IL-17+IL-13. IL-6 and IL-8 were measured by ELISA in culture supernatants.

Results:
1) A pathogen was identified in 88% of the 630 children recruited to this study. The most commonly detected pathogens were RSV, adenovirus, bocavirus, and hRV (33%, 29%, 24% and 19%). Co-detection occurred in 43% of samples. Influenza prevalence increased in the second year (3% vs. 15%) because of the H1N1 pandemic. Children in whom H1N1 was detected were more likely to be admitted to hospital and to be co-infected.
2) hRV was detected in 19% of ARI. We found no clinical or demographic differences between hRV ARI and non-hRV ARI. hRV ARI was more likely to present as a co-infection. The majority (84%, n=99) of samples were successfully analysed with hRV-A being the most common subtype (71%) and all remaining samples being hRV-C (21%). Children in whom hRV-C was detected were more likely to present with the diagnosis of episodic viral wheeze (EVW) / asthma.
3) In the presence of IL-17 significantly elevated IL-6 and IL-8 expression was observed at 48 hours and 24 hours respectively when BEAS-2Bs were infected with RSV. IL-13 caused no increase in IL-6 or IL-8 expression. There was no demonstrable synergy between IL-17 and IL-13.

Discussion:
We were able to detect a viral or atypical bacterial pathogen in most of our cohort. We highlighted the importance of the emerging pathogen H1N1, and the prevalence of co-infection. hRV was shown to be an important cause of ARI and novel hRV-C was associated with EVW/asthma. IL-17 was shown to stimulate inflammatory cytokine production, particularly during RSV infection. These findings highlight IL-17 as a potentially important cytokine in airway inflammation in RSV disease.

Abbreviations

ARI – Acute Respiratory Infection

WHO – World Health Organisation

URTI – Upper Respiratory Tract Infection

LRTI – Lower Respiratory Tract Infection

NPA – Nasopharyngeal aspirate

Hib – *Haemophilus Influenzae*

RSV – Respiratory syncytial virus

hMPV – human metapneumovirus

AdV – Adenovirus

PiV – Parainfluenza virus

hRV – human Rhinovirus

CoV – Coronavirus

hBoV – human bocavirus

Mpp – Mycoplasma pneumoniae

Cpp – Chlamydophila pneumoniae

IL – Interleukin

(RT-)PCR – (Reverse transcription-) polymerase chain reaction

ITU – Intensive treatment unit

EVW – Episodic Viral Wheeze

RNA – Ribonucleic acid

DNA – Deoxyribonucleic acid

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1 Introduction

1.1 Definition

The focus of this project is paediatric acute respiratory infection (ARI). There are a variety of definitions available for what constitutes ARI although all relate to the clinical manifestation of an infection of the airways. The World Health Organisation (WHO) defines ARI as a "presumed pneumonia"[1], concentrating mainly on infection of the lower respiratory tract and producing guidelines for clinical diagnosis of pneumonia in absence of radiology.

Simoes et al describes ARI as infection of the upper and/or lower respiratory tract, defining these as:

"The upper respiratory tract consists of the airways from the nostrils to the vocal cords in the larynx, including the paranasal sinuses and middle ear. The lower respiratory tract covers the continuation of the airways from the trachea and bronchi to the bronchioles and the alveoli"[2].

In the literature, ARI is also often defined by clinical parameters. For example, Regamey et al defined ARI as "...more than two days with cough or wheeze, together with fever >38°C, acute rhinitis, otitis media or pharyngitis"[3].

For the purpose of this project the definition of ARI will be in keeping with that described by Simoes et al with ARI constituting any infection of the respiratory tract, both upper and/or lower. We have chosen this definition so we can investigate nasopharyngeal responses to infection and determine whether they differ in upper and lower respiratory tract infections.

1.2 Clinical manifestations of ARI:

Classification of ARI can be further subdivided based on anatomy and clinical manifestation. Thus upper respiratory tract infections are infections which occur proximal to the entrance of the airways i.e. from the nostrils and mouth to the trachea, and including the paranasal sinuses and the middle ear. The lower respiratory tract is any point past the trachea to the terminal end of the respiratory tract at the level of the alveoli.[2]

In reality many ARIs involve more than one anatomical location, especially in the upper respiratory tract. The further classifications and diagnoses are described as follows.

1.2.1 Upper Respiratory Tract Infections

Infection above the level of the trachea is termed an Upper Respiratory Tract Infection (URTI). Usually these infections are mild but can progress to the lower respiratory tract or cause long term complications. This group can be further subdivided by clinical diagnosis which includes rhinitis, sinusitis, ear infections, acute pharyngitis/tonsillopharyngitis, epiglottitis and laryngitis/ croup[2]. Often a number of these clinical diagnoses are present at any one time.

Our study will classify all infections of the upper respiratory tract as an "upper respiratory tract infection", for instance conditions such as coryza, earache, sore throat or stridor in the absence of lower respiratory tract signs/symptoms. Upper respiratory tract infections/conditions include:

- Rhinitis: Allergic rhinitis is caused by nasal mucosal inflammation in response to an allergen[4 5]. It affects 60 million people in the USA, especially children (estimated cost $11.2billion/year) [6 7 8]. Symptoms include nasal congestion, post-nasal drip, sneezing, watery eyes, ear plugging and frontal headaches[7]

- Sinusitis: The mucosal lining of the paranasal sinuses is inflamed in 87% of URTIs[9 10]. They often resolve with the respiratory infection but can persist as a complication -"acute paranasal sinusitis" [11 12]. Presentation is similar to rhinitis and usually resolves without treatment but represents a large burden on a population - 20 million cases per year in the USA[11].

- Ear infection: In infection of the inner ear ("otitis media") both viral and bacterial pathogens are causative [13-15]. Treatment is debated, antibiotics are often given[16]. Otitis media is estimated to cost $2.8 billion annually in the USA[16]. 70% of children have experienced at least one episode by two years old[14]. Possible complications are chronic otitis media, hearing impairment, mastoiditis, septicaemia, meningitis, formation of abscess and mortality[13]. In developing countries complication rates are higher[13].

- Acute pharyngitis / tonsillopharyngitis: Inflammation of the tonsils and pharynx is common, over 50% are of viral origin[17]. Signs and symptoms include fever, painful throat, reddening of the tonsils and pharynx,

tonsillar exudates, enlarged and tender cervical lymph nodes, pain when swallowing (dysphagia) and headache. Suggestive features of bacterial infection form the “Centor criteria” and include tonsillar exudates, tender cervical lymphadenopathy, fever and absence of cough[18 19]. Bacterial causes respond well to antibiotics.

- Epiglottitis: Epiglottitis can acutely obstruct the trachea and is a life threatening emergency, 88% of cases are in those <5 years[20 21]. Treatment involves oxygen supplementation, intubation, mechanical ventilation and high dose antibiotics[21]. Estimated mortality is 3.6%, however it is now a rare condition in the developed world due to vaccination against *Haemophilus influenzae b* (Hib) [20 22]. In October 1992 (UK) the Hib vaccine was introduced to all children at 2, 3 and 4 months with uptake of 89-96%[23]. Annual attack rates in children under 5 have fallen from 30.9 per 100,000 in 1991/2 to 2 per 100,000 in 1993/4 in the UK, with 96% reduction in other populations[24 25 26].

- Laryngitis / croup: Spasmodic croup and acute laryngotracheitis are caused by viral pathogens especially parainfluenza(PIV) viruses, and less commonly influenza, respiratory syncytial virus (RSV), adenovirus (AdV) and rhinoviruses(hRVs)[27 28]. Clinical presentation is inspiratory stridor, “barking” cough, hoarseness and breathlessness[29]. Severe signs include abdominal movement on respiration, chest wall in-drawing, fatigue, hypoxia and respiratory failure[29]. 85% of cases are mild and fewer than 1% present with severe symptoms[30]. Mortality is low, estimated at 1 in 30,000 cases, prognosis is excellent [27 29]. Treatment has been debated but recently a consensus has been

reached; humidified air confers no benefit, mild and moderate patients should be given dexamethasone and severe obstruction requires adrenaline[29].

1.2.2 Lower Respiratory Tract Infections

Any infection below the level of the trachea is classified as a lower respiratory tract infection (LRTI). The main diagnoses are bronchiolitis, pneumonia and viral induced wheeze/asthma. In general a LRTI is more severe than URTI with increased likelihood of mortality. For this reason much of the focus of international agencies is in prevention of lower respiratory infections, especially pneumonia. As mentioned previously it is the scope of this study to look at both upper and lower respiratory infection. More details on the syndromes constituting LRTIs are as follows:

1.2.2.1 Bronchiolitis:

Bronchiolitis describes “an acute respiratory illness that affects infants and young children with coryza and low-grade fever that progresses over a few days to cough, tachypnoea, hyperinflation, chest retraction and widespread crackles, wheezes or both”[31]. Bronchiolitis is a common clinical syndrome in young infants with hospital admission rates of 30 per 1000 for children younger than 1 year[31]. This condition appears almost exclusively in children less than 1 year old, with the majority treated with supportive therapy. Mortality rates are

currently less that 2 per 100,000 in developed countries (UK)[32]. The mortality due to bronchiolitis has been higher in the past however, in the late 1970s the rate in UK was 21 per 100,000[32].

The most common causative pathogen of bronchiolitis is human respiratory syncytial virus (RSV). RSV is detected in 42%-75% of children presenting with bronchiolitis[33-35]. Other pathogens have also been detected such as AdV, influenza, PiV, hRV, hMPV, coronavirus and human bocavirus[33]. Cases present in a clear seasonal pattern with yearly RSV epidemics in temperate climates. In the UK this period is in the winter with presentations of bronchiolitis peaking between December and January[31].

Given the clear epidemic peaks and typical clinical signs of bronchiolitis, extensive investigations are rarely necessary other than confirmation of RSV status by immunofluoresence. Chest radiography demonstrates non-specific signs of generalised hyperinflation and patchy atelectasis and can sometimes be important in excluding pneumonia[31]. Clinical signs of a child with bronchiolitis include wheeze, tachypnoea, fever, hypoxia, cyanosis and crackles on auscultation, with crackles and cyanosis being associated with increased severity[31]. Risk factors for increased severity include prematurity, chronic lung disease, congenital heart disease, neuro-disability or immunodeficiency[31]. In some of these high risk groups, use of the monoclonal antibody palivizumab has been sanctioned, but it is limited due to excessive costs of $5000-$6000 per patient per season[31].

Treatment of bronchiolitis is a subject of ongoing debate within the literature. Bronchodilators have been previously used however a Cochrane Review showed they have no effect on length of hospitalisation, length of

symptom resolution or reduction in hospital admission[31]. Other treatment modalities have also been used with debatable effect such as nebulised epinephrine, dexamethasone and heliox. The most effective intervention is supportive care with fluid replacement, correction of hypoxia with oxygen and other supportive measures, the majority will recover without event[31]. Bronchiolitis is associated with a number of rare complications that are important due to severity including encephalopathy and septicaemia[31 36]. Also importantly in bronchiolitis are the long term effects on respiratory physiology and its links with recurrent wheeze and asthma in later life. Sigurs et al demonstrated that bronchiolitis in infancy was associated with recurrent wheeze/asthma at 7 and 13 years of age[37 38]. However it is still unclear whether this relationship is causative or if a child who is predetermined to have later asthma may have increased risk of bronchiolitis [39]. Current areas of ongoing research in bronchiolitis are into the immunological profile of affected children. It is hoped that this will characterise the pathology behind this common clinical condition[40]. Other areas of research include the identification of clinical, demographic or laboratory detectable risk factors to allow risk stratification of patients presenting with bronchiolitis and may reduce the number of children progressing to severe disease[41].

Comparatively less is known about bronchiolitis in the developing world. A small number of studies have carried out research in Brazil and South America which have suggested a seasonal peak in bronchiolitis during the rainy season and is supported by studies into RSV prevalence[42-44]. The detection of RSV simultaneously with other respiratory viruses has also been shown[42]. Some studies have also reported bronchiolitis obliterans, an

especially severe form of bronchiolitis, to be more prevalent in Brazil[45]. This has highlighted bronchiolitis as an important area of research in South America. In South Africa it has also been established that RSV is the main causative agent of bronchiolitis but does not have such distinct seasonality as Europe and North America, in Cape Town epidemics have been demonstrated both in the rainy season and also in the winter[46]. In Kenya RSV infection was identified in 27% of severe paediatric pneumonia[47].

In this study the diagnosis of bronchiolitis has been defined as a child <18months in whom upper respiratory symptoms preceded lower respiratory symptoms of wheeze, tachypnoea and signs of respiratory distress.

1.2.2.2 Pneumonia:

Of all clinical presentations of ARI, pneumonia is most associated with mortality. Pneumonia related deaths worldwide in children are estimated to be in the region of 1.9 million, not including neonatal mortality[48]. Many pneumonia deaths are in malaria prevalent regions where pneumonia mortality is frequently misclassified as malaria[48]. The WHO reports that pneumonia is the predominant cause of ARI mortality[49]. Pneumonia is highlighted as one of the primary targets to reduce worldwide mortality in children under five as part of the Millennium Development Goal (MDG)[48]. Overall incidence of pneumonia worldwide is estimated to be 151 million cases per year, of which 7-13% require hospital admission[50]. The majority of pneumonia burden is centred around developing countries with 72% of mortality in children from Africa and

Asia[1]. It is estimated half of these deaths could be prevented with vaccination and even more could be treated with inexpensive antibiotics, highlighting the necessity to confront this condition globally[51]

Clinically URTI often precedes pneumonia. Symptoms of pneumonia include fever, rigors, malaise, cough and dyspnoea[52]. The cough can be productive of sputum of a purulent colour. Clinical signs include dyspnoea, chest in-drawing, dullness to chest percussion, crackles and/or wheeze on auscultation[53]. Very severe cases may present with convulsions or coma, organ failure may be present. In contrast to bronchiolitis, chest radiography is important with the WHO producing guidelines on how to diagnose pneumonia by radiography[54]. Signs on radiography include consolidation, infiltrates, effusion, atelectasis and peribronchial thickening[54]. Resource poor countries often do not have access to radiography and so the WHO has also defined severe pneumonia clinically as the presence of lower chest wall collapse/chest in-drawing and treatment requiring hospital admission[53].

Treatment is with appropriate antibiotics and simple supportive care. Initial treatment is with cotrimoxazole or amoxicillin either orally in mild disease or intravenously if severe[53]. Supportive measures are required, these include oxygen supplementation, anti-pyretics, adequate nutrition and supplementation of feeds[52]. Simple treatments delivered properly can reduce pneumonia mortality in children by 10%, and would only cost a few dollars a child allowing widespread use in settings such as Africa or Asia[48]. Risk factors for more severe disease include malnutrition, HIV or other immunosuppressive disease, household smoking and cessation of breastfeeding before 6 months of age[48].

Vaccination is an effective preventative treatment. Vaccines for the most common bacterial agents are already available including *H. Influenzae* type b (Hib) and *Streptococcus pneumoniae* (pneumococcus). These pathogens are discussed in more detail elsewhere, but account for the majority of bacterial causes of pneumonia. There are two differing types of vaccination available for pneumococcus and Hib – conjugate and polysaccharide. The polysaccharide vaccine stimulates an antibody response to specific serotypes of pathogen, however a T-cell response is not caused and so vaccine protection is short with effectiveness of pneumococcus being reduced from 100% to 8% in 3 years[55]. In comparison the more recent conjugate vaccines function by coupling the bacterial polysaccharide to a protein carrier, inducing a T and B cell response which leads to increased longevity and a "booster" effect when re-exposed to the antigen[55]

The Hib polysaccharide vaccine reduces the incidence of childhood pneumonia by 20% in a developing country and has been available since the 1980s[48]. The newer Hib conjugate vaccine is estimated to reduce invasive Hib disease by 80%, it is often combined one or more of; hepatitis B, diphtheria-tetanus-pertussis, and/or inactivated polio vaccines[56]. The pneumococcal conjugate vaccine was developed more recently and there are a number of forms. The vaccine for seven pneumococcal serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) accounts for 80% of pneumococcal infections in children and was first introduced in 2000[57]. Other vaccines with different serotypes have also been developed. Conjugate vaccines are also more effective at eliminating carriage of pneumococcus and Hib, increasing the value of vaccination on a population scale, a phenomenon termed herd immunity[58]. In

the USA where vaccination levels are high the 7-valent vaccine reduced infant invasive pneumococcal disease by 82%, although a large degree of this is generated by herd immunity due to high vaccination uptake[57]. Interestingly nasopharyngeal pneumococcal carriage in children remains constant after vaccination with other serotypes being present[53 59]. Despite this vaccination is effective and is prioritised by the world health authorities to achieve the MDG[60]. Another common area of research is characterisation of pathogens involved in causation of pneumonia. Previous studies into epidemiology have not been able to identify a causative pathogen in all children and new methods. This is important to our study looking at causation of ARI, including pneumonia.

In Brazil and South America pneumonia burden is also significant. In Brazil LRTIs, of which the majority are pneumonia, cause 1.4 deaths per 1000 live births in those <5 years, greater than both diarrhoeal disease (0.8 per 1000) and accidents or injuries (1.13/1000)[45]. This has caused pneumonia to be one of the main targets in the family health programme (FHP) of Brazil[45]. Much of the FHP is involved in improving protective factors for ARI such as low nutrition and breastfeeding, as well as targeting early case diagnosis and treatment of pneumonia and other LRTIs[61]. Similar to other countries, *S. pneumoniae* is a well recognised cause of pneumonia in Brazil, other respiratory pathogens, especially viruses, are less researched[62].

In our study pneumonia will be a clinical diagnosis defined as a child with fever, tachypnoea and respiratory distress where focal or diffuse crackles or decreased vesicular sounds are present on auscultation. Our classification

is based on clinical diagnosis as radiographic investigations are not available for all children in our study population.

1.2.2.3 Episodic viral wheeze / Asthma:

Wheeze is caused by reduction in airway cross section of airway or increase in airway compliance[63]. One third of children in the UK present to a healthcare professional with a wheezing episode by 3 years of age[64]. There is clinical overlap between viral induced wheeze of infancy and childhood asthma. Wheeze is the predominant clinical sign in both and in the majority of cases there is preceding URTI before wheeze appears 1-2 days later. One third of the children with episodic viral wheeze as a young child will go on to develop atopic asthma[64]. Overlap is also seen between episodic viral wheeze and bronchiolitis, with 75% of children admitted to hospital with acute viral bronchiolitis in the first 4 months of life going on to develop subsequent episodic wheeze with viral respiratory infection[63]. Episodic viral wheeze is a series of discreet episodes of respiratory distress characterised by wheeze whereas asthma later develops into a chronic disease with exacerbations of sudden deterioration in airway function, day-to-day variation in airway function and fixed or persistent airway obstruction[63].

Risk factors for development of asthma and episodic viral wheeze include maternal smoking in pregnancy, history of maternal wheeze, prematurity and reduced lung function in the neonatal period[63]. Treatment is an area of debate in which research is ongoing. Bronchodilators and

corticosteroids have been the mainstay of asthma treatment for many years. These medications have been highlighted for use in episodic viral wheeze with Cochrane reviews having been performed on both medications. The review for bronchodilators concluded there was no clear benefit in recurrent wheeze for children under 2 although some trials included both children who did and did not go on to have asthma later in life[65]. A separate Cochrane review was also performed for children under 17 years with episodic viral wheeze and the effect of inhaled corticosteroids which concluded high dose corticosteroids were effective in the treatment of acute viral induced wheeze and reduced the duration of wheeze, however there was no evidence that low dose corticosteroids reduce the effect of episodic viral wheeze, a treatment that has proven beneficial in children with asthma[66]. Both of these Cochrane reviews advocated further research into episodic viral wheeze treatment.

The burden of episodic viral wheeze / asthma in young children is significant with 26% of children under the age of five receiving inhaled therapy for wheezing disorder and a conservative estimate of cost being £91 million ($145 million) in the UK in 2000[63]. Few population based studies into asthma have been undertaken in Brazil, although the Brazilian centres involved in the international study of asthma and allergy (ISAAC) reported a prevalence of 5-22% in children[67]. One of the few studies focusing on Brazil reported an asthma prevalence of 18% in children aged 4 years as well as a EVW prevalence at 21%[68]. This high prevalence is reflected across many countries in South America with asthma / EVW representing a significant burden on paediatric disease[69].

Research is ongoing into management of childhood wheeze. Current themes in literature investigate the immunology involved of wheeze, the aim being to develop targeted treatments or allow detection of children that will go on to develop asthma. Work is also ongoing to further stratify infants suffering wheeze with hope that early detection will allow more targeted treatment and opportunistic prevention and also improve predictions of long term prognosis[70].

All the factors mentioned previously make it difficult to diagnose asthma in a child under five with certainty. Our study will include children under the age of five years who will be given the clinical diagnosis “episodic viral wheeze (EVW) / asthma” and this will be defined as a child in whom discreet episodes of wheeze occurred, often in association with viral URTI.

1.3 Causative pathogens of Acute Respiratory Infections

There are a variety of respiratory pathogens which can lead to a patient suffering from ARI, both viral and bacterial. In some cases no known cause can be found in a patient presenting with ARI. Although some causative agents have already been alluded to when describing the clinical presentation of ARI this section looks to highlight the causative pathogens of ARI.

Our study will focus on the viral epidemiology of ARI. A variety of bacteria have been implicated in ARI previously. In the past bacteria were much more susceptible to culturing techniques allowing research to be performed. Viral pathogens are more difficult to detect and culture leading to late discovery of pathogens. This is also reflected by developments in

preventative treatment of pathogens with vaccines being available for bacterial pathogens. Bacterial pathogens that are frequently discovered in childhood ARI will be described below but may not be mentioned again in this study due to its viral focus.

1.3.1 Respiratory Syncytial Virus:

Human Respiratory Syncytial Virus (RSV) is a member of the paramyxovirus family[71]. The single stranded RNA genome is contained within a lipid envelope with surface glycoproteins[31]. There are two major strains of RSV, A and B. Some studies have reported A strain being responsible for more severe bronchiolitis[71]. RSV is one of the most highly prevalent pathogens in childhood respiratory disease and can present as bronchiolitis, pneumonia, otitis media, rhinitis or sinusitis[72]. The most common presentation is bronchiolitis, being detected in over 70% of children hospitalised with bronchiolitis[31 33]. RSV is highly seasonal presenting in epidemics, these periods are well characterised in the northern hemisphere where they correlate with winter[71 72]. 80% of infants have been infected with RSV by the end of the first year of life, and 100% by the end of second[31 71]. Majority of infections are mild but mortality does rarely occur, in 2% of healthy infected children or as high as 5% in children with risk factors[71]. The importance of RSV in bronchiolitis has also been highlighted in Brazil with a suggested epidemic in the rainy season[43]. Although this is not as well defined as other countries,

Washburne et al observed RSV circulating in Brazilian communities throughout the year[73]. Both subtypes of RSV have been shown to circulate simultaneously in Brazil[44].

Risk factors for increased severity include prematurity (associated with ten-fold increase in risk), congenital heart disease, immunosupression and neurodisability[71]. Environmental factors can increase risk of RSV infection including passive cigarette smoking, crowded living conditions, older siblings at home, early cessation of breastfeeding and low socioeconomic status[72]. Another consideration that increases RSV burden is long term outlook for patients who suffer RSV bronchiolitis at a young age. Sly et al has showed that if a child has RSV LRTI before 3 years of age then lower lung function and airway hyper responsiveness can be demonstrated at 13 years old[74]. The interpretation of these findings is still debated whether RSV causes late asthma or whether RSV infection is an early sign of a child's predisposition to asthma[31].

The immune response to RSV is atypical compared to other respiratory viruses. Symptoms begin 3-5 days after inoculation. It is highly contagious virus that can be transmitted through direct contact with respiratory secretions and indirect inoculation from contaminated surfaces[71]. Once infected the immune system activates T-helper cells that react with expression of cytokines. Some research has shown that the immune response to RSV is consistent with T helper-2 (Th-2) class of T-helper cell activation such as interleukin-4 (IL-4) and interleukin-5 (IL-5) and interlukin-13 (IL-13)[31 75]. Although a Th-2 response is witnessed in other respiratory viruses, such as Adenovirus, the majority of other viral infections are normally associated with a T helper-1 (Th-1) response

characterised by cytokines such as interferon gamma (IFN-γ)[76] [31]. It is of interest that the Th-2 class of cells are also closely associated with patients suffering asthma or atopy in later life. However this opinion has been disputed by others with some suggesting Th-1 cells and the cytokine IFN-γ plays an important role in pathogenesis of RSV bronchiolitis[77] [78]. Further groups have suggested that cytokines outside the Th-1/Th-2 classification are important, so called "beta cytokines"[79] [80].

A second distinguishing feature of immune response to RSV is the ability to re-infect, with the same strain being able to infect the same host time after time. This is of interest as the immune memory response to viruses usually prevents re-infection. The exact reason for this is unknown, it has been hypothesised that RSV inhibits CD8+ T cells and so the immune systems memory response is ineffectual[81]. This explanation is debated however with Chang et al suggesting this feature is not specific to RSV and so may not give adequate explanation[82].

An RSV specific humanised monoclonal antibody (palivizumab) was developed a decade ago. Its development was a breakthrough in RSV prevention and it is effective in reducing episodes of bronchiolitis. The vaccine however is costly. Thus palivizumab is only used in children who are at high risk of severe RSV disease – those born before 32 weeks gestation, or with significant cyanotic heart disease or chronic lung disease[31] [71].

1.3.2 Adenovirus:

Adenoviruses (AdV) are non-enveloped DNA viruses of the family *Adenoviridae* first discovered in 1953[83]. They most commonly present as respiratory infections but can cause conjunctivitis and gastroenteritis[84]. They are responsible for 5-10% of respiratory tract infections in children[83 85]. In 2010 there were 53 know subtypes of AdV although this is expected to rise[85]. Specific serotypes are associated with different clinical manifestations. Common subtypes involved with respiratory infection include HAdv-3, HAdv-7, HAdv-1, HAdv-2, HAdv-5 and HAdv-4[85]. The prevalence of different serotypes is known to vary geographically and seasonally, with AdVs often appearing as endemics especially in overcrowded spaces[84].

AdV infection commonly presents as a LRTI, detected in 73% of cases of pneumonia in Korea and 65% in the USA[85 86]. It is estimated 5% of children presenting to hospital with ARI and AdV infection will result in mortality, with risk factors including age less than 1 year or presence of co-morbidities[85]. Specific subtypes are associated with severe respiratory infection in children, the most common being HAdv-7[85 87]. In contrast to RSV, AdV infections present throughout the year with no clear seasonal variation[85 88].

There are few studies into AdV prevalence in South America and Brazil. Estimates of prevalence in the South America region have shown AdV to be present in 3%-8% of ARI samples[89 90]. Moura et al also demonstrated AdV to predominantly present as LRTI in Brazil, especially pneumonia[90]. Also AdV has shown to be an important pathogen involved in viral gastroenteritis in Brazil, presenting in epidemic outbreaks, however it is separate serotypes that cause respiratory and GI infection[91].

The immune response to AdV infection involves airway epithelial cells and numerous cytokines and chemokines such as Interleukin 6 (IL-6), Tissue Necrosis Factor alpha (TNF-α), Interferon Gamma (IFN-γ) and Interleukin 8 (IL-8)[83]. AdV infection is capable of lysing infected airway cells and so can directly damage host tissue[87]. Treatment of AdV is usually supportive the use of antivirals is not usually necessary. There is no vaccine widely available, although during the 1960s the USA did produce one for military personnel[84]. Cidofovir is an anti-viral drug that has been shown to be effective against AdV infection, however due to its side effects which include nephrotoxicity, it is used sparingly in disseminated disease or those undergoing stem cell transplantation[92].

AdV has been shown to be detected in respiratory samples for a significant time after initial infection, in some children the same serotype remains for 9 months[93]. It has also been shown to be present in 0.6-3% of children who are asymptomatic[93]. AdV may remain present in a latent form with viral protein remaining in cells and not replicating, this latent infection may be capable of amplifying pathology that causes asthma or COPD in later life[87]. This prolonged latent phase has been advantageous in other areas of research, with AdV being a popular vector for genetic therapy[94].

1.3.3 Rhinovirus:

Human rhinovirus (hRV) belong to the family of viruses picornaviridae along with poliovirus, hepatitis A and enterovirus[83]. This family of viruses share

a non-enveloped structure with single stranded RNA genome (ssRNA)[84]. First discovered in 1950s during research into the cause of the common cold[84]. Viral culturing techniques were used to determine serotypes so by 1987 it was discovered there were 101 serotypes of hRV that separated into two – hRV group A and hRV group B[95]. These methods were unreliable and slow, with samples often unable to be typed. It was regarded that hRV was a "common cold virus" and was limited to the upper respiratory tract meaning little significance in causing morbidity. Mild URTIs and the common cold are often regarded by physicians as a mild clinical problem but in children accounts for 22 million days absence from school each year in the USA[96].

In recent years, research brought a different perspective on hRV infection. hRVs can reproduce in and infect the lower respiratory tract and cause pneumonia[97]. hRVs are also the commonest cause asthma exacerbations in the young and COPD exacerbation in adults[95 98]. These findings were largely made possible because of the increasing availability of RT-PCR, allowing quick and reliable assessment of infective pathogens. These findings significantly increase the morbidity of hRV species. hRVs are also one of the most common causative pathogens of upper respiratory infections, found in up to 90% of URTI, with direct and indirect socio-economic burden in both the developed and developing world[95 97]. In Brazil hRV has been highlighted as an especially important pathogen in paediatric patients. De Freita Souza et al documented the high prevalence of respiratory viruses of children attending day care and suffering ARI in Salvador, hRV was the most common virus involved and detected in 21% of samples[99]. To date there has

been no study in Brazil or South America which describes the role of different hRV groups.

Since the introduction of PCR, many more than the original 101 serotypes have been described. Including a recently described group of hRV strains – Rhinovirus C, whose role in respiratory infection is has yet to be fully described[95]. This new subgroup of hRV has been shown to be associated with severe asthmatic exacerbations compared to traditional groups A and B, being detected more frequently in severe paediatric asthma than previous strains[100]. One of the main aims of this study is to described the clinical and demographic features of hRV infection and define the role of novel hRV-C in paediatric ARI.

1.3.4 Bocavirus:

Human bocavirus (hBoV) was described in 2005 by a team in Sweden[101 102]. It belongs to the family parvovirus and is the first of such viruses to be discovered in humans. Despite its recent discovery hBoV appears to have an important role in ARI, with antigen studies showing almost all children have been exposed by the age of five years[101]. Exact prevalence of hBoV reported in ARI has varied with it being described in 2.9-19% of ARI[103].

Due to its recent discovery it was initially difficult to fully characterise hBoV, requiring developments in ELISA and PCR techniques to fully describe its serology. ELISA techniques have been developed to identify hBoV antibodies in serum, of both IgG and IgM classes, as well as PCR allowing detection in nasal aspirates[104]. Variation in DNA can further sub-divide hBoV

into 4 viral species (hBoV1 – 4) of which hBoV2 appears to be the most common[105].

Clinical presentation of hBoV is similar to other infective agents with fever, rhinorrhea, cough and wheeze. hBoV has been detected in both upper and LRTIs[101]. Its exact role is still unclear with few epidemiology studies testing for hBoV. Those few studies with a control comparison have demonstrated higher prevalence in ARI compared to controls[106]. Further difficulty has arisen with hBoV being frequently detected with other pathogens, co-detections in 50-60% of cases and in some publications as high as 78%[106]. Possible roles that have been suggested include hBoV having a direct pathogenicity, having low pathogenic effect or augmenting the effect of other respiratory pathogens[106]. This lack of knowledge highlights hBoV as an important pathogen for further investigation. These limits in knowledge are further demonstrated by the lack of epidemiological data on hBoV in Brazil or South America. Albuquerque et al showed hBoV to be present in paediatric ARI samples that were further analysed after being found negative for other respiratory pathogens[107].

Transmission of hBoV is currently being studied. There are no permissive cell lines or animal models available. It is most likely transmitted by aerosol means like other respiratory pathogens, but its detection in 0.8-9.1% of childhood gastroenteritis cases suggests it could potentially be transmitted by faeco-oral route[106]. To date there are no specific treatments or vaccines available for hBoV.

1.3.5 Human Metapneumovirus:

Human Metapneumovirus (hMPV), discovered relatively recently in 2001, is of the paramyxovirus family and is the first virus within this family that has been shown to infect non-avian hosts[101]. hMPV is difficult to grow in culture making it difficult to analyse its pathogenesis. It was discovered in nasopharyngeal aspirates of children with respiratory infection in whom no other pathogen could be discovered. It has been reported that hMPV is present in between 2% and 20% of ARI and antibodies to HMPV are present in 100% of children by 5 years of age, which some believe indicate that all children are mildly or sub-clinically infected by this pathogen[106].

Clinically hMPV can present as both URTI and LRTI, although LRTI is more common in children under one year of age, being one of the most common causes of bronchiolitis second only to RSV[106 101]. Two distinct genetic groups of hMPV have been classified (A and B) each with two separate subtypes. Murine models of hMPV have demonstrated that it readily replicates in the lower respiratory tract and inflammation peaks 5 days after infection and can persist for up to 21 days[106]. Infection causes activation of both Th-1 and Th-2 cytokines although Th-1 cytokines are activated in a lower amount compared to RSV infection[106].

Clinically hMPV has similar presentation to other respiratory viruses with wheeze, hypoxia and fever being predominant features[101]. The majority of cases resolve without healthcare presentation or with outpatient care, however 2% require hospital admission with some progressing to ITU treatment[106]. No specific treatments are currently available. Factors that increase the risk of

severe disease are age <1 year, underlying co-morbidities or prematurity[106]. hMPVs interaction with other respiratory pathogens is currently unclear but is an area of future interest, as one study has shown co-infection with hMPV and RSV increases the risk of a child requiring mechanical ventilation ten-fold compared to single infection[106]. hMPV has been implicated clearly in respiratory disease and is only found in 0-1% of asymptomatic children's airways[106]. Similarly to the recently discovered pathogen hBoV, reports on the prevalence of hMPV in Brazil and South America are lacking. Albuquerque et al also demonstrated the presence of hMPV in respiratory samples in which no other pathogen was detected[107]. Cuevas et al also showed hMPV to be present in 24% of children presenting to hospital with ARI, the only other pathogen tested for was RSV[42].

1.3.6 Parainfluenza:

Parainfluenza viruses (PIV) are enveloped single stranded RNA viruses of the family *Paramyxoviridae*[108]. There are four separate types of PIV designated PIV-1, PIV-2, PIV-3 and PIV-4. PIV is detected in between 9% and 30% of childhood ARI presentations and causes both URTI and LRTI clinical manifestations[108]. There is some variation in clinical presentations between PIV types. PIV-1 commonly presents as croup in childhood and can be detected in up to 50% of children presenting to hospital with the condition[108]. PIV is also commonly detected in bronchiolitis, although in much smaller amounts than RSV, being detected in 10-15% of cases with the most common subtypes being PIV-1 and PIV-3[108]. PIV-1 often presents in epidemics

biennially with clinical manifestations of croup or URTI[109]. These two subtypes are similarly the most common PIV subtypes detected in childhood pneumonia specimens, where PIV is detected in 10-18% of pneumonias, PIV-1 is usually associated with secondary bacterial infection pneumonia[108 110]. Relatively little is known about PIV-4 subtype compared to other groups.

Transmission methods of PIV have not been confirmed although it has become apparent that it is unlikely to be transmitted by aerosol methods, contaminated surfaces may provide a vector with PIV surviving on surfaces for up to 10 hours[108]. The respiratory epithelium appears to be the major site of viral replication with cytokines such as IL-2, IL-6 and IL-10 being detected in elevated levels in samples from PIV positive patients[111]. Research into specific targets for prevention and treatment of PIV is ongoing but has produced positive results. Development of a vaccine for PIV has been discussed by a group from the USA, with PIV-3 being the most likely target but the limitation to one sub-type has raised questions on impact if this were to be created and development has not been forthcoming [110].

1.3.7 Coronavirus:

Coronaviruses (CoV) are enveloped positive single stranded RNA viruses of the family *Coronaviridae*[84]. CoV are divided into 3 subgroups which are then further subdivided, groupings are based on serological and genetic analysis and are called Group 1/2/3[112]. Many of the groups of coronaviruses however do not infect humans. Human infection and transmission has been

described in five subtypes – HCoV-229E, HCoV-NL63, HCoV-HKU1, HCoV-OC43 and novel SARS-CoV[112]. The discovery of CoV was relatively early compared to many other respiratory viruses, in the 1960's. However the study of these viruses has increased greatly in recent years after the emergence of a novel coronavirus in 2002 that caused acute respiratory symptoms and exhibited pandemic spread– severe acute respiratory syndrome (SARS)[113].

The reason for limited research into CoV prior to this was because only two human CoV had then been described (HCoV-OC43 and HCoV-229E)[112]. These two viruses were known to cause URTIs but not LRTIs, except in elderly immuno-compromised patients[112]. However the novel strain found to cause SARs was involved in severe LRTI and demonstrated rapid spread. It was reported to cause 8098 human infections of which 774 resulted in mortality (10%) and spread to 32 countries during the pandemic[113]. The novel virus was found in animals including civet cats and raccoon dogs, although the source of transmission is thought to be bats[114].

Since the SARS pandemic two new subtypes, HCoV-NL63 and HCoV-HKU1 have been isolated from hospitalised children with severe respiratory disease[113]. CoV has also been shown to be detected in 4-10% of childhood ARI[47 115]. Transmission of CoV is by aerosol means and findings from the SARS pandemic showed that transmission did not occur until after symptoms had began to appear, adding to the effectiveness of quarantine and containment procedures[112]. Transmission from animal to human is also a great area of interest with the majority of CoV virus family infecting animals and capable of causing severe multi-organ disease, it is thought SARS was transmitted this way[112 113]. Studies into the immunological response of CoV-

SARs have been carried out and demonstrate a Th-1 response with elevated levels of interferon as well as a variety of inflammatory cytokines and chemokines including IL-6, IL-8 IL-10 and IL-2[112]. Due to the severity of the SARS pandemic a target treatment for CoV or vaccine has been a publicised goal but remains elusive with no effective method for vaccination being highlighted[113].

1.3.8 Influenza:

Influenza virus is an enveloped, single stranded negative RNA virus of the family *Orthomyxoviridae*. Influenza viruses are divided by groups with the three types being influenza A, influenza B and influenza C. All influenza A viruses express haemagglutinin (H) and neuraminidase (N) antigens upon their surface which are used to sub classify the virus with 16 H subtypes (H1-16) and 9 N subtypes (N1-9)[116]. Influenza B viruses also demonstrate mutation but to a lesser extent, and influenza C viruses are rare and of little clinical significance. The influenza A group of viruses are by far the most common and are responsible for the seasonal epidemics of influenza infections[117]. The genome of all influenza viruses are highly plastic and susceptible to point mutations, this is demonstrated in influenza A viruses that regularly show changes in their H and A molecules. These mutations change seasonally altering the ability of the virus to evade a hosts immune system, this phenomenon is described as “antigenic drift”[117]. However periodically dramatic alterations in influenza A antigens are observed, usually every few decades. Significant changes in antigen expression results in increased susceptibility of

the population to influenza infection and results in distinctly different antigenic character of influenza virus termed “antigenic shift”[117]. This characteristic mutation in antigens has also allowed influenza to pass between species in the past with avian and swine transmission leading to novel influenza subtypes.

Seasonal influenza is traditionally seen each winter time and represents a large population burden. This is especially true in children, between 1997 and 2001 attack rates of children presenting with influenza-like-illness in the USA ranged from 58 to 90 cases per 100 children in those under five years, 23 to 52 per 100 for those aged between 5 and 11 years and 13 to 27 per 100 in those aged 12 to 17 years[118]. In all age groups in the USA it is estimated the direct healthcare cost of influenza A is $10 billion and the burden on USA economy is $87 billion a year[119]. Morbidity and mortality is most significant in the young and elderly persons or those with underlying conditions or chronic medical conditions, the average mortality each year in the USA is 51,000[117]. Influenza infection can cause a spectrum of disease severity varying from a mild URTI to fatal pneumonitis, although severe disease is much rarer than mild disease[117].

Unlike many other viral respiratory infections, treatments are available for severe influenza infection. Traditional antiviral medications such as amantidine and rimantadine can be used for patients that present to hospital with severe acute symptoms, within 48 hours of onset however are not usually the current treatment of choice[117]. Neuraminidase inhibitors are preferred as they have been developed for specific treatment of influenza, however they are costly and their use is limited to high risk patients such as pregnant women or those with co-morbidities[117]. Oseltamivir is an example of a commonly used

neuraminidase inhibitor that has proven effective in severe influenza infection, including novel H1N1 influenza[120]. Other examples of neuraminidase inhibitors include zanamivir, lininamivir and permaivir[121]. One of the most effective means of reducing the population burden of influenza has been the adoption of seasonal vaccination to high risk groups in many developed countries. Vaccines utilise both inactivated and live attenuated strains of influenza and the most effective choice has varied in the literature, as well as depending on patient age and demographics[122]. Guidelines are updated regularly by the centre for disease control and prevention (CDC) in America where currently vaccination is recommended in children aged 6 months – 18 years, adults over 50 and anybody with high risk conditions[117]. In the UK the Department of Health (DoH) recommends vaccination in high risk groups which include; age over 65, chronic respiratory/ heart/ kidney/ liver/ neurological disease, diabetes, immunosupression, pregnancy, people in long stay residential homes and carers[123]. Each year the vaccine is altered and contains three strains of influenza that are selected to be the most likely to circulate the following influenza season[122].

Novel strains of influenza that have been formed by antigenic shift represent new challenges to influenza management. These strains have the ability to spread rapidly and can easily reach pandemic levels. Novel strains can represent altered clinical presentations of influenza or increased severity of clinical manifestations. The first recorded pandemic was the 1918-1919 “Spanish” influenza (H1N1 subtype) that killed approximately 50 million people worldwide with mortality rates of 2.5%[116]. Similar pandemics have occurred regularly since then, with the “Asian” influenza of 1957 (H2N2 subtype), “Hong

Kong" influenza in 1968 (H3N2 subtype) and "Russian" influenza (H1N1 subtype) of 1977[116].

Most recently, in the spring of 2009, antigenic shift was once again observed in influenza virus of H1N1 subtype when a novel influenza virus (H1N1) appeared in Mexico causing cases of severe viral pneumonitis[103]. This strain rapidly spread throughout the world and by June 2009 the WHO declared that this novel influenza virus had reached the level of global pandemic[124 125]. This newly formed virus was thought to be a product of four previously known strains of influenza A one from humans, one from birds and two from swine origin[126]. Early on in the pandemic it was shown that children were more likely to suffer with infection and over sixty percent of cases in the US were patients under the age of eighteen[127]. The severity of the infection has also been found to be greater in children. An age of less than five years being a high risk factor for severe illness, and risk was especially high if under two years of age[128]. Other comparable risk factors included age greater than 65 years, pregnancy or pre-existing medical condition[128]. This novel pathogen represented a large burden on healthcare, estimated to cause 59 million illnesses and 265,000 hospitalisations in the USA[126]. These features were also reflected in UK data where hospitalisation was highest in those aged <5 years and 59% of in hospital deaths occurred in previously healthy patients[129]. These findings together highlight novel H1N1 as an important pathogen in paediatric ARI.

In summary influenza represents one of the most common and also one of the most variable viral respiratory pathogens involved in ARI. Its predictable seasonal nature has led to developments in vaccination and prevention which

have helped to reduce burden. However significant shifts in antigen structure can lead to varying clinical presentations and severity of this pathogen, most recently demonstrated by the novel H1N1 pandemic.

1.3.9 Bacterial infections:

This study mainly focuses on the viral pathogens that cause ARI in children. Bacterial infection is also an important cause of many types of ARI. Research into bacterial causes of ARI has been ongoing for a greater length of time than viral studies, mainly due to the techniques of culturing bacteria for investigation.

The most common bacteria detected in paediatric airways is *Streptococcus pneumoniae* (pneumococcus). It was first characterised more than a century ago and has always been linked with respiratory disease[57]. Pneumococcus is commonly found in the upper respiratory tract where it is not associated with disease, however spread to the lower airway can result in severe clinical manifestations of ARI especially pneumonia[57]. It is also associated with meningitis and septicaemia, leading to an estimated worldwide mortality of between 700,000 and 1 million children each year[130]. Similar to other pathogens of ARI these deaths are significantly higher in the developing world with the majority being in Africa of Asia[131]. Significant developments in treatment and prevention of invasive pneumococcus disease have been made in recent year. Development of a vaccine (firstly polysaccharide and more recently developed into conjugate) and its utilisation worldwide have reduced

mortality, with estimates suggesting proportions of deaths from pneumococcus has declined from 36% to 26% worldwide[130]. Newer versions of the conjugate vaccine are also being introduced with varieties of vaccine preparations available that target different serotypes of pneumococcus, continued research is ongoing to the impact of vaccination strategy and whether elimination of common strains may allow more unusual serotypes to the airway[132].

Although pneumococcus is the most common bacterial pathogen involved in paediatric ARI, Hib is also a common pathogen. Together pneumococcus and *H. Influenza*e are estimated to be involved in 50% of childhood pneumonia in the developing world[53]. *H. Influenzae* is estimated to cause 371,000 childhood deaths worldwide being involved in the pathology of pneumonia and also epiglottitis[56]. Similarly to pneumococcus however a conjugate vaccine has been developed since the 1990s (after the polysaccharide vaccine showed only modest impact in the 1980's) which has shown promising results worldwide. The *H. Influenza* conjugate vaccine (Hib) has demonstrated to be have high efficacy and be cost effective[56]. Vaccination for both pneumococcus and *H. Influenzae* has been highlighted as high priorities for widespread use in the developing world in order to achieve the millennium goals of paediatric mortality reduction. Use of Hib vaccine is more widespread worldwide than pneumococcus having been recommended by WHO in 2005 and Pneumococcus in 2008[133].

Much literature focuses on the impact of ARI caused by pneumococcus and *H. Influenzae* infection with many well documented reports on its worldwide epidemiology. This is understandable due to their common occurrences. However, many other bacteria have been associated with ARI,

especially pneumonia. Infections such as the fungi *Pneumocystis jirovecii or the bacteria Mycoplasma pneumoniae, Chlamydia trachomatis* and *Chlamydophila pneumoniae* have all been shown to be involved with paediatric ARI with comparatively less known than the two most common pathogens. The impact of these atypical bacterial pathogens varies in different studies on ARI and also with conditions the child may have such as HIV[53]. Research into these less common pathogens is important as the more common bacteria have shown treatment and vaccination strategies to be an effective method of targeting bacterial disease. The interaction of pneumococcus with other respiratory pathogens is also an area of research interest. Especially its interaction with viral pathogens as some studies have suggested an increased severity with pneumococcus and viral respiratory infection[134 135].

1.3.10 Other pathogens and novel pathogens:

Despite ARI being recognised for a significant amount of time as one of the leading causes of childhood mortality worldwide, pathogen classification is still incomplete. Novel pathogens have been discovered with the development of new detections techniques. Examples of this include hMPV and hBoV which were discovered as recently as 2005 and are difficult to cultivate in a viral culture model[101]. The spectrum of pathogens involved is also further complicated by mutations and developments in previously defined viruses that lead to novel clinical manifestations, such as the CoV that caused SARs and novel H1N1 influenza[136]. These pathogens can mutate de-novo or also can be transmitted from animal hosts, raising the potential for new infections. Co-

detection of pathogens is also common which brings into question the role of different infective agents, and whether they are causative in ARI, present as a coincidence or if there is interaction between two pathogens that can cause pathology.

Overall no study has purported to detect all pathogens in individuals presenting with ARI. There are still questions raised about previously discovered pathogens and it remains important to fully assess novel pathogens after their emergence.

1.4 Risk factors for ARI:

There are a number of recognised risk factors that increase both the probability of suffering ARI and the likelihood of severe infection. Recognised risk factors of both bacterial and viral infections have led to development of a targeted approach to prevention of disease. Children and adults with risk factors can be selected for vaccination regimes or preventative treatments to decrease overall disease burden of ARI.

Frequency of ARI correlates closely with age, children being much more likely to be infected than adults. It is estimated that children suffer 6-8 respiratory infections a year compared to 2-4 in adults[96]. The majority of these infections are mild and confined to the upper airway, resolving without presentation to a healthcare professional. However, each one of these infections has the potential to progress into a more severe clinical presentation or systemic illness. There are also observable differences in children that occur

with age, with infants under 2 being at especially high risk of severe LRTI due to the small calibre of their airways and their naivety to viral infections once maternal antibody has wained[137].

Previous respiratory and cardiovascular pathology is also associated with increased risk of severe ARI. In children conditions such as asthma or Cystic fibrosis can increase the likelihood of respiratory infection, whereas in adulthood chronic obstructive pulmonary disease (COPD) can increase risk of hospitalisation with ARI[2 129]. Pulmonary hypoplasia is a recognised as a risk factor for severe viral infection, especially RSV disease in infancy, it also correlates closely with prematurity which also is recognised to be associated with ARI[138].

Worldwide distribution of ARI severity is also skewed towards the developing world, especially Africa and Asia. It is estimated 70% of ARI burden is represented by the developing world with the majority of pneumonia mortality worldwide being present in 42 countries[2 139]. There are multiple reasons for this including lower levels of vaccination and reduced availability of hospital care[2 60]. Vaccination levels for Hib and Pneumococcus are lower in the developing world due to problems with cost and distribution, however international organisations such as WHO and the Global Alliance for Vaccines and Immunisation (GAVI) in recent years have aimed to make these more accessible[133]. Two highly prevalent conditions in the developing world, especially Africa, are malaria and human immunodeficiency virus (HIV). Both of these are associated with increased severity of ARI and it has been reported that malaria may cause underreporting of ARI mortality due to misdiagnosis[131]. Immunodeficiency is a well documented cause of increased ARI susceptibility

with the most common reason worldwide being HIV, infection with HIV can also allow infection with novel infections which are rarely seen in immuno-competent patients e.g. *Pneumocystis jiroveci* which pose treatment difficulties for physicians due to the immunocompromised patients relative inability to clear infections[140]. Malnutrition is also an important factor which is more prevalent in the developing world and is associated with increased severity of ARI and increased risk of mortality in children[141]. In the developing world it is also much more prevalent to cook with biomass fuels in an enclosed indoor setting, which is associated with increased risk of ARI[142].

Lifestyle factors can also affect a child's risk of ARI. The most recognised factor is parental smoking, both during pregnancy and infancy. It has been shown that parental smoking is associated with increased frequency of respiratory infections as well as higher prevalence of adolescent asthma, wheeze, middle ear infection and reduced lung fuction[143]. In the post-natal period environmental tobacco exposure causes airway release of pro-inflammatory cytokines and induces hyper-reactivity[143 144] A second suggested explanation is prenatal smoking being associated with other ARI risk factors such as prematurity and reduced birth weight[143]. A household environment with postnatal smoking is also associated with respiratory infections and asthma in the absence of prenatal smoking[143].

Finally certain combinations of respiratory infections have been shown to produce more severe ARI in children. These findings are much debated in the literature but one combination that is agreed upon is influenza infection in the presence of pneumococcus infection[145]. The interaction of respiratory

pathogens increasing risk of severe ARI is important as it further highlights the need for full assessment of pathogens present during ARI.

1.5 The treatment and prevention of ARI

Interventions to reduce the burden of paediatric ARI have involved both the acute management of the child and preventative measures such as vaccination strategies. Vaccination has become available for a number of pathogens involved in ARI, some of which are accessible worldwide. The most widely used vaccines for ARI pathogens are Hib, pneumococcus and influenza[2]. Conjugate Hib vaccine is effective in reducing prevalence of both pneumonia and meningitis caused by infection, both in industrialised countries and also in developing countries[146]. All studies have shown the vaccine to protect in greater than 90% of cases, resulting in conjugate Hib vaccine being utilised in all industrialised countries as part of routine childhood vaccination schedule[2]. Various preparations are available and the Hib vaccine is often included with other preparations to immunise against multiple pathogens in on injection e.g. diphtheria, tetanus, polio. In the UK vaccination schedule four doses of Hib are given at 2, 3, 4 and 12-13 months[147]. In contrast Brazil gives four doses at 2, 3, 4 and 6 months[148]. Interestingly the Hib vaccine was initially developed to target invasive meningitis, but has also shown to reduce childhood pneumonia hospitalisations by 20 – 30%[2].

Pneumococcal disease is one of the most commonly detected bacterial pathogens in childhood ARI. A number of vaccines are available for this

bacterium which target differing numbers of strains. In the UK the conjugate 13-valent preparation is part of the vaccination schedule and is administered at 2, 4 and 12-13 months[147]. Previous preparations of the vaccines were shown to be highly effective in both developed world and developing world, with 59% reduction in LRTI mortality in children under five by use of the polysaccharide vaccine[2]. However vaccine coverage is not currently included in every country. Brazil has very recently added the conjugate pneumococcus vaccination to the childhood vaccination schedule with the main limitation being cost and economic assessment being necessary before uptake[56 149 150].

Influenza vaccination is not utilised as frequently as the previous two, mainly due to costs. The CDC recommended during the H1N1 pandemic that all children between 6 months and 18 years and adults over 50 should be universally vaccinated[117]. However cost and vaccine shortages meant that this was not achieved or attempted in many countries. Influenza vaccination does not form a part of standard childhood immunisation in UK or Brazil. High risk groups may however be targeted for vaccination, these include many risk factors mentioned previously but in children reasons may be chronic medical conditions, prematurity or immunosupression such as HIV[117].

Acute management and treatment of individual presentations of ARI is usually dependent on diagnosis. Many URTIs are self limiting and may not present to a physician, also with the majority being viral treatment options are limited. Bacterial infections for diagnoses such as tonsillitis or otitis media may highlight the need for antibiotics, but it is difficult to ascertain with certainty whether a bacterial or viral pathogen is implicated although tools such as the Centor criteria have aimed for clarification(where fever, tonsillar exudates,

cervical lymphadenopathy and absence of cough are suggestive or bacterial infection requiring empirical antibiotic treatment)[18]. Pneumonia has been the target for guidelines produced by the WHO to reduce mortality in the developing world. WHO clinical guidance has highlighted that the diagnosis of pneumonia is likely based on rapid breathing and chest wall in-drawing, allowing the diagnosis to be made in the absence of radiological investigation[2]. The choice of treatment is then made on the evidence that the majority of bacterial pneumonias are caused by *S. pneumoniae* or *H. Influenzae* and so penicillin based antibiotic is first line treatment, by oral route if mild or intramuscular route if severe[2]. Other measures for both pneumonia and ARI in general are supportive, with correction of fluid balance and hypoxia being primary targets[2] (**Figure 1.1)**.

1.6 The global burden of acute respiratory infections in children

ARIs represent a large worldwide burden in paediatric mortality and morbidity. The burden of individual diagnoses that constitute ARI have been mentioned previously. WHO data recognises ARI as the largest identifiable cause of mortality in children under 5 years, accounting for 19% of deaths, higher than both malnutrition and malaria deaths in this age group (**Figure 1.3**)[1]. This equates to an estimated 3.9 million deaths per year, with some opinions suggesting this may be an underestimate due to misdiagnoses such as malaria and variable definitions of ARI being used for classification[1 131 151].

The burden of mortality is significantly higher in the developing world with 70% of ARI mortality in Africa and South East Asia and the majority of deaths occurring in 42 countries of the world[151 152]. Reasons for this include lower vaccination coverage and reduced access to healthcare[2]. The most common diagnosis to result in mortality is pneumonia[2].

ARI morbidity is also significant worldwide. WHO estimates ARI to cause over 94 million disability adjusted life years (DALYs) worldwide in all age groups[153]. The majority of pneumonia cases do not result in mortality and in developing countries more than 25% of children will suffer pneumonia each

1.7 Diagnostic methods for Acute Respiratory Infections

In the past much viral and bacterial diagnostics were done by culturing techniques, growing pathogens collected from patient samples in a controlled environment. This method was more effective in bacterial diagnosis which could be viewed on microscopy. However for viruses many additional stages were involved including antibody staining and washing, viruses also were generally more difficult to cultivate and often resulted in negative samples or contamination[95]. Large studies into viral epidemiology were therefore unrealistic with high negative rates and laborious methodology.

Immunofluoresence assay are one method of detecting viral pathogens in respiratory infection. This method is divided into indirect and direct immunofluoresence where an indirect assay requires a secondary antibody and is processed in a lab. More recently developed immunofluoresence kits

have the fluorescing antibody attached to the primary kit and can be administered at the point of care[154]. Both of these techniques are faster and more reliable than viral culturing techniques, however are more expensive. Cost is less than RT-PCR and unlike RT-PCR a dedicated virology centre is not necessary. Immunofluorecent tests are available for a variety of pathogens including RSV, hMPV and influenza[154-156]. In direct comparison studies have shown RT-PCR to be more sensitive than direct florescence however sensitivity is higher than cell culture techniques[155 156]. Detection of multiple pathogens is not possible with direct fluorescence and kits are not available for all viral pathogens, they are also costly and a large number would be required to screen for multiple pathogens and so they are not appropriate in this study.

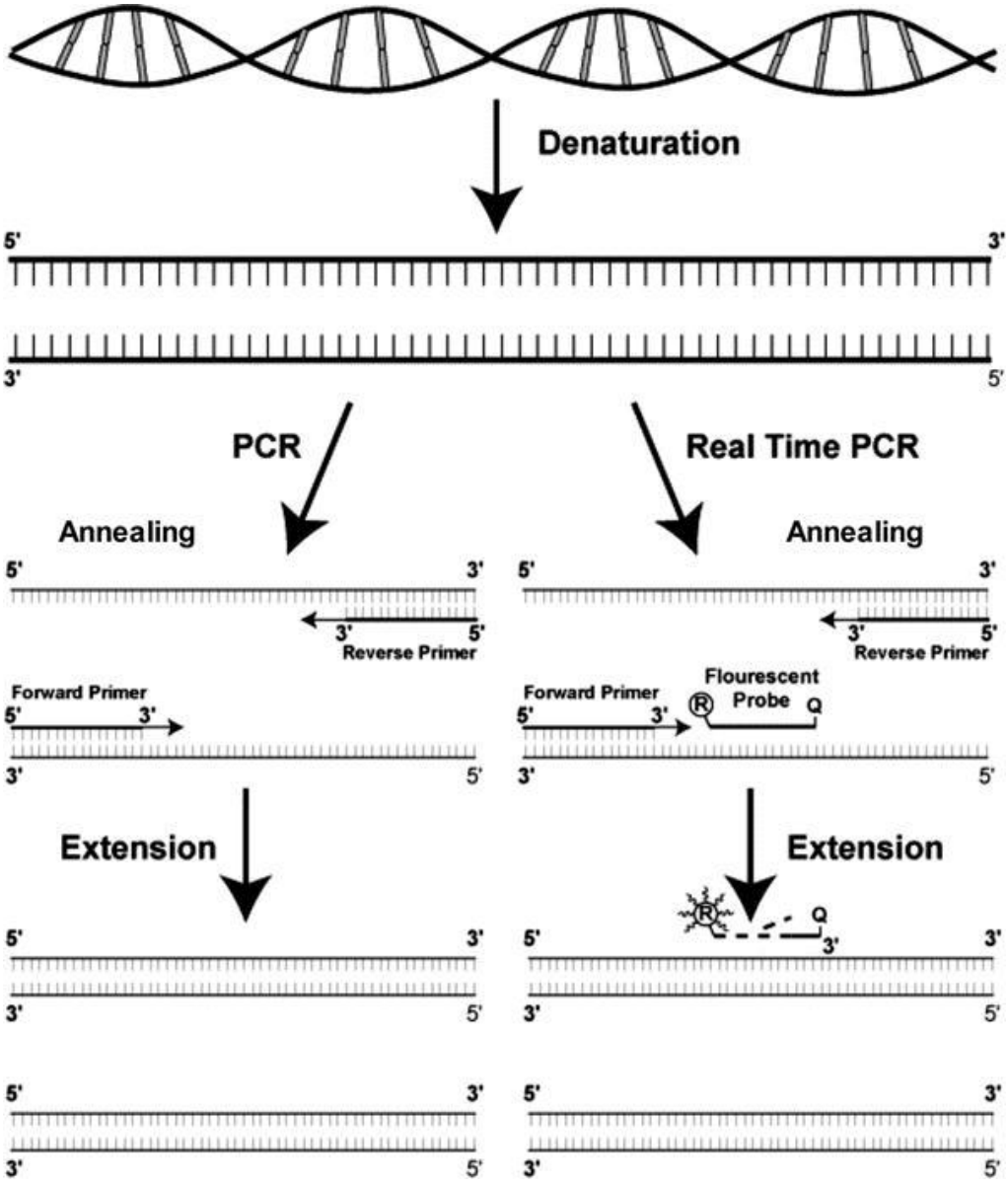
Since the turn of the century the use of polymerase chain reaction (PCR) has become widespread in viral diagnostics. The process represented a significant research breakthrough earning a Nobel prize in 1993[157]. The process of PCR aims to replicate the process of a normal cell cycle in a controlled environment with the aim of amplifying genetic material[158] . The three stages of thermal denaturation, primer annealing and primer extension allow identical copies of genetic material to be made, with selective amplification possible due to the design of primers (**Figure 1.2**) [158]. Use of

Figure 1.1: A child receiving nasal CPAP for bronchiolitis in Recife, Brazil.

An example of acute ARI management (Photo taken by Dr Paul McNamara and used with permission)

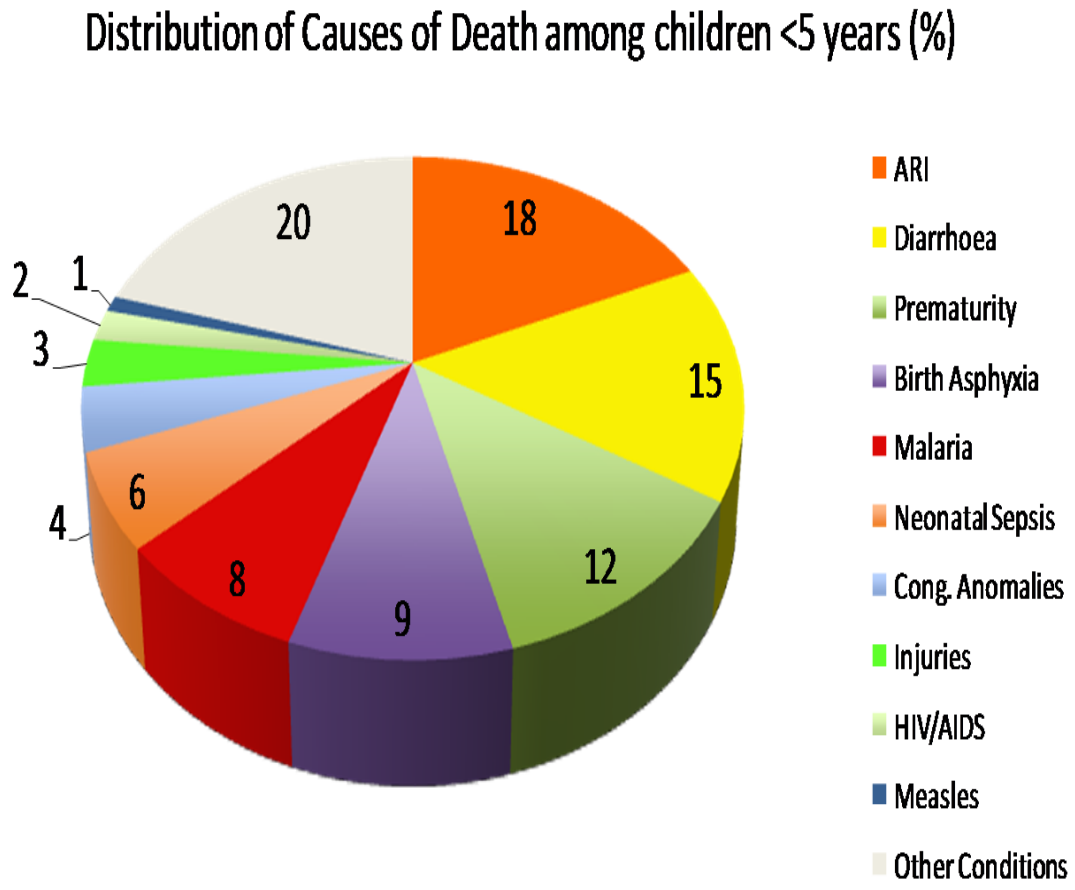
Figure 1.2: (taken from Garcia et al[159]) "Three steps in polymerase chain reaction (PCR) and real-time PCR."

Step 1, Denaturation: Template DNA mixture is heated to 90–95°C, allowing the DNA double strand to denature and separate into two single strands. Step 2, Annealing: Template DNA mixture is cooled to 50–60°C to allow primers (for PCR) and primers plus the TaqMan probe, which consists of an oligonucleotide with a 5-reporter dye (R) and a downstream, 3-quencher dye (Q) (for real-time PCR), to anneal to the single-stranded DNA. Step 3, Extension: The temperature is raised to 72°C to allow Taq polymerase to add deoxynucleotides to the target DNA (for PCR) and complete the amplification. For real-time PCR, the TaqMan probe containing a reporter dye at the 5 end and a quencher dye at the 3 end is separated on cleavage by DNA polymerase, resulting in increased fluorescence of the reporter as well as the newly amplified target DNA. These three steps are repeated 30–40 times to replicate 2n copies of DNA region of interest where n = cycle number."[159]



fluorescence in PCR primers can allow quantification of genetic material in comparison to known “housekeeping” genes expressed in samples. Further developments in PCR have allowed multiple sections to be analysed simultaneously in one sample with separate wavelengths being indicative of specific sections – multiplex PCR[160]. Overall these developments have allowed new insights to viral infections, with rapid and sensitive analysis being possible of large numbers of samples. Utilisation of PCR techniques is costly though, especially the machines which make high throughput analysis possible. Due to the expense the utilisation of PCR is limited worldwide, especially in the developing world.

Figure 1.3: Worldwide mortality causation data in children <5 years (WHO data 2010)[1]



year throughout the first five years of life[53]. Overall 2-3% of all children have pneumonia severe enough to require hospitalisation[53]. URTIs are also highly prevalent in this age group and have the potential to progress to significant disease. Children are estimated to experience 4-8 upper respiratory infections a year, twice as many as adults[88]. Mortality is rare with URTIS but they are still a common cause of morbidity. Even in developed countries URTIs are estimated to cause 20 million days of absence from work and 22million days of absence from school in the USA alone[96].

1.8 Pulmonary immune response to ARI:

The immune response to infection is an important aspect of pathology in ARI. The immune response to infection in the lung is vitally important, with many respiratory pathogens causing pathology due to the inflammation and mucus production that is caused by the immune system clearing infection. Classically the immune responses to all infections are divided into the innate and adaptive immune systems. The effector mechanisms of the innate immune system include antimicrobial peptides, phagocytes, alternative complement pathway as well as natural barriers such as the skin[161]. These mechanisms can be activated rapidly in response to a pathogen, immediately after its detection.

The innate immune response is non-specific compared to the adaptive immune response, with pattern recognition receptors in non-self pathogens being detected and triggering the innate immune system to respond

immediately while recognising self molecules and not responding. Activation of an innate receptor can mediate a number of functions, including phagocytosis, release of chemoattractant substances or release of effector molecules that initiate the adaptive immune response[162]. Receptors of the innate immune system are germ-line encoded and therefore limited, this means the function if to recognise receptors that are found on common pathogens and are widespread[161]. These “pattern-recognition receptors” are expressed on the effector cells of the innate immune system which include macrophages, dendritic cells and B cells. The best characterised receptor is mannan-binding lectin[161]. Toll like receptors (TLRs) are also an important range of receptors involved in innate immunity.

In contrast the adaptive immune response produces a highly specific response targeted at an individual pathogen. The main cell classes involved in adaptive immunity are T cells and B cells. It takes time for this response to be activated, three to five days, and so highlights the importance of overlap between the innate and adaptive response. The receptors of the adaptive immune response differ to that of the innate system. Receptors for the innate system are coded for in the germline genome of the cell limiting their potential range. Whereas cells of the adaptive immune systems receptors are produces from a large array of gene segments, arranged in different sequences and that then undergo a process of somatic recombination to increase variability[163]. Once a receptor binds to a complimentary pathogen, which must be presented to T cells by an antigen presenting cell (APC) or a major histocompatability complex (MHC) protein on the cell surface, the adaptive immune cell replicates rapidly to produce numerous identical cells with similar complimentary

receptors – the process of clonal expansion[163]. B-cells are involved in the production of antibodies which are important for increasing uptake of pathogen by phagocytotic cells and causing agglutination of pathogen molecules. T cells are further subdivided by role into T helper cells, cytotoxic T cells, memory T cells, regulatory T cells, Natural killer T cells and γδ T cells. They can also be divided by their expression of the surface proteins CD4 and CD8[163]. The surface protein is important for T cell function as it defines which class of MHC the cell can bind to, CD8+ T cells bind to class I MHC cells which are expressed on the surface of almost all cell types and can express endogenous antigen, such as virus, within the cell. CD4+ T cells in contrast bind to MHC class II receptors which are present on APCs and inducible by stimuli from the innate immune response e.g. TLRs[163].

The largest group of T cells within the body is the T-helper subclass (Th)[163]. These cells play a vital role in directing immune response by secreting a range of cytokines that can increase or regulate specific aspects of immune response. Cytokines can be defined as “a diverse group of protein signal molecules that are produced by a wide variety of cells, they activate or influence adjacent cell movement differentiation, growth and death”[164]. In 1986 it was found that not all Th cells expressed identical types of cytokines and with analysis demonstrating two distinct groups; T-helper 1 cells (Th-1) and T-helper 2 cells (Th-2)[165]. Th-1 cells were characterised by expression of interferon gamma (IFN-γ) whereas Th-2 cells expressed interleukin 4 (IL-4) which was important in inducing immunoglobulin E (IgE)[165]. Both IL-4 and IgE were found to be elevated in asthmatic and atopic patients leading to the hypothesis that and imbalance between Th2 and Th1 helper cells was

important in the generation of asthma in children[164-166]. More recently the discovery of new cytokines (e.g. IL-17) which were not explained by the Th1/Th2 classification led to re-evaluation of the classification of Th cells. It has since been shown that numerous other Th cells are present which differentiate in response to the presence of specific cytokines and express individual cytokines respectively (**Figure 1.4**)[163]. One subclass of T-helper cells that have gained special attention is the T-helper 17 (Th-17) cells which have been shown to play an important role in linking the adaptive and innate immune system, they have also been demonstrated to play a role in autoimmune diseases including rheumatoid arthritis[165]. This study aims to assess the role of IL-17 in RSV infection, an important infant infection that is associated with asthma and wheeze in later life.

1.9 Work that has led to this study

Previous work by Dr Katie Rose (MPhil student 2009/10) highlighted the importance of viral pathogens in paediatric ARI in Brazil. In collaboration with the Instituto Materno-Infantil Professor Fernando Figueira (IMIP) Children's Hospital, Recife, North Eastern Brazil, her findings on the viral and atypical bacterial epidemiology of children <5 presenting to hospital with ARI over a one year period have been published[167]. This publication documented the prevalence of pathogens in this cohort and showed that a pathogen could be detected in 86% of samples, the most prevalent pathogens being RSV, AdV, hRV and hBoV (**Figure 1.5**[167]). This manuscript also highlighted the extent of co-infection in this group and characterised the pathogens involved (**Figure**

1.6)[167]. Through continuation of this previous work we hope to further characterise the pathogen epidemiology in this group, specifically with regard to H1N1 and hRV.

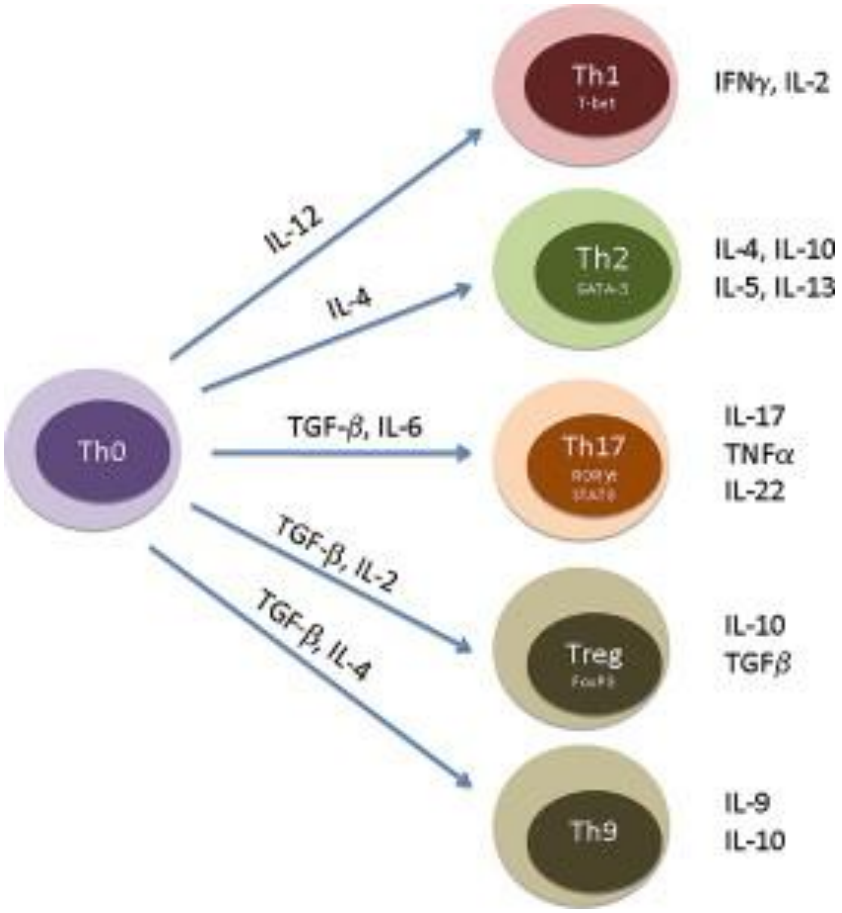


Figure 1.4: (taken from Bonilla et al[163]) " $CD4^+$ T_H cell subsets."

Antigen-specific naive T_H0 T cells are stimulated to expand on interaction with APCs expressing MHC class II/peptide complexes. Depending on the type of APC and the cytokine milieu *(arrows)* at the site of antigen encounter, T_H0 cells can be driven down one of several differentiation pathways. The T_H populations that arise retain the TCR specificity of the parent T_H0 cell but secrete unique constellations of cytokine products that mediate distinct effector functions, including activation for killing of microbes (T_H1), production of antibodies and expulsion of helminths (T_H2), induction of inflammatory responses (T_H17), and dampening of immune activation (regulatory T *[Treg]* cells). Specific transcription factors (indicated in the nuclei) stabilize lineage commitments and dictate the specific cytokine secretion profiles. *FoxP3*, Forkhead box protein 3; *RORγt*, (retinoic acid receptor related orphan receptor γt); *STAT3*, signal transducer and activator of transcription 3; *T-bet,* T-box expressed in T cells."[163]

Figure 1.5: (Taken from Bezerra et al[167]) “Pathogen prevalence (%) in nasopharyngeal aspirates from children less than five years with acute respiratory infection.”

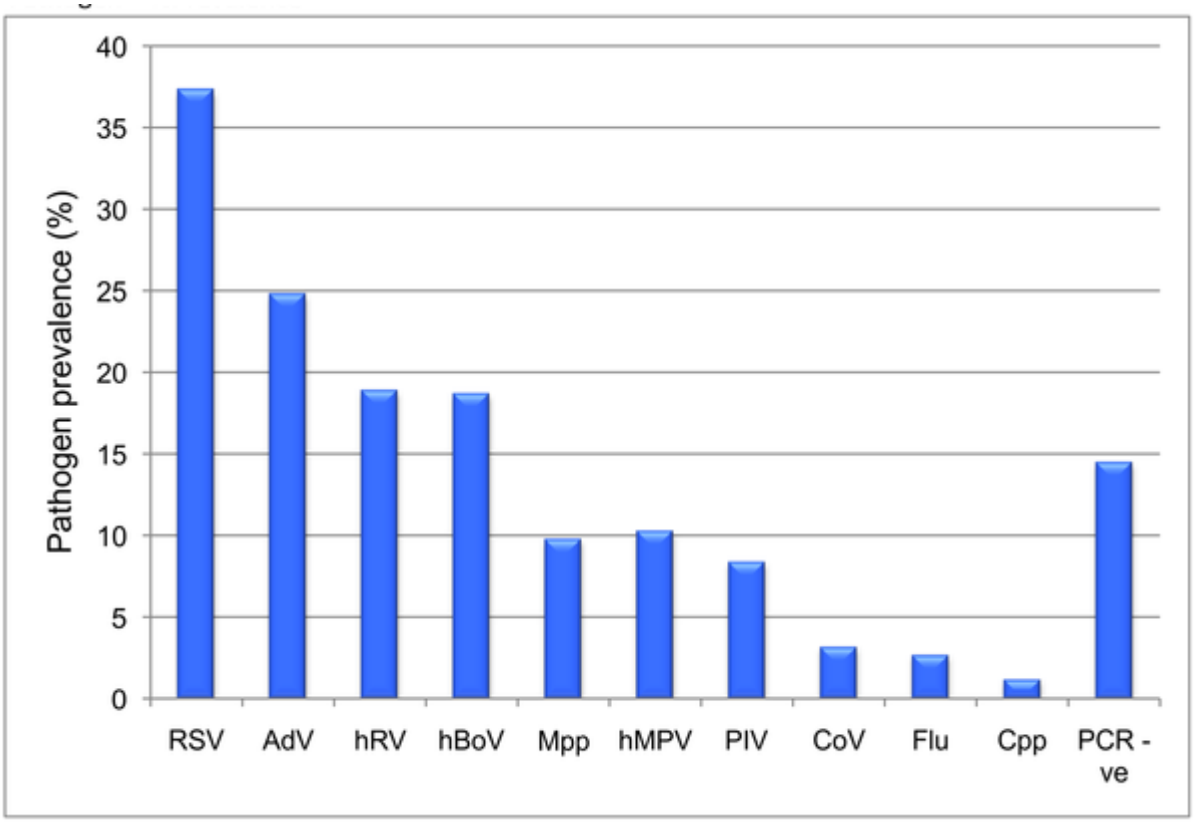
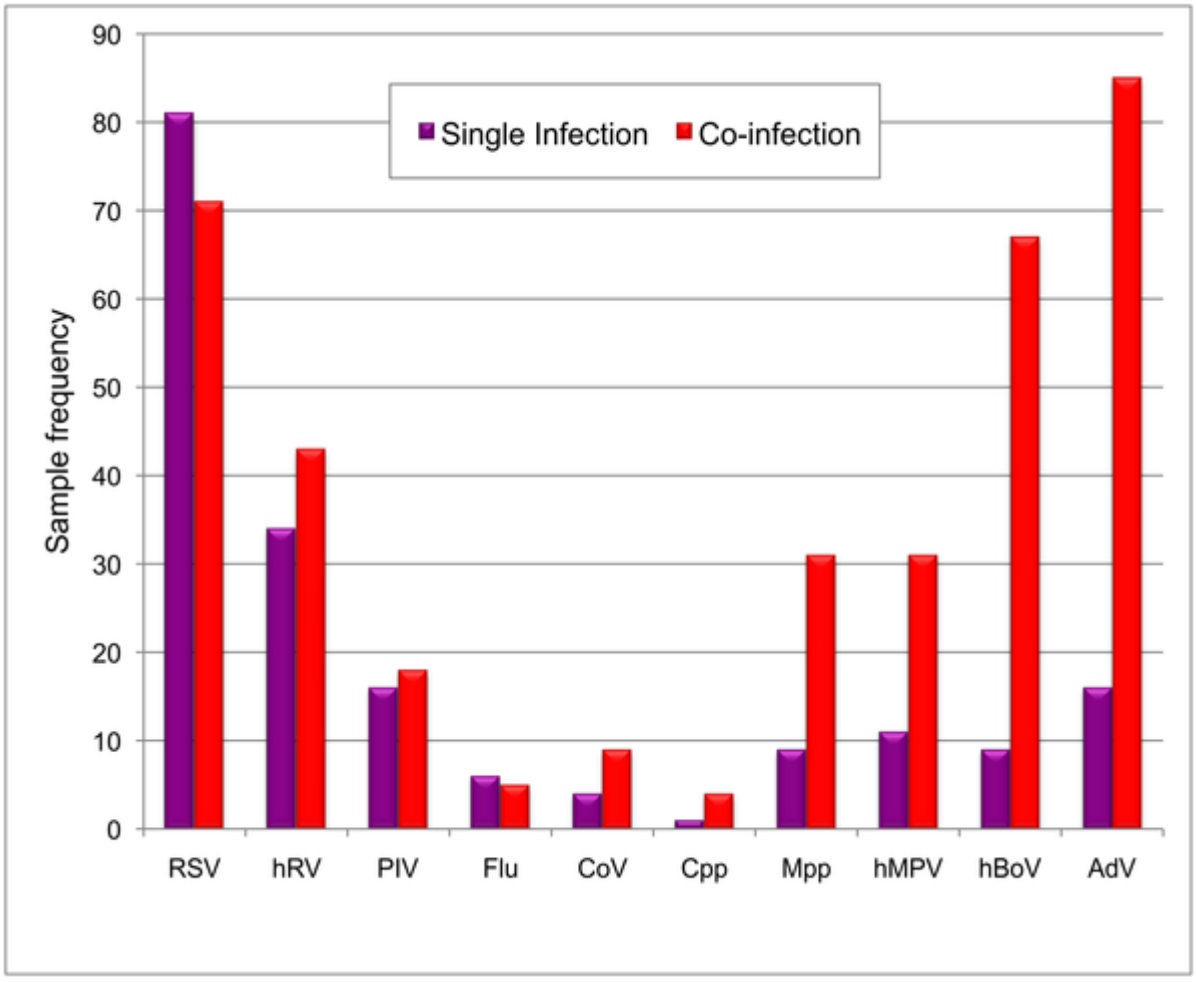


Figure 1.6: (Taken from Bezerra et al[167]) “Pathogen frequency in singly infected (red) and co-infected (pink) nasopharyngeal aspirate samples.”



1.10 Aims and objectives of this study:

This MPhil thesis describes three discrete studies, all investigating different aspects of viral and atypical bacterial ARI in paediatric patients. The titles/aims of these studies are as follows:

An investigation into the causes of childhood ARI and the impact of H1N1 influenza

Aims:

1) To characterise the viral and atypical bacterial pathogens detected in children <5 years presenting to hospital in Recife, Brazil.

2) To characterise the clinical and demographic features of children suffering H1N1 infection compared to non-H1N1 ARI.

An investigation into the prevalence of Rhinovirus-C in paediatric acute respiratory infection

Aim:

1) To describe the role of the novel hRV group, hRV-C, in paediatric ARI.

An investigation into the interaction between Interleukin 17, Interleukin 13 and RSV infection in airway epithelial cells

Aim:

1) To determine the role of interleukin 17 (IL-17) in RSV infection of airway epithelial cells and measure for synergy with IL-13.

2 An Investigation into the causes of childhood ARI and the impact of H1N1 Influenza

2.1 Introduction

ARI represents the largest single identifiable cause of mortality in children under five years old worldwide accounting for 19% (3.5 million) of identifiable mortality, the vast majority occurring in just 42 coutries[152] [49]. ARI has been defined in Section 1.1.

Despite such a large global burden, some studies have suggested initiatives to reduce ARI impact have been met with limited success and ARI remains the largest cause of childhood mortality[152]. The pathogenic causes of ARI have been studied, but previous techniques made this a slow and laborious process[168]. New techniques such as reverse transcription polymerase chain reaction (RT-PCR) have now made it possible to analyse a range of pathogens simultaneously with high specificity. It has also facilitated discovery of newer pathogens (e.g. hMPV, hBoV)[106]. These techniques are however rarely available in the developing world.

There is still much that is unknown about the interaction between respiratory viruses in the airway and how they cause symptoms/signs of childhood ARI. Some viruses, e.g. RSV, have been studied in detail but more novel viruses are less defined or rarely included in studies. Epidemiological studies do not identify a pathogen in all cases of ARI, many report positive

rates of 35%-47%[169-171]. Further complexity is encountered when multiple pathogens are detected on analysis, causing confusion as to which pathogen is the cause of the acute episode or if there is interaction between multiple pathogens.

There is much heterogeneity between studies into paediatric ARI epidemiology. RT-PCR has allowed analysis of ARI pathogens in a number of publications. However, the choice of pathogens, age of participants, clinical inclusion criteria and sampling methods have tended to vary (**Table 1.1**). There are few studies of this nature in Brazil or South America. This study follows on from the first year's results into paediatric ARI epidemiology in Brazil previously documented by her MPhil studies in 2009/10. Her publication in PLoSOne described a high level of pathogen detection (87%) and highlighted the importance of co-infection in this population(39%)[167].

Pathogens are also capable of mutation, changing their clinical properties. Children suffer 6-8 respiratory viral infections a year compared to 2-4 in the average adult, making novel pathogen emergence especially important in this group[96]. Recently a novel strain of H1N1 influenza A (H1N1) emerged. First identified in April 2009 in Mexico, H1N1 rapidly spread throughout North America[127]. In June 2009 the WHO issued a pandemic alert on H1N1[127]. Initial reported cases of H1N1 presented with viral pneumonitis caused severe respiratory distress[116]. Demographic and epidemiological data demonstrated children, especially those under age five, were at high risk of severe disease and accounted for 18% of hospitalisations[126 127].

With the burden of ARI burden being so great in children it is important to fully document the pathogens involved and to fully assess novel pathogens.

This study aims to assess the pathogens involved in children under five presenting with ARI to a paediatric A&E in Recife, North East Brazil. Two thirds of children in Recife live in slum or favela population and viral diagnostic techniques are not usually available. Following on from the first year of this study, RT-PCR for 18 pathogens was performed on samples collected over two years (2008-2010). The timing of this study had the unique fortune of covering the emergence of H1N1 influenza and so the secondary aim was to assess the impact of the H1N1 pandemic on ARI presentations in this cohort.

Table 2.1: An overview of studies into viral ARI epidemiology.

Study	Bezerra[167]	Berkley[47]	Wang[115]	Do[172]	Jartti[173]	Singleton[174]	Sung[169]	Kaplan[175]
Country	Brazil	Kenya	Shanghai	Vietnam	Finland	USA (Alaska)	Hong Kong	Jordan
Age Group	<5 years	<12 years	<9 years	<15 years	<16 years	<3 years	<5 years	<5 years
Setting	A&E	Admission	Outpatient	Admission	Admission	Admission	Admission	Admission
ARI included	All ARI	Pneumonia	All ARI	All ARI	Acute wheeze	LRTI	All ARI	All ARI
Sample method	NPA	Nasal Wash	Swab	Swab + NPA	NPA	Swab	NPA + Swab	NPA
Length of Study	12 months	12 months	24 months	35 months	20 months	24 months	12 months	6 months
N	407	759	817	309	293	440	475	326
Median age	8 months	7 months	36 months	24 months	19 months	64% <1 year	24 months	5 months
Detection:	87%	56%	60%	72%	88%	90%	47%	78%
RSV	37%	34%	19%	24%	27%	23%	8%	43%
AdV	25%	4%	7%	5%	5%	30%	5%	37%
hBoV	19%	2%	19%	16%	-	-	-	18%
hRV	19%	-	12%	4%	24%	44%	4%	11%
hMPV	10%	3%	7%	7%	4%	15%	2%	3%
PiV	4%	8%	13%	7%	6%	18%	9%	0
Mpp	10%	-	-	-	-	-	2%	0
Flu	3%	5%	17%	17%	2%	5%	10%	1%
CoV	3%	10%	4%	8%	1%	6%	4%	1%
Cpp	1%	-	-	-	-	-	0%	-
Co-infection:	39%	-	14%	20%	19%	-	4%	22%

2.2 Methods

This chapter details the methods used to identify respiratory pathogens present in the airways of children presenting with ARI. These findings include the second year of a project into this topic with the findings of the first year recently published[167].

2.2.1 Patient Recruitment

Patient recruitment took place in the emergency department at the Instituto Materno-Infantil Professor Fernando Figueira (IMIP) Children's Hospital, Recife, North Eastern Brazil between April 2008 and March 2010.

Children below the age of five, presenting to the accident and emergency department with signs or symptoms of ARI lasting less than seven days were eligible for inclusion. This included both upper respiratory and lower respiratory presentations. Patients were excluded if they had been re-admitted within the course of the same infection or had cyanotic heart disease. Participants were included whether they did or did not require hospital admission.

Consent was obtained from all participants parents before recruitment. Ethical consent was obtained both from the IMIP ethics committee and the National Research Ethics Office of Brazil.

2.2.2 Clinical observations and collection of demographic data

Upon recruitment, each participant's parent/guardian was asked to complete a clinical and demographic questionnaire of 95 questions. This gathered information including demographic information such age, gender and date of birth. It also collected medical information including birth history, history of asthma, prematurity previous history of pneumonia or tuberculosis, allergies and vaccination history. Questions also covered social factors such as housing, number of siblings, fathers' occupation, number of children sharing a room, mean family income and smoking in the household were also included.

Clinical data such as heart rate, respiratory rate, temperature and oxygen saturation were also recorded on admission. The presence of identifiable clinical signs including cyanosis and chest in-drawing were noted. If the patient was admitted, clinical observations were recorded daily for the duration of hospital stay.

Upon discharge each participant was assigned a discharge diagnosis by the attending physician who was not directly involved in the study. Diagnosis categories included; URTI, bronchiolitis, pneumonia, episodic viral wheeze/asthma and "other". Diagnosis was classified as URTI if symptoms were confined to the upper respiratory tract, such as coryza, otitis media, sinusitis or tonsillitis with no obvious extension to the lower respiratory tract. Bronchiolitis was defined by lower respiratory infection signs such as wheeze or tachypnoea having been preceded by upper respiratory signs in children aged less than 18 months. Pneumonia was defined as the presence of tachypnoea, fever and crackles or decreased air entry on auscultation. For the

most part, pneumonia was diagnosed clinically and not radiographically although some patients did have a chest x-ray. A participant was classified as episodic viral wheeze if they had a history of discreet episodes of wheezing associated with an assumed viral infection. “Other” was any other diagnosis that was not suitably covered by other categories and included rarer presentations such as bronchiolitis obliterans.

2.2.3 Sample Collection

Once a child had been recruited, a Nasopharyngeal Aspirate (NPA) was collected in accordance with standardised protocol[176]. The process involved aspirating nasal secretions from both nostrils using a soft catheter with two lateral eyes and a port for finger tip suction control. The catheter was attached to a conical trap on a medium pressure vacuum and was advanced to a depth of 5-7cm and then withdrawn. Children were held by parents to reduce discomfort. Sample collection was undertaken by a single researcher during working hours (9am until 3pm, Monday until Friday) in accident and emergency (A&E) department.

2.2.4 Sample preparation and storage

Once collected all samples were processed within 3 hours of collection. Processing involved dilution of the sample within 3mls of sterile normal saline (0.9% NaCl in H_2O) by aspiration through the catheter, so as to remove all

residual secretions. The solution collected was then centrifuged at 500g for 10 minutes at 4°C. This allowed each sample to be divided into a supernatant and a cell pellet. They were separated and the cell pellet was re-suspended in a solution of 2-mercaptoethanol and RNA Lysis buffer (Qiagen, UK) in order to extract nucleic acid from each sample. This solution was then divided into two aliquots. All samples pellet and supernatant were then immediately placed at -70°C and stored at this temperature until being transported to the Institute of Child Health at Alder Hey children's hospital on dry ice. The timing from sample collection until freezing was less than four hours in all cases.

2.2.5 Nucleic acid purification

Upon arrival in Liverpool samples were stored at -70°C until nucleic acid purification was performed. The timing between NPA sample being collected and nucleic acid purification ranged from 2 months to 12 months. Nucleic acid purification was performed with an automated kit, the QIAsymphony Virus/ Bacteria Mini Kit (Pathogen Complex 200 protocol) according to manufacturers' instructions using the QIAsymphony automated machine (QIAGEN, Crawley, UK). For each patient 200µl of sample supernatant was utilised and eluted 60µl of both DNA and RNA which were co-extracted. Once extracted the purified nucleic acid was stored at -70°C until PCR was performed.

2.2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After nucleic acid was extracted and purified RT-PCR was performed for 18 different pathogens using six channels of the LightCycler 480 real time PCR machine (Roche Diagnostics, Burgess Hill, UK). The 18 pathogens were divided into 5 separate master mix preparation. The first included Flu A, Flu B, hMPV, RSV and novel pandemic H1N1 influenza (H1N1). The second included the CoV subtypes – HKU1, NL-63, 229E and OC43. The third included master mix primer probes for PiV types 1-4 and hRV. The fourth included primer probes for AdV, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. The final master mix included the primer probe set for Bocavirus. Master mixtures were made up on ice and nucleic acid was heated to 95°C before addition of master mix in order to allow denaturation. 5µl of sample was used in each sample for pathogens with RNA targets and 10µl for pathogens with DNA targets.

RT-PCR was then performed on the LightCycler 480 real time PCR machine. The PCR method, including primer probe genetic sequence is described by Hopkins et al (for complete primer sequences see **Appendix A.1**) [177]. Briefly, the reverse transcription process for Flu, H1N1, hMPV, RSV, CoV, PIV and hRV was carried out at 50°C for 20 minutes, denaturation at 95°C for 2 minutes and then 50 amplification cycles of 95°C for 15 seconds, 58°C for 45 seconds and 72°C for 1 second[177]. Each well contained 20µl of master mix and 5µl of purified RNA. Reverse transcription for AdV, *Mpp* and *Cpp* was similar and differed by removing the 50°C hold step and the enzyme activation step at 95°C was extended to five minutes. The contents of the wells were altered

slightly with 15µl of master mix and 10µl of sample. RT-PCR for other pathogens was performed using the Roche LC480 Probes Master kit (Roche Diagnostics, Burgess Hill, UK). Bocavirus differed in that it used the Qiagen Quantitect Probe PCR kit (Qiagen, Crawley, UK). The bacteriophage MS2 replicase gene was present in each master mix, this acted as an internal control to ensure amplification of genetic material had occurred, it has previously been demonstrated that this internal control is suitable for viral respiratory pathogen identification[178].

2.2.7 PCR Analysis

For each pathogen a positive control and negative control was contained on each plate. Individual pathogens on the same plate were analysed by use of the multi-channel analysis of different wavelengths. The LightCycler 480 machine provided analysed results with amplification plots. These amplification plots were then checked by two researchers to confirm positive and negative samples in any amplification plots that were unclear, this was done by comparing each sample individually to the control positive and negative samples to decide the cycle and magnitude of amplification of a sample.

2.2.8 Statistical analysis

All results were then added to the statistics package SPSS 18.0.0 (SPSS Inc, Chicago). Frequencies of pathogen prevalence were calculated using SPSS and graphs produced with Microsoft Excel 2007 (Microsoft Inc). Differences in prevalence, admission rates and presence of co-infection were calculated using the Fisher Exact test for smaller numbers and chi squared for larger samples. Variation between groups was assessed with the Kruskall-Wallis test. Significance in all cases was considered as $p<0.05$.

For analysis purposes pathogens were grouped. PiV types 1 – 4 were considered as “Parainfluenza”; “Coronavirus” included the subtypes CoV OC43, CoV HKU1, CoV229E and CoV NL63. Influenza A and B were considered as “flu” for comparison between the two years, in the second year of the H1N1 pandemic the novel H1N1 was also considered separately.

2.3 Results

2.3.1 Recruitment and patient demographics

Over the two year study period 630 children presented with ARI and were recruited to the study. The cohort contained more males than females (n=345, 55%). The median age of children presenting with ARI was 7 months (range 0-57 months) with 530 (84%) children below 18 months and 452 (72%) children being less than 12 months. Hospital admission was required in 339 (54%) of children due to severity of their ARI, with the median length of stay for those requiring admission being 4 days (range 0-38 days). A member of the household smoked in 263 (42%) of children. Family mean income was recorded in 65% of patients and was found to be US$ 276; this was significantly lower than the Brazilian mean (US$ 450).

Recruitment was higher in the first year (n=407, 65%) compared to the second year (n=223, 35%) (**Table 2.2**). In the second year the gender distribution was more equal and the age of children presenting with ARI was lower (median 7 months vs. median 6 months p=0.005). Otherwise, all other demographics were similar between the two years.

Table 2.2: Demographics of children recruited to study. (*=significant difference, p<0.05)

	Entire study (April 2008-March 2010)	Year 1 (April 2008-March 2009)	Year 2 (April 2009-March 2010)
N	630	408	222
Gender	55% Male	58% Male	50%*
Median age (range)	7 months (0-57)	8 months (0-57)	6 months (0-45)
Birth weight <2500g	15%	14%	16%
Breast Fed	92%	92%	92%
Co-Morbidities	5%	5%	6%
Smokers at Home	42%	43%	40%
Hospital Admissions	54%	52%	57%
Co-Infection	43%	40%	50%*

2.3.2 Clinical information

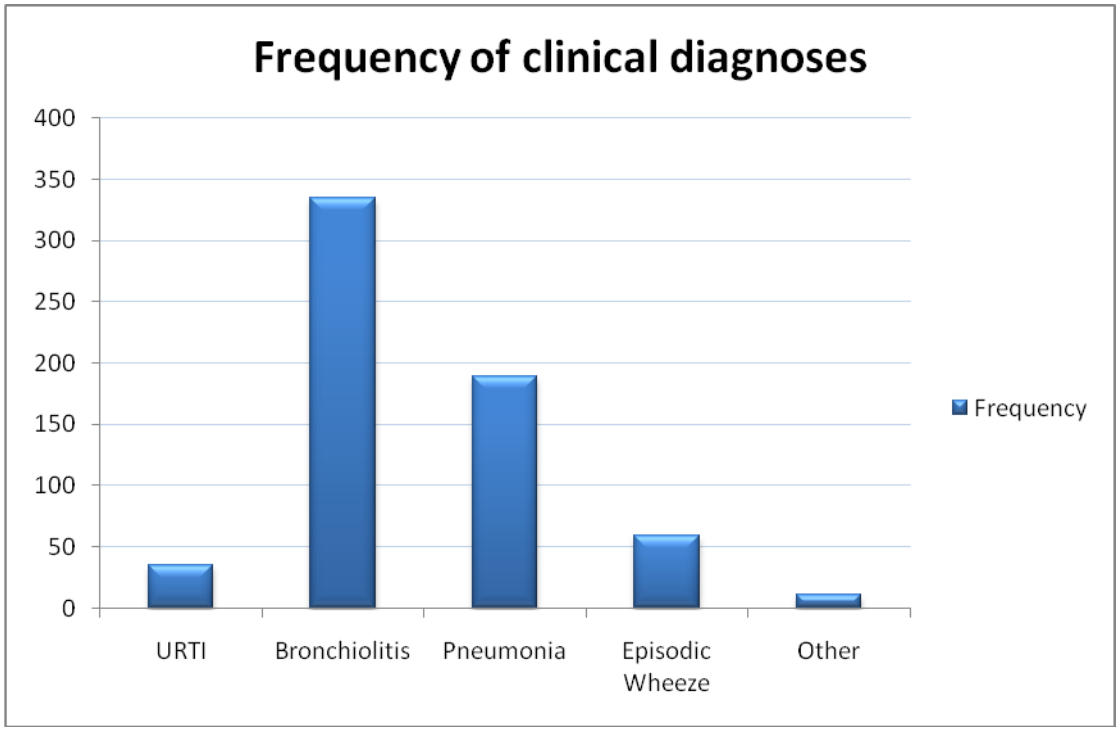
The most common discharge diagnosis was bronchiolitis (n=335, 52%) followed by pneumonia (n=189, 30%) and episodic viral wheeze/asthma (n=59, 9%) with the least common diagnoses being upper respiratory tract infection (n=35, 5.6%) and “other” (n=11, 2%) (**Figure 2.1**). “Other” diagnoses included conditions such as bronchiolitis obliterans and whooping cough. A total of 32 (5%) children had a medical co-morbidity e.g. non-cyanotic heart disease or neurological disability. Breastfeeding was common with 580 (89%) of parents reporting the child was breastfed or weaning.

Clinically 87 (13%) of children had evidence of hypoxia (peripheral oxygen saturation <90%) and 43 children (7%) required oxygen supplementation during their hospital visit. Hypoxia was clinically evident in a small number of children, with 48 (7%) having chest in-drawing documented on admission. A fever (defined as temperature >38°C) was present in 105 (16.2%) of children. There were 3 children (0.5%) within the study who died during their admission to hospital for ARI.

In the second year the diagnosis of pneumonia was more common than in the first (27% vs. 35% p=0.02) and the diagnosis of EVW / asthma was made less frequently (13% vs. 2% p<0.01). There were no significant differences in the frequency of children presenting with URTI (6% vs. 6%), bronchiolitis (53% vs. 56%) or “other” (2% vs. 1%).

Figure 2.1: Frequency of clinical diagnoses of all children recruited to study.

(n=630, URTI = Upper Respiratory Tract Infection. Episodic Wheeze = Episodic Viral Wheeze / Asthma)



2.3.3 Pathogen detection

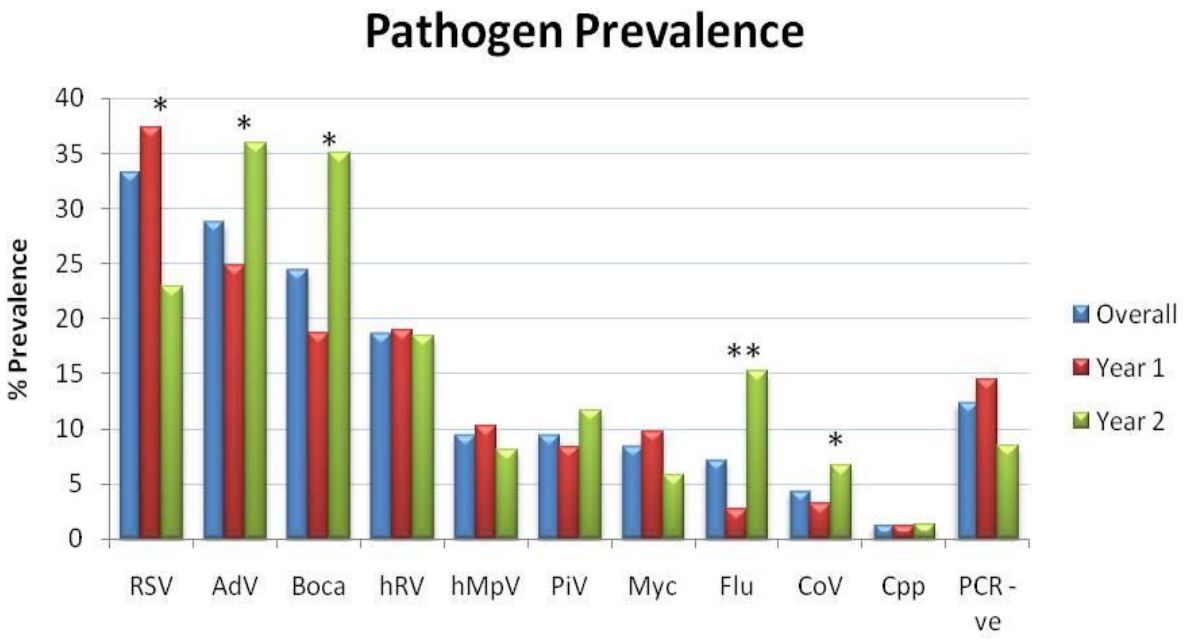
All samples were successfully tested for 18 different pathogens by RT-PCR. At least one pathogen was detected in 87.6% of samples with detection rates being higher in the second year (91.5% PCR positive) compared to the first year (85.5% PCR positive).

Overall the most commonly detected pathogens over the two year study period were RSV, AdV, hBoV and hRV (33.2%, 28.7%, 24.4% and 18.7% respectively) (**Figure 2.2**). The pathogens hMPV, PiV, *Mpp* and Flu were detected in smaller amounts (9.5%, 9.5%, 8.4% and 7.1%) with the least prevalent pathogens CoV and *Cpp* being detected infrequently (4.4% and 3.1% respectively).

By dividing the study into two twelve month periods (April 1st 2008 – March 31th 2009 and April 1st 2009 – March 31st 2010) significant differences in the annual prevalence of some pathogens were observed. A significant decrease in the prevalence of RSV was detected in the second year (37.3% vs. 22.9% x^2 = 13.82 p=<0.001). Other pathogens showed significant increases in prevalence with the most significant increase being that of influenza (2.7% vs. 15.2% x^2= 34.17 p=<0.001) (see section 2.3.6). Other pathogens that demonstrated increased prevalence in the second year included hBoV (18.7% vs. 35.0% x^2=20.74 p=<0.00001), AdV (24.8% vs. 35.9% x^2=8.60 p=0.003) and CoV (3.2% vs. 6.7% x^2=4.23 p=0.04).

Figure 2.2: Percentage prevalence of pathogens in entire study

(April 2008-March 2010, light blue), 1st year of study (April 2008-March 2009, red) and 2nd year of Study (April 2009-March 2010, green) (* = p<0.05 for differences in frequency of pathogens between years, ** = p <0.01) NB sum of prevalence is >100% due to frequency of co-detection



2.3.4 Co-detection

Co-detection / co-infection is when more than one pathogen being detected on PCR from one sample. High levels of co-detection of multiple pathogens were found. More than one pathogen was detected in 272 (43.2%) samples. This was higher in the second year compared to the first year (50% and 40% respectively p=0.009). The highest number of pathogens simultaneously detected was five and this occurred on three occasions (0.5%) (**Figure 2.3**). Co-infection was not associated with increased severity of ARI, with there being no association between co-infection and hospital admission in our group (x^2=0.23 p=0.63).

In patients samples where more than one pathogen was detected ("co-infections") the most common pathogens detected were AdV, hBoV, RSV and hRV (54.4%, 48.1%, 37% and 29.9% respectively of total co-infection frequency (**Figure 2.4** and **Figure 2.5**). When observing overall detection of a pathogen as a proportion of samples which were detected as co-infections, the pathogens with the highest proportion of positive results being present in co-infections were *Cpp* (87.5% of *Cpp* detected as a co-infection), hBoV (84.4%), AdV (81.2%) and *Mpp* (79.2%). In contrast, the pathogens that were least common to present as co-infections were RSV (59.3% of RSV detected as co-infection), hRV (59.3%) and PiV (63.3%).

Figure 2.3: Prevalence of co-detection as a percentage of all samples.

(PCR –ve = no pathogens detected on PCR)

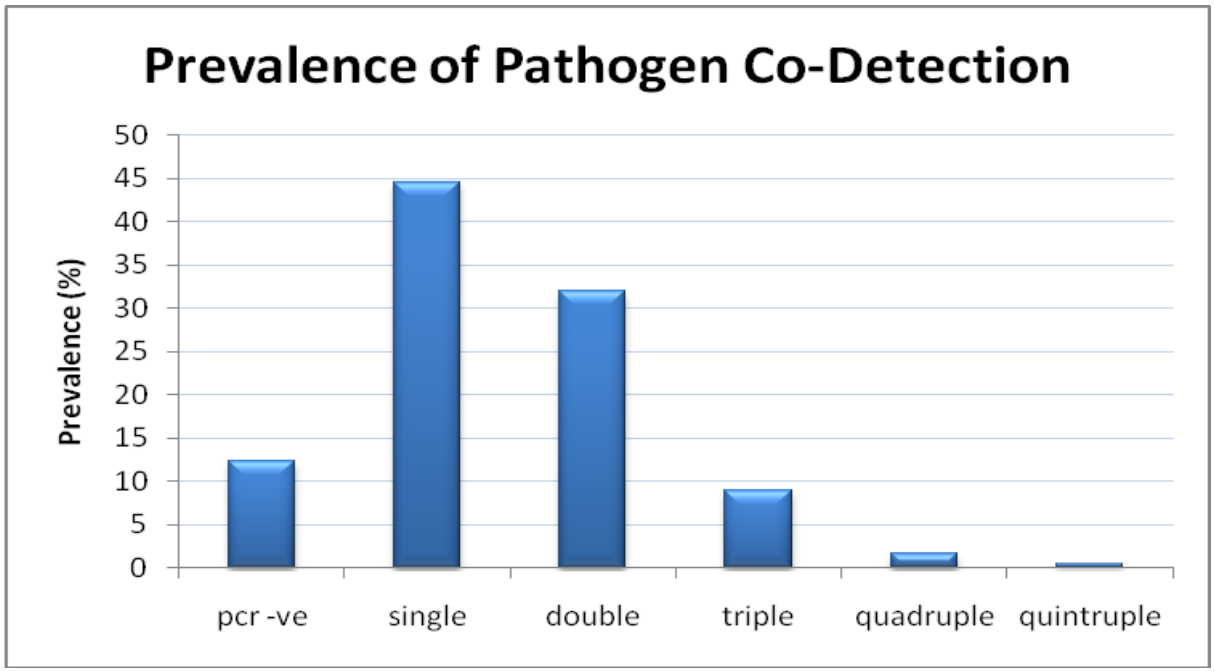


Figure 2.4: Prevalence of pathogens presenting with co-infection as percentage of all co-infections.

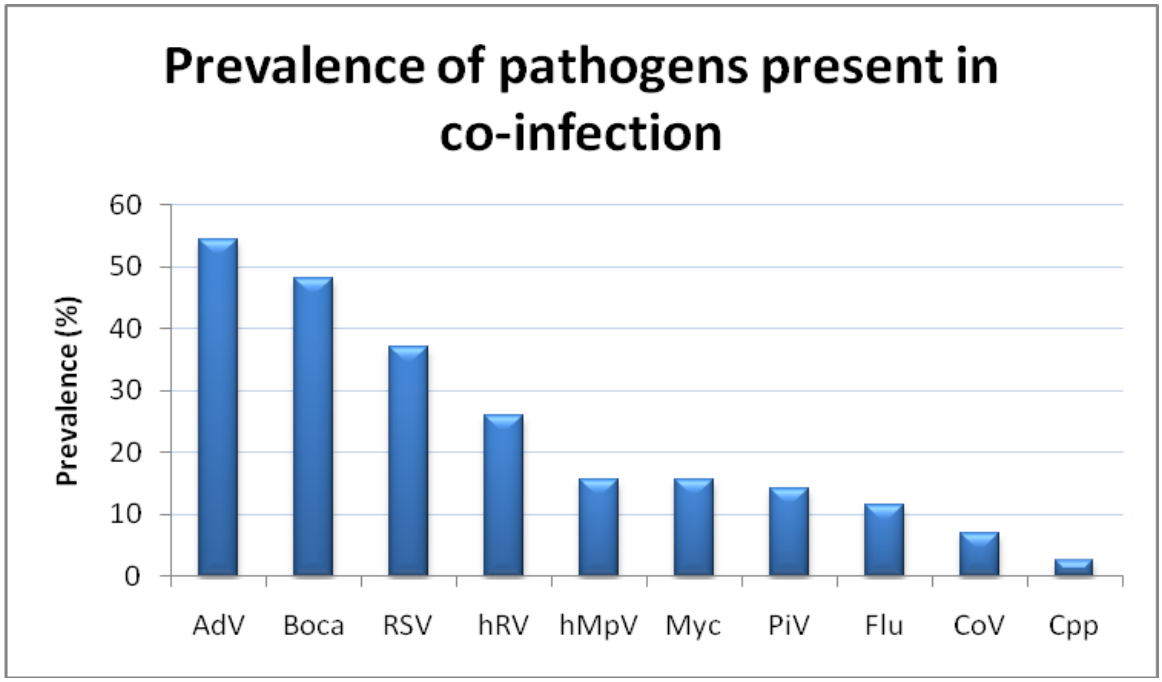
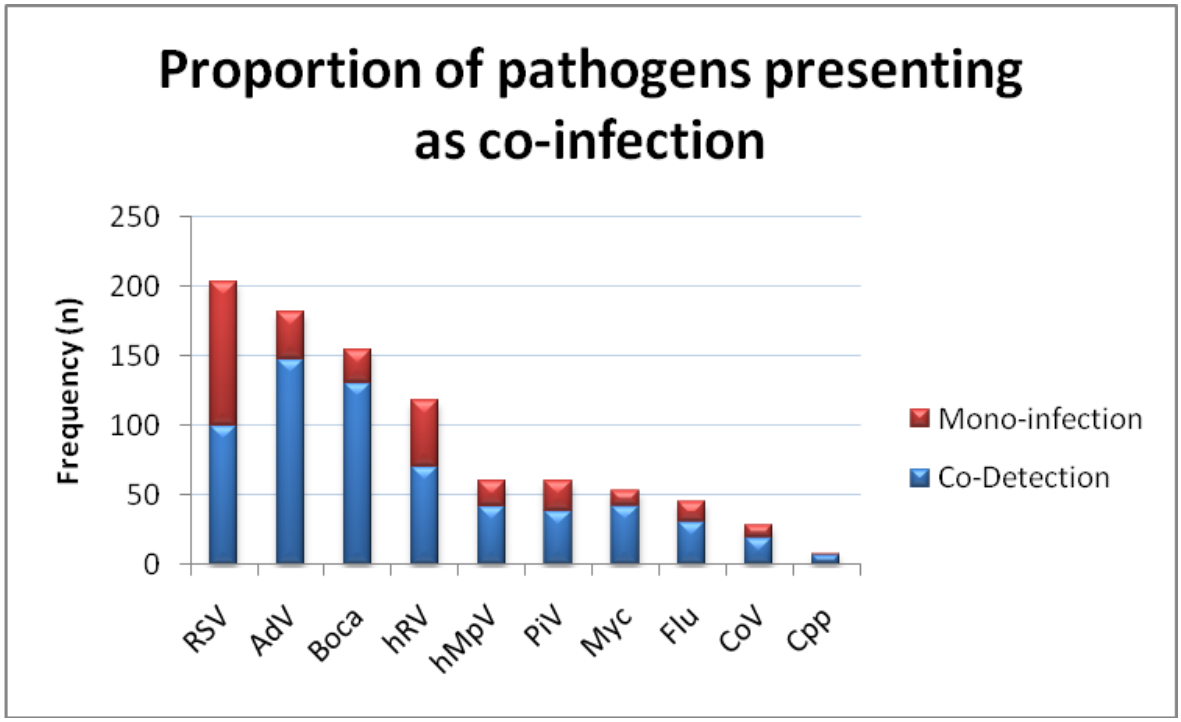


Figure 2.5: Frequency of pathogens presenting as mono-infection and co-infection shown as proportion of total pathogens detected.

(Red = mono-infection Blue = co-infection Total size of bar = frequency of pathogen detected)

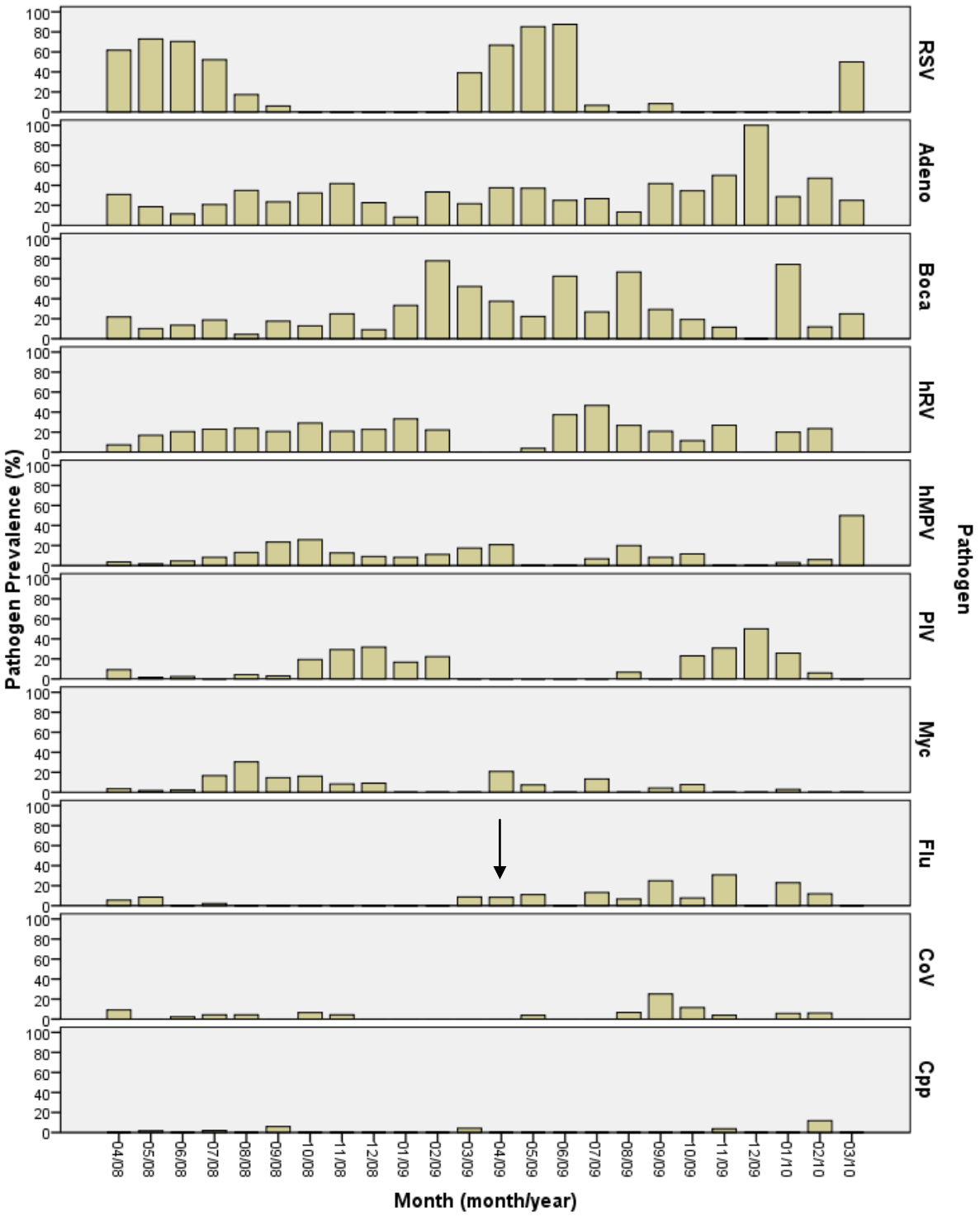


2.3.5 Temporal relationships

This study collected clinical samples over two years and so analysis of temporal patterns was possible (**Figure 2.6**). The rainy season in Recife occurs between April and July. Seasonality was demonstrated most clearly for RSV with a seasonal increase being apparent between March and August of both years. There was also variation in the monthly prevalence of influenza which will be discussed in the following section in more detail. Some pathogens seem to be endemic such as AdV and hBoV.

Figure 2.6: Temporal relationships of pathogens detected divided into monthly percent prevalence each month

(arrow represents emergence of pandemic H1N1 in Mexico)



2.3.6 H1N1 Pandemic

Of the 45 samples that were positive for Flu on RT-PCR, 27 were of the novel H1N1 influenza subtype (4.3% overall) with the first case being detected on the 20th August 2009. The demographics of both non-H1N1 influenza and H1N1 influenza was comparable to the demographics of the study population with regards gender, age, birth weight, breast feeding and parents smoking at home (**Table 2.2**). Significant differences were seen however with children in who H1N1 was detected being more likely to require hospital admission compared to non-flu ARI (78% vs. 53% x^2 = 6.48 p=0.01) and compared to non-H1N1 flu ARI (78% vs. 44% x^2=5.24 p=0.02).

Rates of co-infection were also significantly higher in the H1N1 group compared to non-flu ARI (78% vs. 41% x^2=14.4 p<0.001) and was increased compared to non-H1N1 flu but not to the level of significance (78% vs. 56% p=0.12). The most common pathogens detected simultaneously with H1N1 were AdV and hBoV, both being detected in 40.7% of H1N1 positive samples (**Figure 2.5**). There was a single child with five pathogens detected simultaneously and these included AdV, H1N1, PiV, CoV and hBoV.

With regards clinical presentations all children found to have H1N1 on RT-PCR presented with lower respiratory manifestations of ARI (48% bronchiolitis, 52% pneumonia) (**Table 2.2**).

The temporal relationship of H1N1 was clearly defined throughout our study period (**Figure 2.8**). This shows the emergence of novel H1N1 influenza compared to non-H1N1 influenza. There was no month in which the two were detected simultaneously and H1N1 appears to present outside of the influenza

season, with cases of non-H1N1 flu being detected between March and July of both years. The monthly prevalence of H1N1 is also higher than non-H1N1 flu. Prior to the H1N1 pandemic, flu represented an infrequent pathogen detected in few cases of ARI with the highest prevalence being 13%. After the emergence of H1N1 the prevalence increased with a peak prevalence of 30% in December 2009. In the six-month period of October 2009 until March 2010 H1N1 was detected in 20% of samples collected.

Table 2.3: Demographics of children presenting with novel H1N1 influenza compared to Non-H1N1 influenza and Non-influenza ARI.

(* = p<0.05 comparing H1N1 influenza positive ARI to non-H1N1 ARI)

	2008-2010 (n=630)		
	Non-Flu	Non-H1N1 Flu	H1N1 Flu
N	585	18	27
Gender	Male 56%	Male 39%	Male 48%
Median age	7 months (1-57)	8.5 months (0-31)	8 months (range 0-44)
Birth Weight <2500g	14%	22%	15%
Breast Fed	92%	83%	100%
Co-Morbidities	5%	0%	7%
Smokers at home	41%	44%	48%
Hospital admission	53%	44%	78%*
Co-infection	41%	56%	78%*
Clinical diagnosis:			
URTI	6%	11%	0%
Bronchiolitis	54%	50%	48%
Pneumonia	29%	28%	52%*
EVW	10%	11%	0%
Other	2%	0%	0%

Figure 2.7: Percentage prevalence of pathogens detected as co-infections with novel H1N1 influenza.

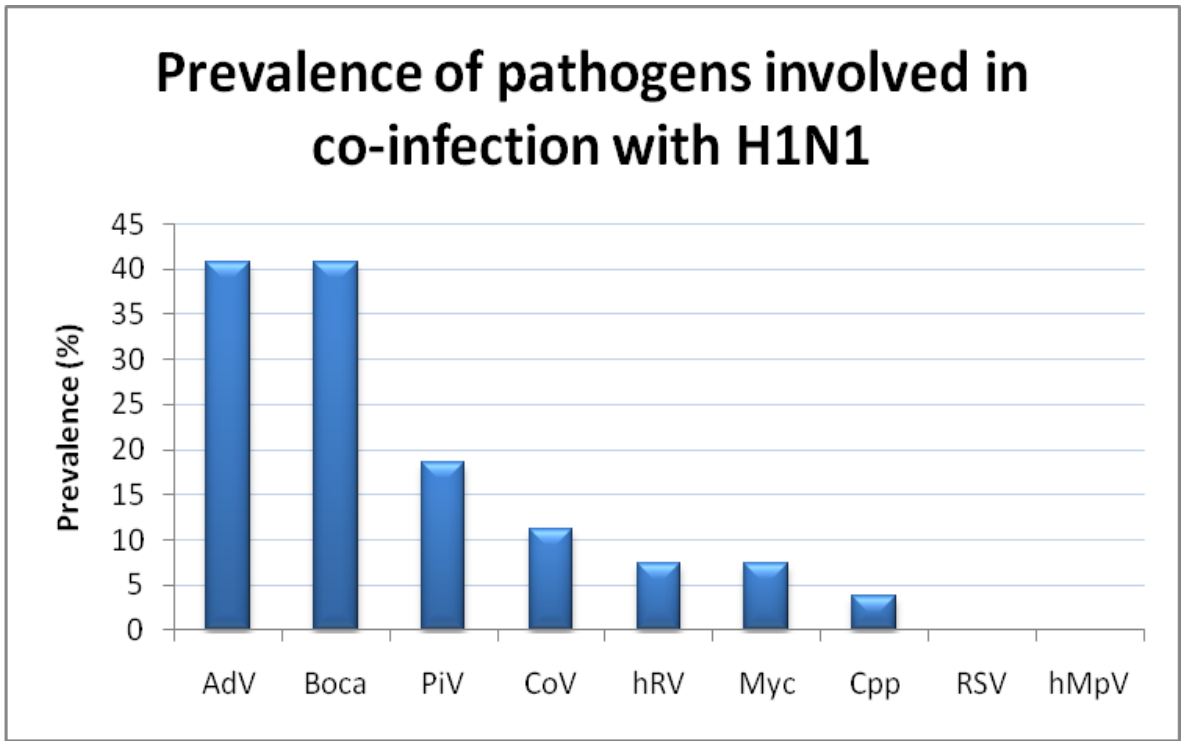
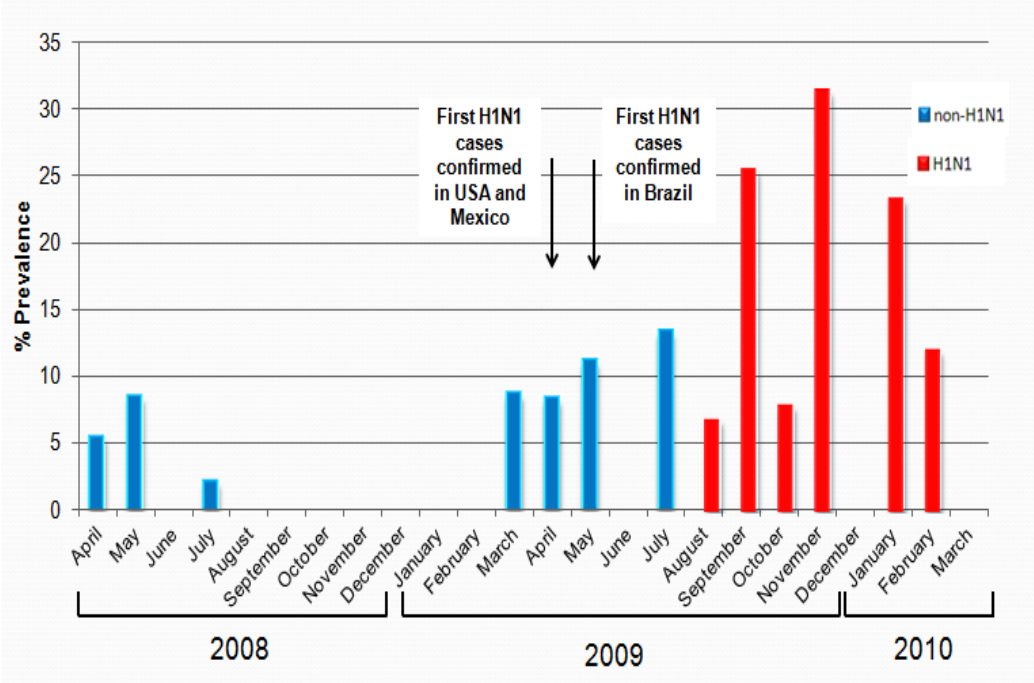


Figure 2.8: Monthly percentage prevalence of non-H1N1 influenza (blue) and novel H1N1 influenza (red) over the 24 month study period.



2.4 Discussion

2.4.1 Overview

This findings of this investigation are divided into two sections; the first analysing the pathogens present in NPA of recruited children, the second assessing the role of the novel H1N1 swine influenza pathogen in this age group.

2.4.2 Pathogen Detection

Over the two-year period, we clearly described the pathogens involved in ARI in pre-school children. At least one pathogen was identified in 87.6% of patients. This detection rate was generally high compared to the literature, with many similar studies finding pathogens in only 47-60% of samples [47 115 169]. However, similar levels of detection have been reported in a number of studies with rates of 88-90%,[173 174]. A possible reason for our high levels of detection may be the wide range of pathogens that we tested for. There was no difference in methodology or handling of samples in the two years.

We recorded the clinical diagnoses, with the majority of ARI being LRTI. The most common manifestations were bronchiolitis (53%) and pneumonia (30%) (**Figure 1.1**). EVW and URTI were less common (9% and 6% respectively) and 2% of children were classified as “other”. The most common diagnoses being bronchiolitis is not surprising due to the low median age of

our study (7 months). Bronchiolitis is common in this age group, 2% of children under two years are hospitalised with the condition[31].

The most commonly detected pathogens were RSV, AdV, hBoV and hRV (33%, 29%, 25% and 19% respectively). RSV has previously been described as one of the most common pathogens detected in ARI in young children. Berkely et al found RSV to be present in 34% of PCR positive samples in children presenting with LRTI[47]. This probably reflects the young age, bronchiolitis being the most common clinical diagnosis in children recruited (52%). We also showed the seasonal nature of RSV (**Figure 2.6**), a feature which is well documented in the developed world but less so in South America. The seasonal nature correlates closely with Recife's rainy season (April-July). RSV prevalence was significantly lower in the second year of the study. This is due to the second years "RSV season" spanning the artificial cut-off between the first and second year of the study.

Another interesting finding was the high prevalence of Adv in our cohort, previous reports on AdV prevalence in childhood ARI has varied. Both Sung et al and Do et al found AdV present in 5% of samples from children <5 years and <15 years respectively[169 172]. However higher levels of AdV have been reported, 37% of ARI in children <5 years in Jordan and 20% in children <3 years in Alaska[174 175]. Our findings support AdV being a highly prevalent pathogen in paediatric ARI, found in 29% of samples. There was also no clear seasonal variation (**Figure 2.6**). Reasons for disparity in the literature are unknown. The median age studies finding low levels of AdV is higher, with both Sung et al and Do et al's median age being 24 months, compared to studies detecting high levels with Kaplans et al's median age being 5 months

and Singleton et al focusing on children <3 years with 64% <12 months[169 172 174 175]. Our cohort's median age was 7 months and median age in AdV positive children was significantly higher than AdV negative ARI (6 vs. 8 months $p<0.01$). A second possibility for high prevalence is cramped living conditions, the mean number of children sharing a room was 3.1 in the 407(65%) children where information was collected. Historically AdV infection is associated with living in high density conditions[84]. Also it is possible AdV infection may persist in the airway, some studies have shown the same strain of AdV can be present for over 200 days in the same patient[93].

High prevalence of hBoV is also of interest as there has been no consensus on the exact prevalence of hBoV in childhood ARI. Previous studies have suggested prevalence of 3-19%[106]. Our study reports a higher level of hBoV. It has been suggested transmission of hBoV may be by faeco-oral route, being present in 9% of children with gastroenteritis[106]. A possible explanation for our high prevalence is the majority of our cohort lived in favela or slum housing in Recife where faeco-oral transmission of pathogens such as hBoV may occur more frequently. Data collected for our cohort suggested the mean family income (US$276) was significantly lower than the Brazilian mean (US$450)[179].

Less prevalent pathogens were found in frequencies in keeping with the literature on paediatric ARI. PIV was found in 10% of our cohort which is similar to other reported findings of 7-13% of paediatric ARI[47 115 172]. CoV was detected in 4% of samples, similar to reported literature of 1-10%[47 173 180]. In the first year we found high levels of *Mpp* with a demonstrable peak in infections and an association with pneumonia presentations[167]. The

prevalence of *Mpp* was lower in the second year (6%) (**Figure 2.2**) and there was no observable seasonal peak (**Figure 2.6**) 15% of *Mpp* infections did present with the diagnosis of pneumonia.

Co-infection was common in our study (43%) of children, other studies have reported co-infection in 14-22% of ARI presentations[115 172 175]. The most common pathogens present in co-infection were AdV, hBoV and RSV. Wang et al described co-infection rates of 14% with the most common pathogens being hBoV, RSV, hRV and AdV[115]. Our finding of AdV being the most common pathogen involved in co-detection is most probably related to its high prevalence, which was discussed previously. Similarly, RSV being a common pathogen in co-infection is probably due to its high prevalence. hBoV has previously been reported to be associated with co-infection, presenting as a multiple pathogen in 50-70% of cases[106]. Our study found this to be true, with 84% of hBoV positive samples being co-infections.

Overall our results found no correlation with co-infection and hospitalisation (p=0.63). Other studies have found the converse to be true, Semple et al described hMPV and RSV co-infection being associated with severe bronchiolitis[181]. Some pathogens are documented to cause increased severity when simultaneously present e.g. influenza and *S pneumoniae*[182]. Our results do not seem to agree with this but a possible explanation is the large range of pathogens we tested for compared to other studies. Also hBoV is not tested for frequently in similar studies, and was a common cause of co-infection in our cohort. It is plausible that certain combinations increase severity in our cohort but are not apparent due to the large number of pathogens we tested for.

Finally there was significant difference in co-infection between the two years of the study. The most likely explanation for this is the increased prevalence of AdV and hBoV in the second year with both pathogens presenting commonly as co-infections.

2.4.3 H1N1 Pandemic

The timing of our study coincided with the emergence of the H1N1 influenza pandemic. When comparing two years of the study, the pathogen with the most significant change in prevalence was influenza, going from the ninth to the fourth most prevalent pathogen. Most (79%) of these cases in the second year were due to H1N1. Demographic data for individuals with H1N1 were comparable to those with non-H1N1 ARI. We found that the proportion of children with H1N1 who had co-morbidities was not significantly different to non-H1N1 flu and non-Flu ARI. Other studies documenting the pandemic showed children with cardiovascular or respiratory co-morbidities to be at higher risk of severe H1N1 ARI[126]. We did not find this in our cohort, possibly due to type 2 statistical error as many of the studies finding this feature were studying larger populations and the percentage of co-morbidities was higher in the H1N1 group although not significant. Baker et al used data from South Africa, South America and Australia, over 21,000 reported cases, and highlighted was the presence of co-morbidities[183].

In our study median age of children found to have H1N1 was also 8 months, and 63% were below 1 year old. The Flu-CIN cohort reviewed 631 cases of H1N1 and demonstrated 16% of all cases were under 5 years of age,

7% under 1 year[129]. The finding that young children are at high risk is well documented in the setting of H1N1[126]. Our findings would be in agreement. We found no significant difference between age of presentation in the H1N1 positive children compared to non-H1N1, most likely because young age is a well recognised risk factor of ARI in general.

All children in our cohort who suffered H1N1 infection had lower respiratory infection (48% bronchiolitis and 52% pneumonia). H1N1 was significantly more likely to present with pneumonia compared to non-H1N1 ARI. However, the clinical presentation of H1N1 varied from mild febrile illness to diffuse viral pneumonitis[126]. In our study, a significant proportion of children presented with pneumonia, showing the severity of this novel pathogen. The Flu-CIN group found that 35% of children with H1N1 presented with pneumonia[129]. Other groups have described pneumonia frequency in H1N1 positive patients of all ages to range from 18-66%[184]. We demonstrate pneumonia is a common manifestation of H1N1 in a child which implicates it as an important pathogen in severe childhood ARI.

Infection with H1N1 was also associated with hospital admission compared to non-H1N1 ARI. Literature suggests the majority of infections are mild and do not require hospitalisation[116]. Bryant et al analysed the prevalence of H1N1 in <16 year olds and found that in 119 confirmed cases 5 required hospitalisation (4%)[185]. Within our cohort the majority of those presenting with H1N1 (78%), and indeed all causes of ARI (overall 54%) required hospital admission. This finding is larger than Bryant et al and could potentially be the young age of our cohort and the large proportion presenting with pneumonia.

Co-infections were significantly higher in the H1N1 population compared to the non-H1N1 population in our study. The most common pathogens detected simultaneously were AdV and hBoV. In comparison to overall co-infection RSV was not a common pathogen detected with H1N1, due to the H1N1 pandemic being separate to the RSV season (**Figure 2.6**). Co-infection was not associated with increased likelihood of hospital admission. We are unable to comment upon co-infection with bacterial pathogens, with much of the co-infection literature on H1N1 focusing on this topic. Koon et al demonstrated high levels of co-infection in the H1N1 pandemic with *S. aureus* and *S. pneumoniae* being the most commonly co-detected pathogens [186]. Our findings would be in agreement that co-detection is a common feature of novel H1N1 infection.

There was a striking difference in influenza prevalence between the two study years. When further analysed 79% of influenza infections in the second year were of H1N1 subtype. There is little literature on the change in prevalence with the emergence of H1N1, as many studies began collection of H1N1 samples after the pandemic was declared. In Brazil it was reported H1N1 was widespread in July and peak incidence was in August[187]. We report these features to be later in our population with initial appearance in August and peak prevalence in November. We also found no influenza-B (flu-B) infections were detected. This is reasonable as the prevalence of Flu-B is documented to be lower than flu-A, especially in seasonal epidemics. There have been reports of flu-B increasing in prevalence after the H1N1 pandemic[188]. Our findings were unable to show this trend, although our study

only continued for one month after the initial wave and so this change may not yet have become apparent.

2.4.4 Limitations

Our study was able to achieve its original aims and describe the viral pathogens detected in the airways of children presenting with ARI to hospital in Brazil, there were a number of possible limitations which must be acknowledged. The techniques we used were not able to quantify the amount of pathogen that was present in the airways with results being qualitative in nature. This was due to the technique of NPA not guaranteeing to collect the same amount of aspirate fluid at a specific concentration. It would have been of interest to know quantity of pathogens in order to decide whether an acute infection was occurring or the participant had a low level of pathogen present. The nature of PCR technique means that pathogens that may not be involved in active ARI but are present within the airway and this feature must be acknowledged, especially in multiple infections. A possible means of accounting for this would have been the use of a control group but very few studies in the literature recruit such a group. One of the largest studies that achieved this to date was by Singleton et al in Alaska, their findings suggested that the involvement of some pathogens such as hRV, CoV and AdV may not be associated with ARI compared to controls[174].

A second limitation is that we did not test for a wide variety of bacterial pathogens. Although we aimed to assess the viral and atypical bacterial causes of ARI and tested for more pathogens than many studies in the

literature, we did not investigate bacterial causes of ARI, such as Pneumococcus and Hib. It would be reasonable to assume that many patients recruited would have been infected with some of these pathogens. It has been previously shown that C-Reactive protein in the airway sample is associated with bacterial infection, however it was not in the protocol of this study to test for this[189]. It also is of further interest as streptococcus has been shown to interact with influenza infection and an association with respiratory viruses and invasive pneumococcal disease has been previously shown[117].

RT-PCR is a highly sensitive and specific technique, NPA is also shown to have high diagnostic yield[176]. NPA has been shown to be superior to sampling with nasal swab, although some studies have performed both with literature suggesting this may produce higher sensitivity for infections[190]. A possible limitation in our sampling method is that we only assessed the viruses and atypical bacteria within the upper airways and these results may not be representative of the lower airways. Despite these limitations the technique is relatively non-invasive (compared to sampling the lower airways) and can be used in a large number of participants.

Classifications of clinical diagnosis in this study may have varied with different physicians. It is feasible that making definitive diagnoses may have been difficult, especially when differentiating bronchiolitis and pneumonia in a young infant. It is not clear how this possible limitation could have been avoided as further diagnostic tools were not available in the setting of this hospital, but it is acknowledged that this may have occurred.

One final possible limitation is the difference in recruitment between the two years in the study. The reason for this was due to one researcher taking

NPA samples and her availability being less in the second year. There were some differences in demographics with median age being significantly lower in the second year. The difference in median age was one month however with both years having a large proportion of children under the age of 1 year so the difference may be minimal. Details on overall hospital presentations is not collected by the hospital so we cannot comment if the proportion of ARI presentations recruited was comparable between the two years.

2.4.5 Further Work

Our work has been successful in its original aims of analysing viral and atypical bacterial pathogens present in paediatric ARI. It has also raised a number of ideas for further work. Some of the pathogens we found in high levels are in agreement with current literature such as RSV. However others have been reported in higher levels than previously documented. It would be of interest to assess more fully the reasons for high levels of AdV and hBoV. If the reason for this is the slum conditions and high population density in our group this could be achieved by comparing with a different population in a more developed setting.

Co-infection was a common finding in our study and due to the range of pathogens tested for and variety of combinations we were unable to analyse this fully. Further analysis of this could be achieved by comparison to a control group with no respiratory symptoms. Few studies have made this comparison although the few that are able to have raised interesting findings on the role of pathogens, with some established pathogens being suggested to have no

association with ARI[174]. Our study found no association with co-infection and increased severity of ARI although this has been found in previous work, it would also be of interest to analyse this in children.

Finally our study was able to demonstrate the emergence of the novel pathogen H1N1 swine influenza; the clinical differences that were shown show the importance of analysing new pathogens. Possible further work could be to analyse how this pathogen presents in the next flu seasons to assess the populations change in response over time.

2.4.6 Conclusions

The first aim of this study was to assess the causative pathogens of ARI in this population where viral diagnostics are not traditionally available. In this we found a variety of viral pathogens to be detected in the airways of children with ARI. Of the most prevalent pathogens some have been highly studied and were in keeping with the literature, such as RSV although we demonstrated the epidemic nature of this pathogen in a temperate climate where it is relatively less proven. However we identified a number of pathogens presenting at levels that differed from previous literature. We found AdV and hBoV to be common pathogens in our study which could potentially be due to the young age of our patients or the social conditions from which we recruited. Co-infection was a common occurrence in our study and highlights the complexity of pathogen interaction within paediatric ARI suggesting possible areas for further work. Overall we have managed to give both an overview of the pathogens involved in ARI, reporting a high positive rate of 88%, as well as

demonstrating the seasonal nature of specific pathogens. We highlight the severity of ARI in the paediatric population with the majority presenting as lower respiratory infections and hospital admission being required in 54% of patients who presented.

The second aim was to assess the impact of H1N1 swine influenza. We have shown that in one of the populations most at risk of the disease that H1N1 infection presented predominantly as LRTIs and often with more than one infection compared to non-H1N1 ARI. H1N1 infections appeared to be associated with more severe ARI with more children requiring hospitalisation who were infected. Furthermore, the pandemic did not appear to significantly affect the prevalence of other common circulating respiratory viral and atypical bacterial pathogens. Of note, the H1N1 pandemic in this population was dwarfed by the annual RSV season epidemic.

3 An investigation into the prevalence of hRV-C in paediatric acute respiratory infection

3.1 Introduction

3.1.1 Chapter overview

The third chapter of this thesis assesses the prevalence of human Rhinovirus (hRV), including hRV-C, in our preschool cohort with ARI from Recife, Brazil. In hRV-positive samples, genetic amplification techniques were used to type and categorise hRV groups – A, B or C. Demographic and clinical features of all hRV samples were recorded to characterise any features specific to these groups.

3.1.2 Introduction

Human Rhinovirus (hRV), first described in the 1960s and has traditionally been implicated as the most common causative pathogen of URTIs and the common cold (see section 1.3.3)[84]. A cure for the common cold was a much publicised goal in the 1960s due to its frequent nature and large economic burden. This led to much research focusing on hRV and showed its replication cycle to occur at temperatures between 33°C and 35°C. This finding

led to the belief that hRV could only be present in the upper respiratory tract and would not be able to infect the lower airways[97]. Research into hRV continued and by 1987, 100 serotypes of hRV had been characterised by use of viral culture and neutralising antibody studies[95]. These serotypes consisted of two groups based on partial genetic sequences and responses to certain antiviral medications – hRV-A and hRV-B[97]. The hRV serotypes were also divided into “major” and “minor” groups depending on the two main receptors to which the virus would bind; intracellular adhesion molecule-1 (ICAM-1) and low-density-lipoprotein receptor respectively[97]. The discovery of appropriate receptors led to production of targeted antiviral medications but due to excessive cost and low efficacy these were never widely accepted. After this, interest in hRV dwindled and it was widely regarded as a minor respiratory pathogen with effects limited to the common cold.

In recent years the impact of hRV has been re-evaluated. It has been shown that hRV infection plays an important role in lower respiratory infections. Originally it was hypothesised that detection was contamination from the upper airway. However, analysis of secretions from tracheostomies were able to demonstrate definitively that hRV can be found in the lower airway without contamination[191]. The increasing availability of advanced viral diagnostic techniques such as RT-PCR also allowed increased sensitivity compare to previous viral culture techniques. These studies highlighted that hRV was commonly detected in lower respiratory presentations of ARI, with many cases in children who were negative for other pathogens[192].

hRV has also been shown to be one of the most common pathogens detected in asthma exacerbations, found in up to 85% of samples[97]. Young

children infected with hRV have also shown to be at increased risk of episodic viral wheeze in later life with some studies reporting this risk to be even greater than RSV infection[193]. With these newly discovered features of hRV, it is now accepted that it is an important pathogen in paediatric ARI.

In 2005/2006 molecular diagnostic techniques found that some strains of hRV were significantly dissimilar from the previous classification system. Groups in Australia and New York all reported that a new hRV group had been detected in paediatric respiratory disease[194 195]. Despite different strains demonstrating significant variation specific genetic regions have been identified for characterisation of hRV strain, namely the Viral Protein (VP) regions (VP2, VP4) and the traditionally used 5' NCR region[95]. These new strains constituted a new group classified as "Rhinovirus-C" (hRV-C) [95]. The discovery further highlighted the hRV family of viruses as an evolving and changing set of viruses with many undiscovered strains.

Further studies have shown that hRV-C is a common cause of ARI. In some studies, hRV-C has been shown to be more prevalent than hRV-A accounting for 64% - 81% of hRV detected in ARI or asthma exacerbations[100 196 197]. Some studies have also reported clinical differences between hRV-C and other hRV groups. One study showed that it was associated with more severe asthma exacerbations than other groups, although other studies have showed no such difference[198 199].

Overall the novel group hRV-C represents a newly identified family of respiratory pathogens which have commonly been found in paediatric ARI and especially asthma presentations. Recent advances in molecular diagnostics have given the opportunity to analyse these strains in detail. Reports of

increased severity and differing clinical presentations raise concerns that this subgroup may represent an important pathogen that causes burden in childhood respiratory disease. This highlights the need to assess the clinical impact of this novel group of hRV in paediatric ARI.

3.1.3 Aims of this study

This study follows on from the previous chapter detailing pathogens found to be present in paediatric patients presenting with ARI from Brazil. As part of that larger study we will further analyse the samples that were found to have hRV infection present. As stated in Section 1.10 the aims of this investigation is to describe the role of the novel hRV group, hRV-C, in paediatric ARI.

3.2 Methods

hRV strain analysis was performed by members of Professor Peter le Souef's group in Perth, Western Australia.

3.2.1 Reverse Transcription

RNA samples from children in which hRV had been identified by RT-PCR were reverse transcribed to cDNA so that they could be transported easily to Australia. Reagents used included random primers, AMV-RT, RNAse inhibitor and dNTPs (all Promega, USA) which were used to make a master mix. Master mix 30µl was then pipetted into 20µl of each sample, the entire process being performed on ice. Once prepared, sample and master mix were reverse transcribed using a thermocycler TC-512 machine (Techne, UK) with conditions of 35 minutes at 42°C, 20 minutes at 50°C and finally 5 minutes at 85°C. All sample cDNA was then stored at -30°C prior to transportation to Perth, Australia for further analysis. This process was in keeping with that described previously in the literature[200].

3.2.2 Transportation

All samples were packaged in dry ice and sealed; they arrived at their destination within 36 hours when they were returned to storage at -30°C. A

Material Transfer Agreement (MTA) from Liverpool, UK to Perth, Australia was obtained prior to dispatch.

3.2.3 Analysis of Rhinovirus Subtype

The method used by the Perth group to analyse hRV strain and group has been published[100] and is based on a technique pioneered in Professor Jim Gern's laboratory in Wisconsin, USA[200 201]. The method uses a molecular assay that targets the 5' Non-Coded Region (NCR) of the HRV genome to identify strain, a sequence of 260 base pairs which is the most conserved genomic region between different hRV strains[200]. Briefly, cDNA was amplified using two-step amplification using specifically designed. PCR primers and cycling conditions described previously[200]. PCR products were sequenced by the Australian Genome Research Facility. The amplified sequences were then compared to a database of known strains, including both the original 101 hRV strains and the newly identified strains using phylogenetic tree analysis software (ClustalX) to determine strain. The hRV group was assigned based on the 420 bp VP4/VP2 sequence which was also amplified in these strains. Due to the close genetic similarity between hRVs and Enteroviruses (EnV) sequences were also checked with known EnV sequences.

3.2.4 Statistical analysis:

Once each sample was assigned a strain and group, statistical analysis was performed on SPSS v18.0.0 (SPSS Inc, Chicago). Frequencies of hRV pathogens and prevalence's of subgroups were calculated on SPSS and graphs produced on Microsoft Excel (2007, Microsoft Inc). Differences in hospital admissions, prevalence between years, clinical presentations and co-infection were calculated with chi-squared test. Variation between groups was assessed with Kruskall-Wallis Test. Difference in age was calculated with Mann-Whitney U test. A p-value <0.05 was considered significant.

3.3 Results

3.3.1 Patient Recruitment and Demographics

In total hRV was detected by RT-PCR in samples from 118 (18.7%) children from the Recife cohort making it the fourth most prevalent pathogen in the 630 children recruited. Demographics of children were not significantly different between the hRV positive group and those children presenting with non-hRV ARI (**Table 3.1**). Of the 118 hRV positive children 65 (55%) were male. The median age of these children was 6 months (range 0-48 months), with 113 (96%) being under 18 months and 89 (75%) under 12 months of age. Twenty two children (19%) had a history of low birth weight and 47 (40%) had a member of the household who smoked. Co-morbidities were less common in the hRV group compared to non-hRV ARI (3% vs. 6%) but this difference was not significant. The severity of ARI in 67 patients (57%) necessitated admission to hospital.

The most common diagnosis in children with hRV infection was bronchiolitis, which was diagnosed in 68 (58%) of patients. Pneumonia was diagnosed in 30 (25%) and episodic viral wheeze (EVW) / asthma and URTI were diagnosed in 10 (9%) and 6 (5%) of children respectively.

Table 3.1: Patient demographics and clinical diagnoses comparing those with hRV positive ARI and hRV negative ARI.

(EVW = episodic viral wheeze / asthma. URTI = upper respiratory tract infection. All presented as n (%). *= significant difference $p < 0.05$)

	ALL HRV	NON-HRV ARI
N	118	512
GENDER	64(54%) MALE	281(55%) MALE
MEDIAN AGE (RANGE)	6 MONTHS (0-48)	7 MONTHS (0-57)
BIRTH WEIGHT <2500G	22(19%)	70 (14%)
BREAST FED	107(91%)	473 (92%)
CO-MORBIDITIES	4(3%)	28 (6%)
SMOKERS AT HOME	47(40%)	216 (42%)
HOSPITAL ADMISSION	67(57%)	272 (53%)
CO-INFECTION	70(59%)*	200 (39%)*
CLINICAL DIAGNOSIS:		
URTI	6(5%)	29 (6%)
BRONCHIOLITIS	68(58%)	267 (52%)
PNEUMONIA	30(25%)	159 (31%)
EVW	10(9%)	49 (10%)
OTHER	4(3%)	7 (1%)

Figure 3.1: Percentage prevalence of pathogens detected in co-infections with hRV

(AdV = Adenovirus, hBoV = human bocavirus, RSV = Respiratory syncytial virus, *Myc* = *Mycoplasma pneumoniae,* hMpV = human metapneumovirus, PiV = parainfluenza virus, Flu = Influenza virus, CoV = Coronavirus, *Cpp* = *Chlamydophila pneumonia*)

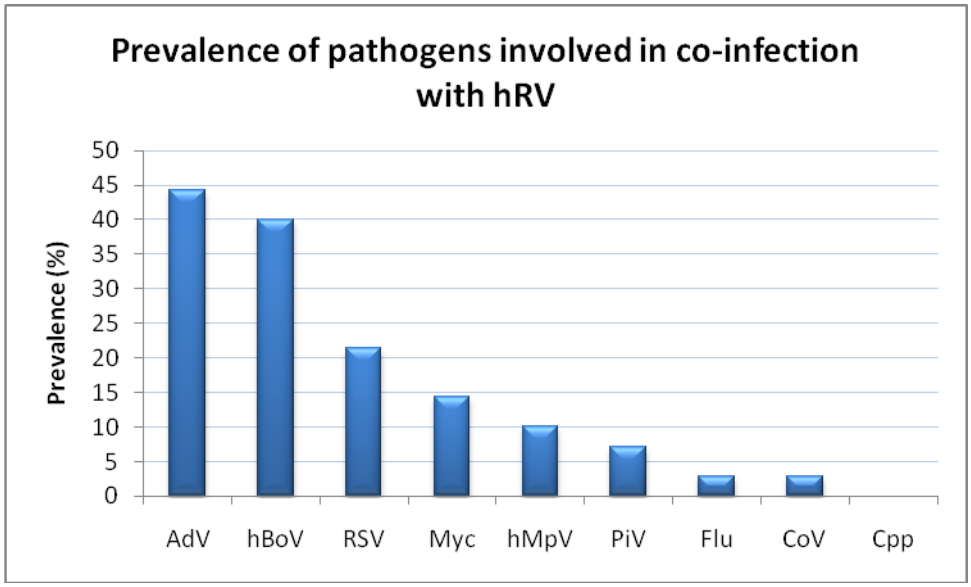
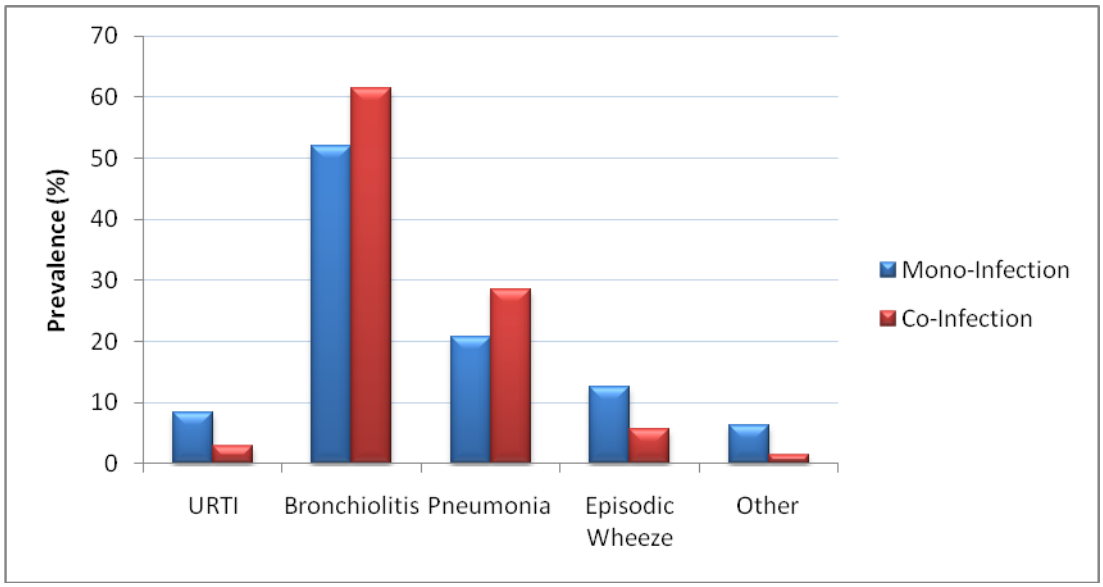


Figure 3.2: Percentage of clinical diagnoses for hRV mono-infections and hRV co-infections (URTI = upper respiratory tract infection. Episodic Wheeze = episodic viral wheeze / asthma. All differences not significant p > 0.05)



Co-infection was a common occurrence in the hRV population with more than one pathogen being detected in 70 (59%) of samples. The most common pathogens co-detected were AdV (n=31, 44%), hBoV (n=28, 40%) and RSV (n=15, 21%), with other pathogens being detected in smaller frequencies (**Figure 3.1**). Children with hRV detected were significantly more likely to have co-infection compared to non-hRV ARI (59% vs. 39% x^2=16.1 p<0.001), this finding remained significant if PCR negative results were removed from the analysis (x^2=6.51 p=0.01). There were no significant differences in clinical diagnoses of those with hRV co-infection compared to mono-infection (**Figure 3.2**). There were no difference in rates of hospital admission in those with mono-infection and co-infection (54% vs. 59% x^2=0.225 p=0.64).

3.3.2 hRV Serotype analysis

All samples had the 5' NCR region amplified and analysed to determine hRV strain and group. Of the 118 samples tested 104 were successfully analysed and characterised. Analysis was unsuccessful in the remaining 14 samples for presumed reasons of human genomic DNA contamination, simultaneous detection of multiple hRV serotype infections, identification of a new strain that required further analysis or failure of amplification (**Figure 3.3**). The successfully amplified samples were shown to contain 49 separate strains. Seventy strains were from the hRV-A group and 29 from the novel hRV-C group (**Figure 3.4** and **Figure 3.5**). Poliovirus or coxsackievirus were found in 5 samples rather than hRV. No hRV-B was detected in and samples. There

was no significant difference in clinical or demographic data between those infected with hRV-A or hRV-C (**Table 3.2**). Co-infection was similarly prevalent in both hRV groups.

3.3.3 Clinical presentations

In both hRV groups the majority of ARI presentations were lower respiratory clinical manifestations with 84% of hRV-A group and 76% of the hRV-C group having ARI of the lower airway. The most common diagnosis was bronchiolitis in both groups, with 56% of hRV-A and 66% of hRV-C being discharged with this diagnosis. Pneumonia was the second most common diagnosis in the hRV-A group with 27% of this cohort having pneumonia. In contrast, EVW/asthma was the second most common diagnosis in the hRV-C group with 21% of children having this diagnosis (**Figure 3.6**). The higher level of episodic viral wheeze in this group was significantly higher than hRV-A with this diagnosis being made in only 3% (x^2=6.676 p=0.01). Upper respiratory tract infection was the least common diagnosis in both hRV groups (7% hRV-A, 3% hRV-C).

Figure 3.3: Reasons for sample exclusion from analysis

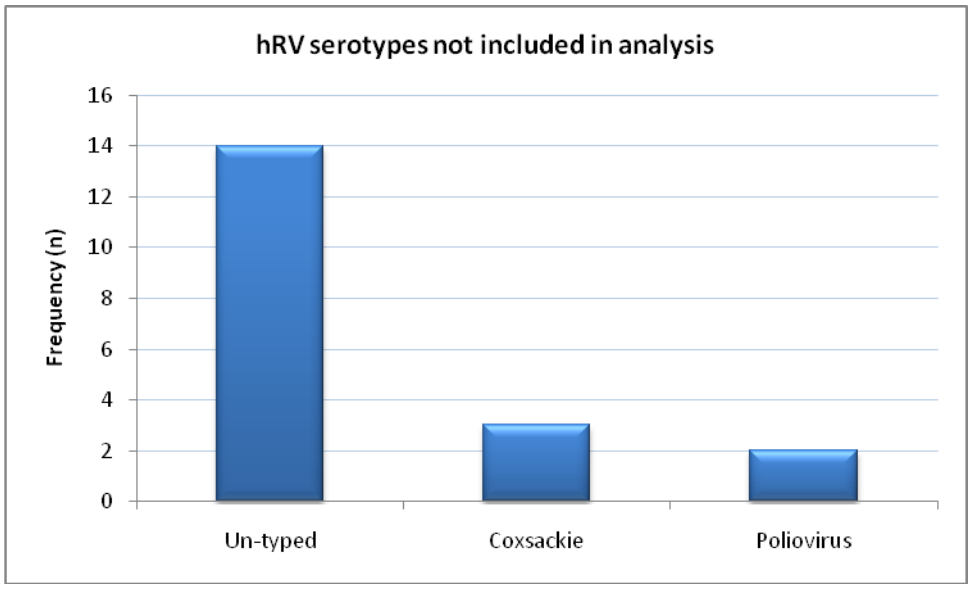


Figure 3.4: Distribution of serotypes from samples of hRV-A group (All shown as frequency (n))

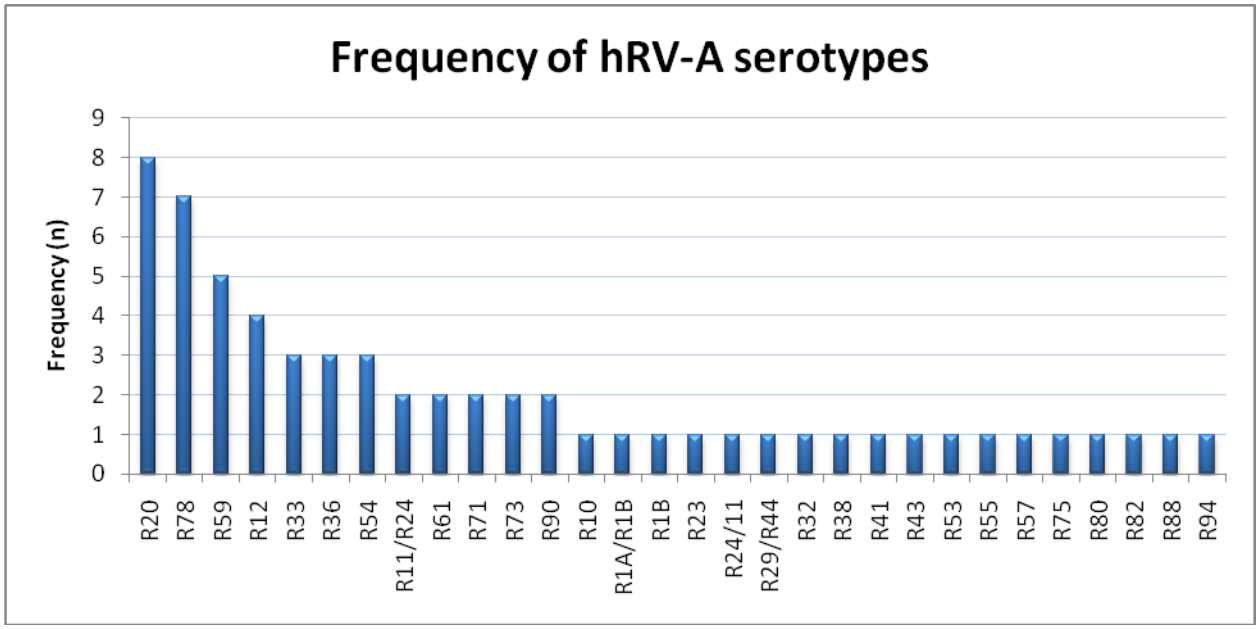


Figure 3.5: Distribution of serotypes from samples of the hRV-C group (all shown as frequency (n))

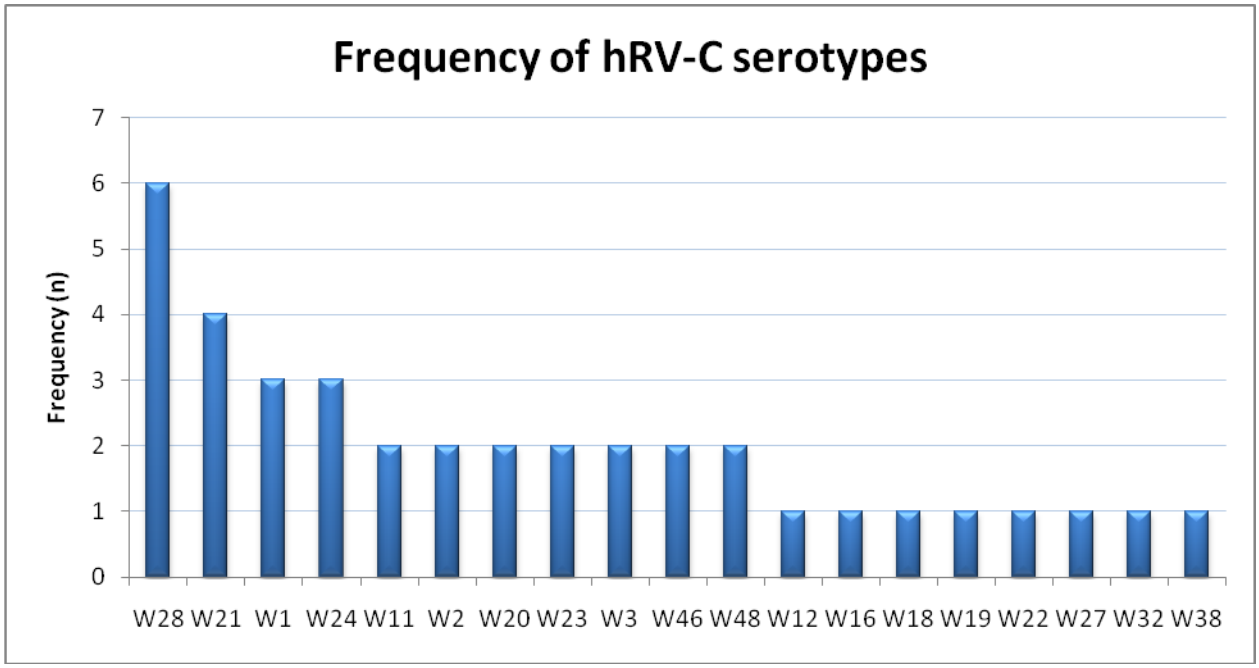
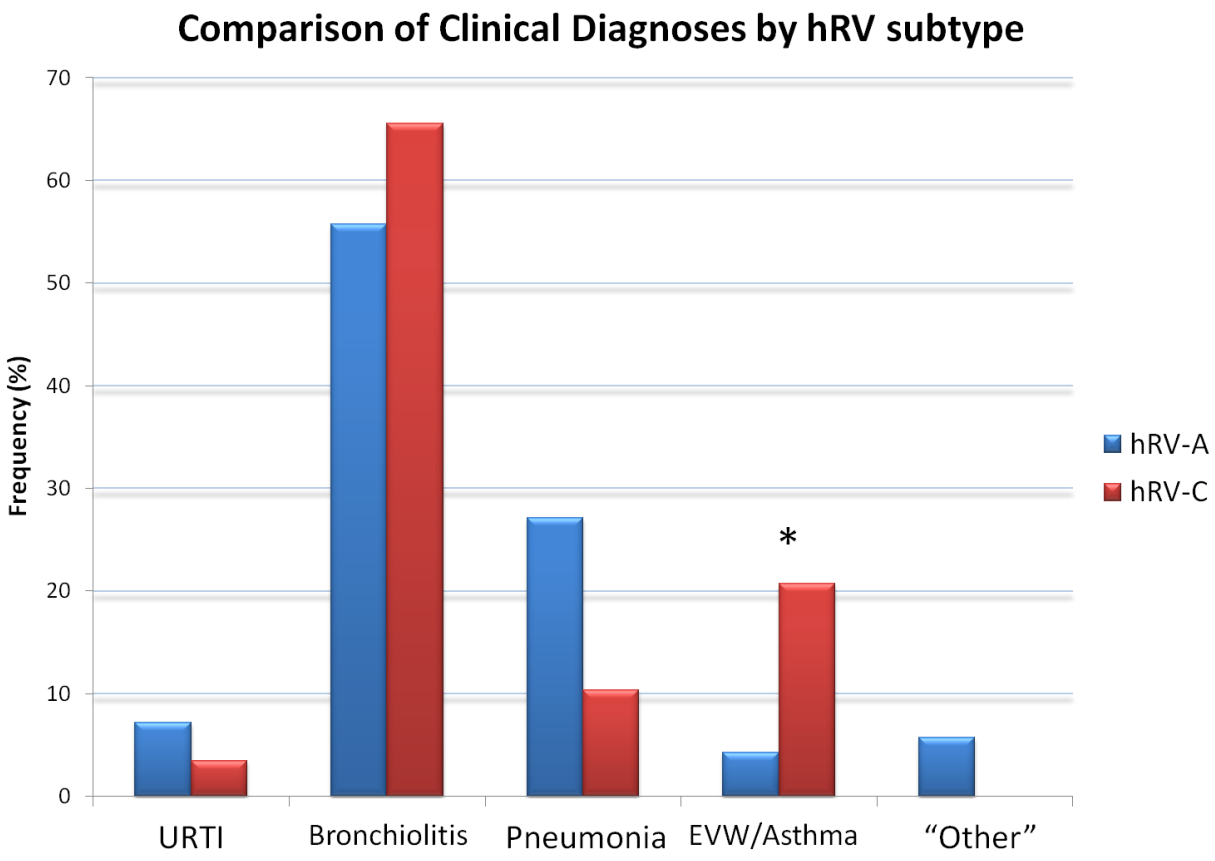


Table 3.2: Patient demographics and clinical diagnoses of those with hRV-A subtype hRV infection and those with hRV-C infection (URTI = upper respiratory tract infection. EVW = episodic viral wheeze / asthma. All presented as n (%). * = significant difference $p < 0.05$)

	HRV-A	HRV-C
N	**70**	**29**
MALE GENDER	**38 (54%)**	**16 (55%)**
MEDIAN AGE (RANGE)	**6.5 MONTHS (0-47)**	**6 MONTHS (0-48)**
BIRTH WEIGHT <2500G	**15 (21%)**	**5 (17%)**
BREAST FED	**61 (87%)**	**27 (93%)**
CO-MORBIDITIES	**3 (4%)**	**0 (0%)**
SMOKERS AT HOME	**32 (46%)**	**8 (28%)**
HOSPITAL ADMISSION	**28 (40%)**	**9 (31%)**
CO-INFECTION	**40 (57%)**	**15 (52%)**
CLINICAL DIAGNOSIS:		
URTI	**5 (7%)**	**1 (3%)**
BRONCHIOLITIS	**39(56%)**	**19 (66%)**
PNEUMONIA	**19 (27%)**	**3 (10%)**
EVW	**3 (4%)***	**6 (21%)***
OTHER	**4 (6%)**	**0 (0%)**

Figure 3.6: Comparison of frequency of clinical diagnoses in each hRV group. (All expressed as percentage (%). URTI = upper respiratory tract infection. EVW/asthma = episodic viral wheeze / asthma. * = significant difference $p < 0.05$)



3.4 Discussion

3.4.1 Discussion

Using genetic amplification techniques we have successfully classified 104/118 (88%) hRV samples in our paediatric population of pre-school children presenting with ARI in Recife. We detected hRV-A in 71% of these samples and hRV-C in the remaining 29%. No hRV-B was detected. Analysis of clinical data within the hRV cohort by group showed that hRV-C was detected more frequently in children with a clinical diagnosis of EVW/asthma. There were 5 samples that were identified to not be hRV and were identified as poliovirus or coxsackievirus. This is understandable due to the genetic similarities between the viruses meaning they were identified as hRV by our probes as they were amplified, and we did not test for these viruses in our 18 pathogen screen to rule out a false positive.

Our findings demonstrate hRV as a common respiratory pathogen. It was detected in 19% of children presenting to Recife with ARI making hRV the fourth most commonly detected pathogen in our study. This is comparable to other ARI epidemiology studies that report prevalence to be 12-26% [115 173 202].

Demographic and clinical features of ARI were similar in children with and without hRV. However, it is interesting to note that the majority of children with hRV infection presented with lower respiratory tract manifestations of infection (bronchiolitis or pneumonia). This further supports the re-evaluation of hRV as an important pathogen of the lower respiratory tract. In the past, hRV has been

viewed as a mild pathogen unable to enter the lower respiratory tract because of its presumed limited ability to replicate only at 33-25°C[97]. Louie et al found that hRV was commonly present in children with pneumonia, bronchiolitis and asthma that required admission to paediatric ITU[203]. Our findings are similar and demonstrate the majority of hRV infections detected to be diagnosed with lower respiratory pathology and only 5% of hRV ARI being confined to symptoms of the upper respiratory tract. These findings should be considered carefully because NPA collects samples from the upper airway and so we are assuming hRV present in the upper airway is involved in LRTI in our patients. A sampling method such as broncheoalveolar lavage or endotracheal aspiration would be required to confirm presence in the lower airway.

In this cohort hRV-A was the most common hRV group (71%) while hRV-C accounted for all remaining hRV strains that were successfully analysed (29%). Considering the large size of our cohort it is surprising that hRV-B was not detected, however this group has been reported in low levels previously (0-3%)[196 197 204]. The 29% prevalence of hRV-C is directly comparable to 35% and 36% of hRV infections in children from Spain and China respectively[199 205]. However, a number of groups have reported larger proportions of hRV infections to be hRV-C, in 56-68%[100 206]. The reasons for this discrepancy are unclear but lie in the presentations of children recruited to the study. Both Arden et al and Bizzintino et al recruited children with asthma exacerbations and found the prevalence of hRV-C to be higher than our results; 56% Arden et al, 68% Bizzintino et al[100 206].

It has been well documented in the past that hRV infection is a significant cause of asthma exacerbation in the paediatric patient and it would seem

reasonable that the burden of novel hRV-C would be greater in this group of presentations. The findings of Bizzintino et al also demonstrate that hRV-C is associated with an increased severity of asthma exacerbation further suggesting that hRV-C plays an important role in asthma and viral induced wheeze[100]. In contrast, and similar to our study, hRV-C prevalence appears to be lower in ARI. Calvo et al analysed NPA samples from children hospitalised with ARI and reported 35% of hRV were hRV-C. Yu Jin et al also reported 36% of hRV to be hRV-C in children hospitalised with ARI[199 205].

We found that hRV infection was present in all clinical presentations of ARI. Interestingly bronchiolitis was the most common presentation in the subgroups detected (hRV-A 56% and hRV-C 66%). In the hRV-A positive group pneumonia was the second most common clinical presentation (27%) whereas EVW was in hRV-C (21%). We found that hRV-C subtype was significantly more likely to present as viral induced wheeze compared to hRV-A. This finding supports the hypothesis that hRV-C is a significant cause of asthma exacerbation and wheeze in the paediatric population.

Few studies have included all ARI presentations and analysed for hRV group associations with clinical diagnoses. A study by Lau et al in Hong Kong included children and adults admitted with ARI and found a similar occurrence, with wheeze being more common in the hRV-C population[207]. Calvo et al undertook a similar analysis in children with ARI in Spain and found a higher proportion of hRV-C positive children to have asthma/recurrent wheeze but this difference was not significant (hRV-C 46% vs. hRV A 39%).

Overall we have demonstrated hRV to be an important pathogen involved with paediatric ARI, being detected in 19% of our population and

being the fourth most prevalent pathogen. Clinically hRV presented as a variety of clinical manifestations, with the majority being either bronchiolitis or pneumonia which further supports the literatures re-evaluation of hRV as an important pathogen in lower respiratory infection. When analysing for the novel group, hRV-C, we found it to be commonly detected in 29% of hRV infections. Demographically there were no significant differences in hRV group but clinically we found hRV-C to be associated more commonly with EVW/ asthma compared to hRV-A. This is an important finding as studies involving asthma exacerbations have reported higher prevalence's of hRV-C[100 206]. Our findings appear to support the current literature and highlight the novel subtype of hRV-C as an important pathogen in paediatric asthma and wheeze.

3.4.2 Further Work:

Since its relatively recent discovery in 2006 much interest has been generated by hRV-C. We reported our findings in paediatric ARI, demonstrating hRV to be frequently detected in children with LRTI and demonstrating hRV-C to be more frequently associated with EWV/asthma than hRV-A.

Further work is needed to confirm these findings of the association of hRV-C with asthma. Although significant, the numbers of children with the diagnosis of EVW/asthma in our cohort was low. To date no research has been conducted on this pathogen in the UK and this would provide an ideal setting for a large investigation to confirm our results. The lack of a control group is especially interesting, other studies have reported hRV to be detected in high

levels in asymptomatic patients[174]. Further work could possibly accommodate this also.

Our findings in context with current literature highlight hRV-C to be associated with asthma pathogenesis. Recent discoveries have allowed hRV-C to be cultured for experimentation[208]. It would be of great interest to analyse why there is an association with asthma, in cell or animal models.

4 An investigation into the role of Interleukin 17 in RSV infection

4.1 Introduction

Infection with RSV commonly occurs in infancy, with the majority of children being infected by 2 years of age. In the developed world, 1-2% of all infants having sufficiently severe infection to require hospitalisation, most commonly with bronchiolitis[137]. Hospitalisation with RSV infection in infancy has been linked to respiratory dysfunction in later life, with the prevalence of wheezing being increased in children up to 13 years of age[37 38].

T-Helper (Th) cells are important in the pathogenesis of RSV bronchiolitis. Originally they were classified into T-helper 1 and T-helper 2 cells (Th-1 and Th-2) according to their secreted cytokine profile. The response of Th-1 cells are characterised by expression of cytokines the Interferon gamma (IFN-γ) and Tissue necrosis factor alpha (TNF-α)[209]. In contrast Th-2 cells express the cytokines IL-4, IL-5, IL-9 and IL-13[209]. The immune response to respiratory viral infections normally favours a response from the Th-1 subclass of T-helper cells[31]. However in the case of RSV bronchiolitis elevated levels of the cytokine IL-4 have been described, suggesting a role for these cells in disease pathogenesis[31].

Findings that children with atopic asthma in later life have elevated levels of Th-2 cytokines, such as IL-13, led to the hypothesis that an imbalance of Th-1/Th-2 cells is involved in asthma pathogenesis and may be linked to the

action of RSV in infancy[210]. IL-13 has a pleotrophic effect with an important role in airway hyper-responsiveness and airway remodelling of asthma[211]. IL-13 has been found in high levels during RSV infection and is a suggested target of bronchiolitis therapy[212 213]. Limited success of Th-2 targeted drugs further suggested cells other than Th-1/Th-2 to be involved in airway pathology[214 215].

In recent years a novel group of T helper cells were discovered that did not match the classical Th-1 or Th-2 classification. This distinct group of CD4+ T helper cells secreted the pro-inflammatory cytokine IL-17 and so were termed Th-17 cells. Th-17 cells have shown to be important in the pathology of many diseases including Crohn's disease, sarcoidosis, rheumatoid arthritis and atherosclerosis[216]. Experiments involving mouse and human models have also shown that IL-17 plays a role in both bacterial and viral infections and provides an important link between the innate and adaptive immune response[216]. The first discovered cytokine, IL-17A has now shown to be one of a family of six which have been classified IL-17A-F[217]. The cytokines IL-17A and IL-17F are produced primarily by activated CD4+ T cells and are the most researched[217 218]. The receptors for both of these IL-17 cytokines are present on the epithelial cells of the lung[218 219]. A raised response to metacholine challenge has also been shown to correlate with levels of IL-17 in the sputum, suggesting that IL-17 is involved in airway hyper-responsiveness[220]. However, in the case of RSV bronchiolitis elevated levels of the cytokine IL-4 have been described, suggesting a role for these cells in disease pathogenesis[31].

IL-17 has shown to be important in both neutrophil recruitment and acute inflammation, causing expression of the cytokines IL-8 and IL-6 from

airway epithelial cells[221]. The presence of IL-17 has also been shown to increase the effect of other inflammatory cytokines. Synergy has been demonstrated with IL-1β and TNF-α causing significant increase in pro-inflammatory cytokines when IL-17 was present[222 223]. Both of these findings highlight IL-17 as an important cytokine in acute inflammation, a key feature of RSV infection of the airways in the lungs of infants. It also raises questions over the role of IL-17 in RSV infection. To date no study has measured the response of airway epithelial cells to RSV infection in the presence of IL-17.

In this chapter, the effects of both IL-13 and IL-17(specifically IL-17A) and IL-13+IL-17 together have been examined on airway epithelial cells in the context of RSV infection. An immortalised airway epithelial cell line (BEAS-2Bs) has been used as the model in which to assess these effects with the inflammatory cytokines IL-6 and IL-8 used as outcome measures.

4.2 Methods

4.2.1 Cell Culture

To measure the response of airway epithelial cells to stimulation with cytokines and RSV, the immortalized airway epithelial cell line BEAS-2Bs was used. These cells were cultured in a T75 flask, previously coated with collagen buffer for 24 hours. BEAS-2Bs were grown in a solution of BEGM, with medium being changed three times per week and a phosphate buffered saline (PBS) wash being performed at each change (Details of reagents and manufacturers for this chapter can be seen in **Appendix A.3**). Once the T75 flask of cells reached confluence, the cells were transferred into a 96-well culture plate at a density of 30,000 cells/cm^2 and grown in BEGM until they were 70% confluent.

4.2.2 RSV infection and cytokine stimulation

Cultures were infected with 0.25 multiplicity of infection (MOI) RSV-A2 and stimulated with IL-17A or IL-13 or IL-17 and IL13 at concentrations of 1ng/mL, 10ng/mL and 100ng/mL. Dr Fonceca and Dr McNamara had previously found that an MOI of 0.25 caused significant inflammation but did not destroy the cultures [224].

Stimulated and infected cultures were harvested at 24 and 48 hours. Cell supernatants were collected and stored at -70°C and RLT buffer added to cultured cells and also stored at -70°C until needed for analysis.

4.2.3 ELISAs

Secretion of IL-6 and IL-8 from stimulated or infected airway epithelial cells was measured by ELISA (R&D systems) according to manufacturer's instructions. Samples were diluted five times with reagent diluent in non-RSV infected supernatants and ten times in RSV infected supernatants. Results were calculated by use of a standard curve as described by manufacturer. The ELISAs were sensitive to a concentration of 30pg/mL.

4.2.4 Statistics

Statistical analysis was performed using the statistics package SPSS (version 18.0.0). Results are presented as average +/- standard deviation or 1 standard error (2 standard errors in graphs). In the case of normally distributed data the student-t test was used to compare variation between samples, with ANOVA utilised to analyse variation across groups. Results were considered significant if $p \leq 0.05$. For ELISA data results were presented as value ± standard error of mean (± 2 in graphs), with results shown to 3 significant figures.

4.3 Results

4.3.1 – IL-6 responses in BEAS-2B cells infected with RSV and stimulated with IL-17 and IL-13

Secreted IL-6 from airway epithelial cells stimulated by IL-17, IL-13 and IL-17+IL-13 in the presence and absence of RSV infection was measured by ELISA.

At 24 hours, IL-6 concentrations were significantly raised in RSV-infected compared to non-infected culture without addition of cytokine supernatants (RSV-infected control: 13,800±6620 pg/ml control: 700±92 pg/ml p=0.02). However, there were no other significant differences between control cultures and RSV infected without cytokine present and/or cytokine stimulated cultures (**Figure 4.1 A**, and **Figure 4.1B**).

At 48 hours IL-6 responses to IL-17 and IL-17+IL-13 stimulation in the presence of RSV infection were significantly greater than those in un-infected cultures where RSV was not present (**Figure 4.2 A** and **Figure 4.2 B**). In RSV infected cultures IL-6 secretion was significantly greater with addition of cytokine than RSV infected cultures without cytokine when stimulated with 10ng/ml IL-17 (109,000±29300pg/mL vs. 25,900±4880 pg/mL p=0.049) and 100ng/mL IL-17 (120,000±19500 pg/mL vs. 25,900±4880 pg/mL p=0.009).Although mean IL-6 production was increased in response to IL-13, no significant differences were observed for any concentration of IL-13 compared to the RSV-infected control.

Figure 4.1 A: IL-6 production (pg/mL) by BEAS-2B cells stimulated with IL-17 and/or IL-13 in the presence or absence of RSV infection at 24 hours.

(* = p<0.05 compared to control in RSV negative / RSV control in RSV positive samples)

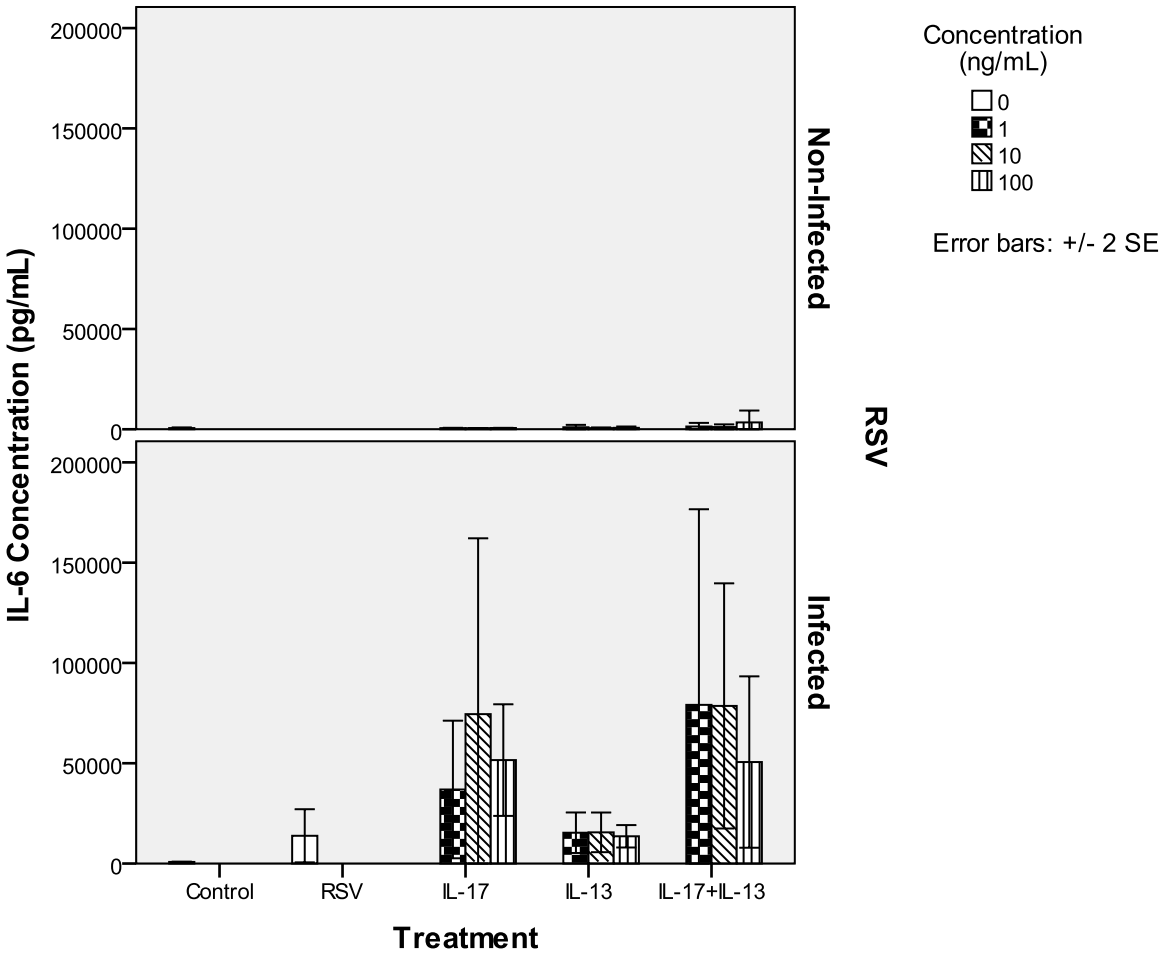


Figure 4.1B: IL-6 production (pg/mL) by BEAS-2B cells stimulated with IL-17 and/or IL-13 in the absence of RSV infection at 24 hours.

(* = p<0.05 compared to control in RSV negative / RSV control in RSV positive samples)

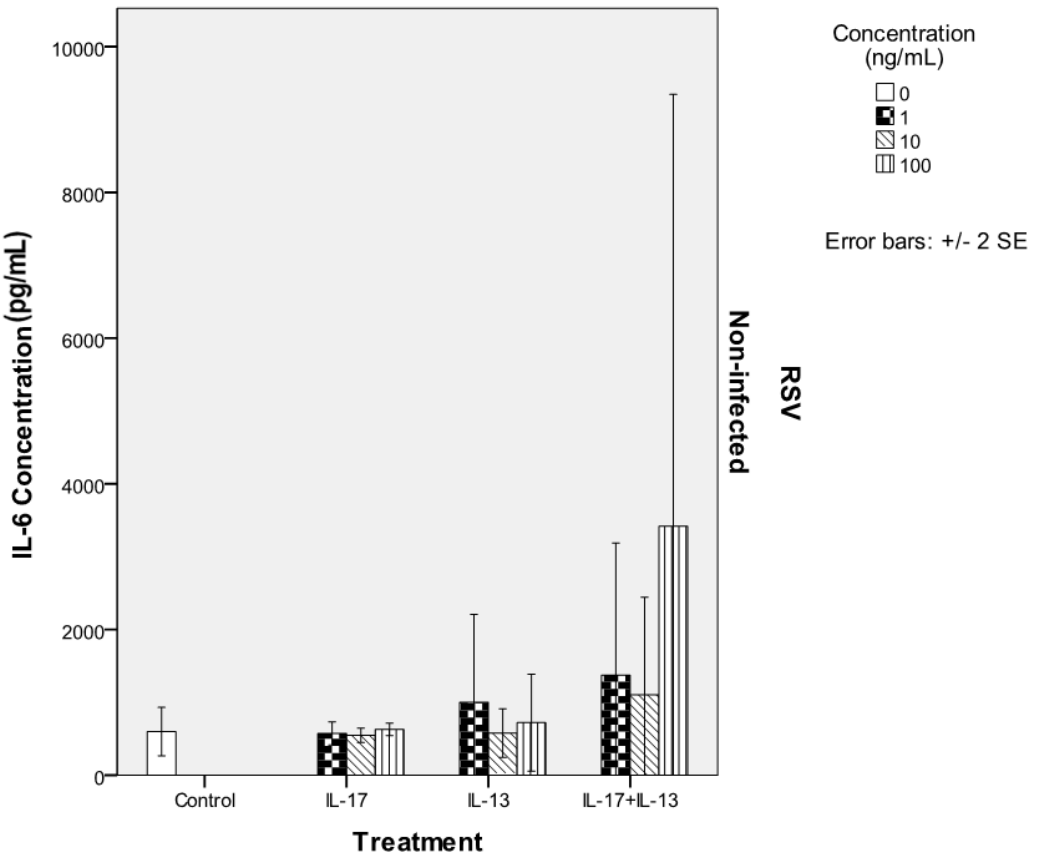


Figure 4.2 A: IL-6 production (pg/mL) by BEAS-2B cells stimulated with IL-17 and/or IL-13 in the presence or absence of RSV infection at 48 hours.

(* = p<0.05 compared to control in RSV negative / RSV control in RSV positive samples)

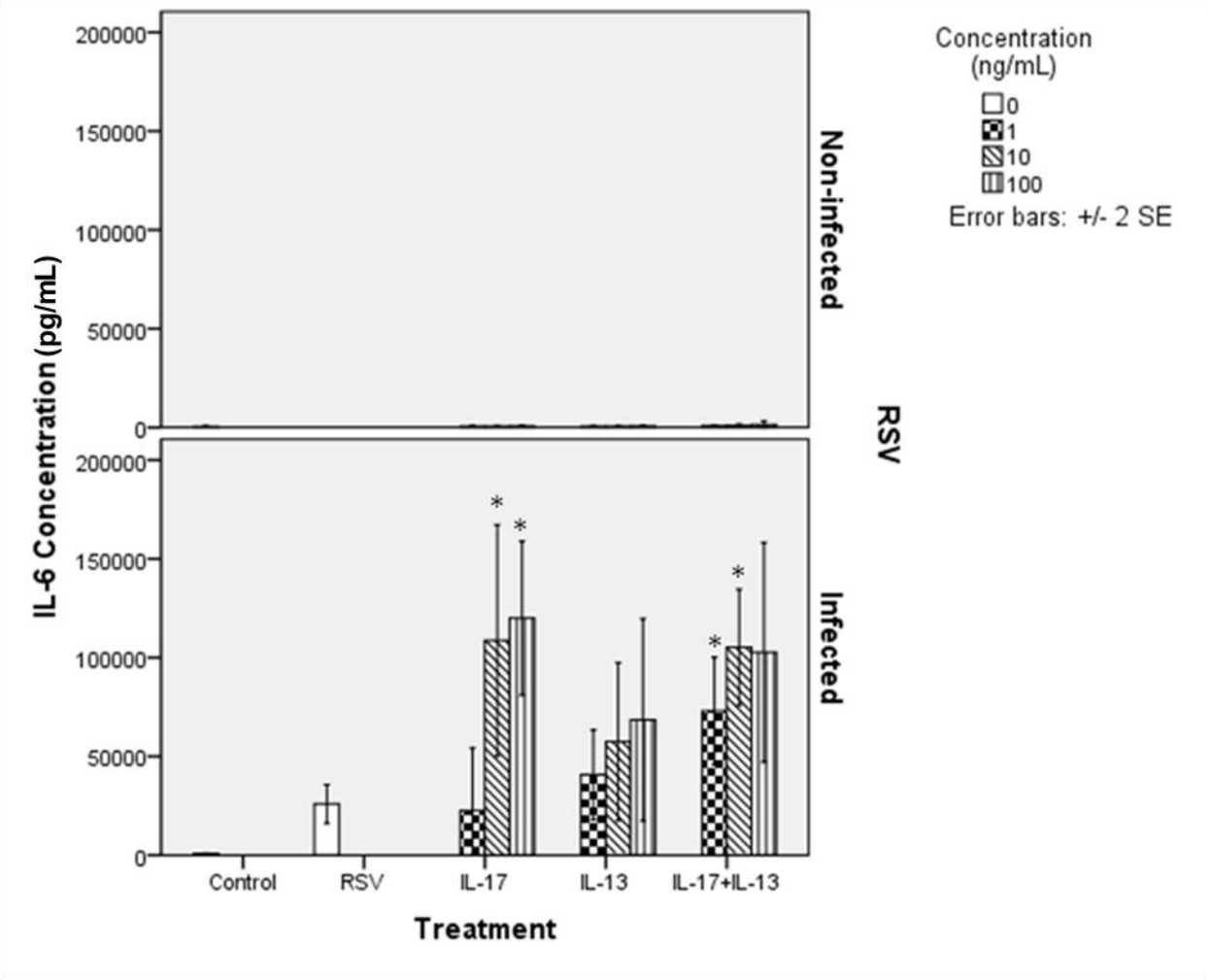
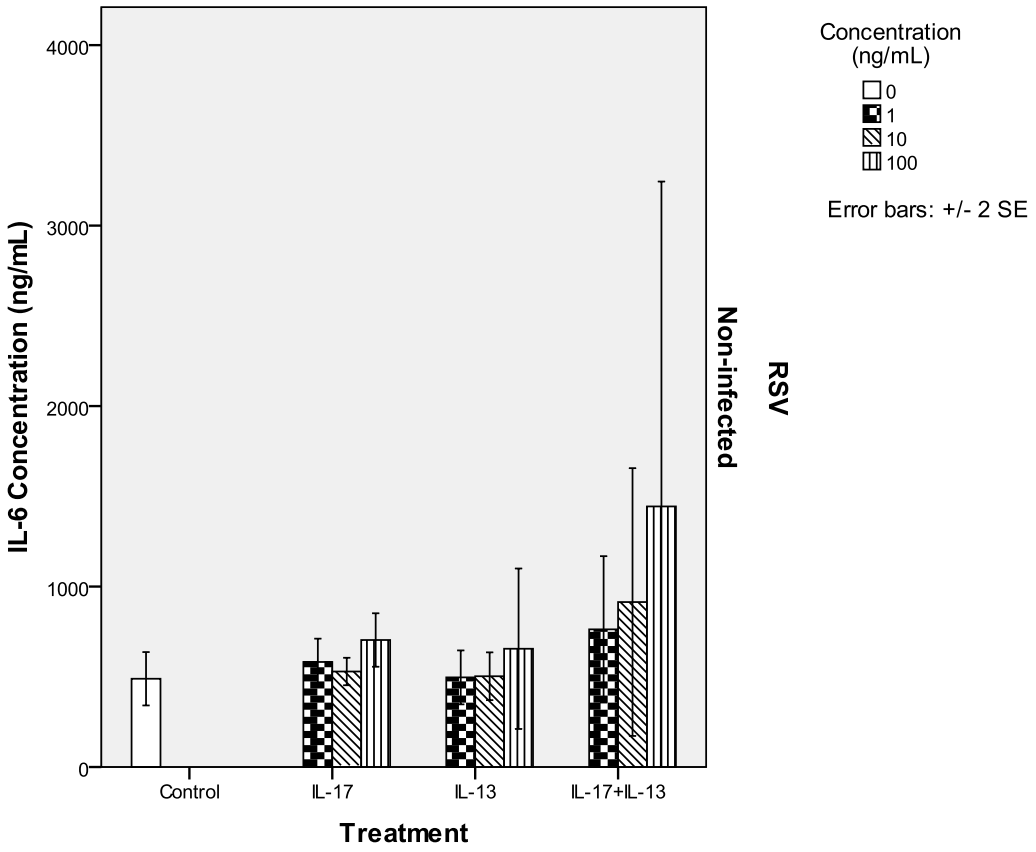


Figure 4.2 B: IL-6 production (pg/mL) by BEAS-2B cells stimulated with IL-17 and/or IL-13 in the absence of RSV infection at 48 hours.

(* = p<0.05 compared to control in RSV negative / RSV control in RSV positive samples)



IL-6 production was increased when stimulated simultaneously with IL-17 and IL-13 in the presence of RSV compared to RSV control at concentrations (both cytokines) of 1ng/mL (73,000±13,600 pg/mL vs. 25,900±4,880 pg/mL p=0.031) and 10ng/mL (105,000±14,700 pg/mL vs. 25,900±4,880 pg/mL p= 0.007) with a trend observed at a concentration of 100ng/mL (10,300±48,100 pg/mL vs. 25,900±4,880 pg/mL p=0.053).

4.3.2 – IL-8 responses in BEAS-2B cells infected with RSV and stimulated with IL-17 and IL-13

Secreted IL-8 by BEAS-2B cells was measured in a similar manner to IL-6.

At 24 hours significant increases in IL-8 expression were observed in the presence of IL-17 and RSV infection (**Figure 4.3**). IL-8 production was greater when stimulated with IL-17 at a concentration of 1ng/ml (799±21 pg/mL vs. 571±75 pg/mL p=0.042) and 100 ng/mL (916±65 pg/mL vs. 571±75 pg/mL p=0.025). The mean IL-8 expression at 10 mg/ml was higher than RSV control but this was not significant. There was no significant response to stimulation with IL-13. In the presence of IL-17 and IL-13 at 1ng/ml significant expression was observed (861±63 pg/mL vs. 571±75 pg/mL p=0.04).

At 48 hours no significant increase in IL-8 expression was observed with any combination of cytokines (**Figure 4.4**). Similarly to 24 hours there was no observable increase in IL-8 expression comparing RSV infected control to RSV non-infected control (429±78 pg/mL vs. 658±60 pg/mL p=0.13).

Figure 4.3: IL-8 production (pg/mL) by BEAS-2B cells stimulated with IL-17 and/or IL-13 in the presence or absence of RSV infection at 24 hours.

(* = p<0.05 compared to control in RSV negative / RSV control in RSV positive samples)

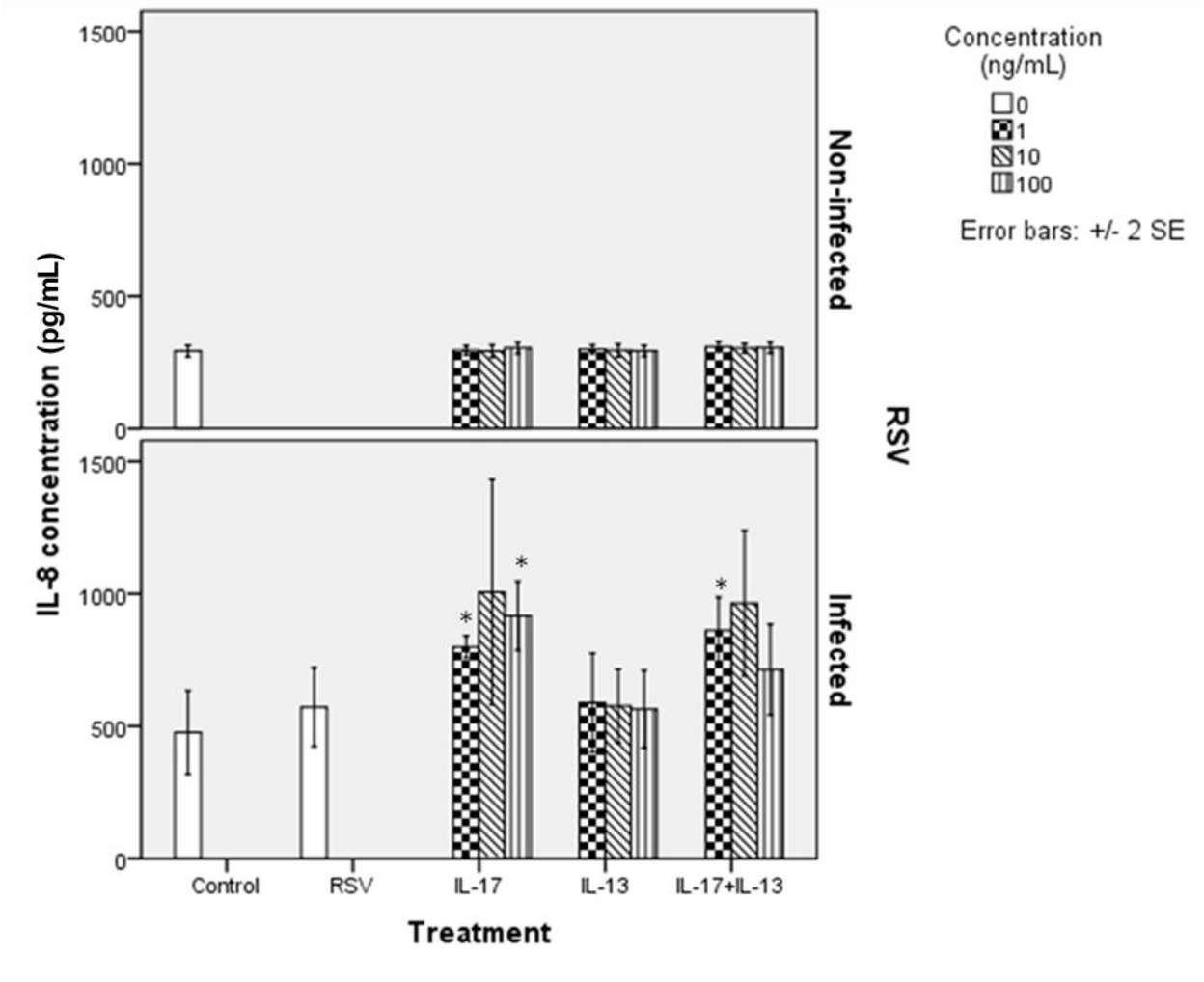
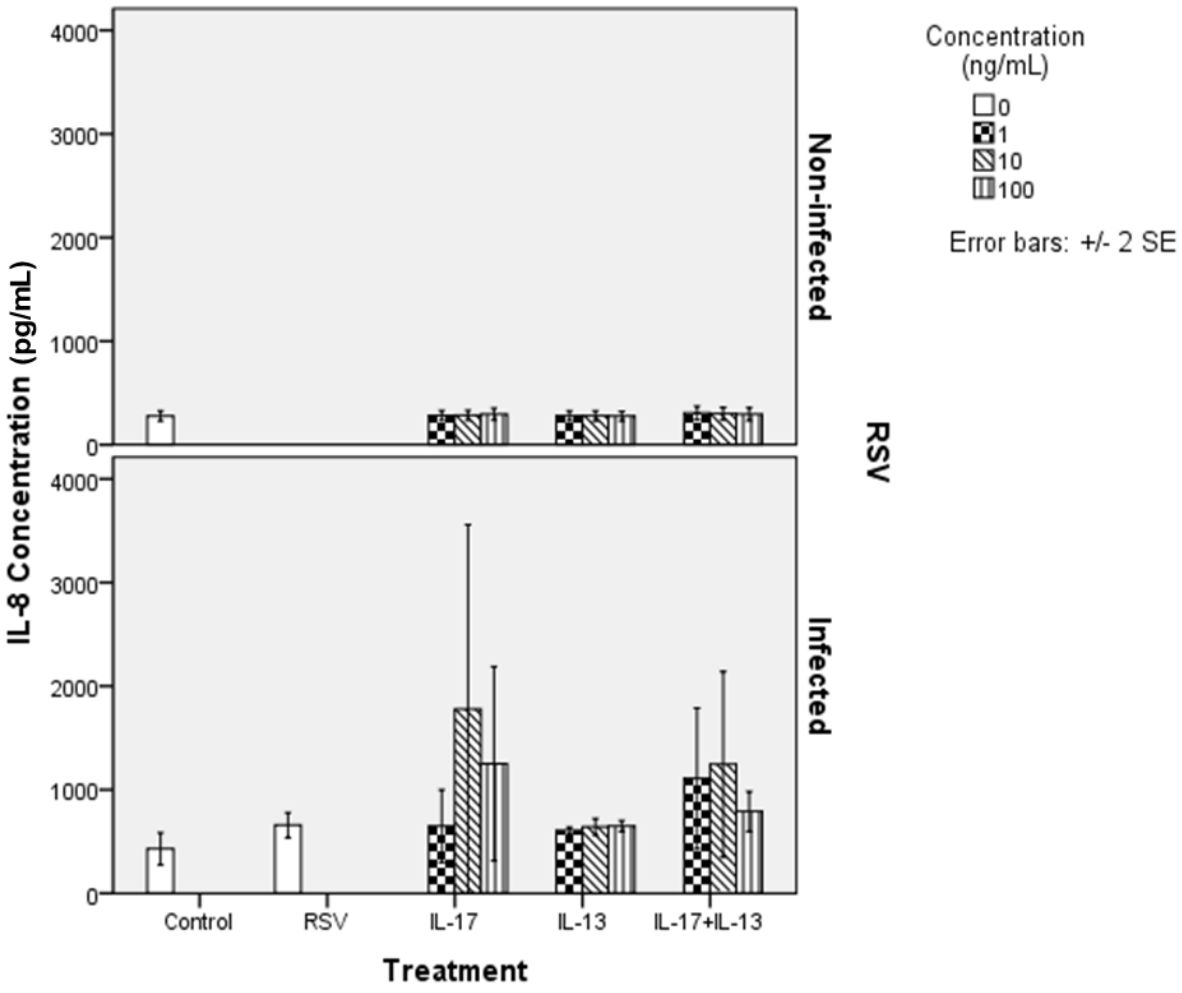


Figure 4.4: IL-8 production (pg/ml) by BEAS-2B cells stimulated with IL-17 and/or IL-13 in the presence or absence of RSV infection at 48 hours.

(* = p<0.05 compared to control in RSV negative / RSV control in RSV positive samples)



4.4 Discussion

In the presence of RSV infection, IL-17 caused significantly increased expression of the pro-inflammatory cytokines IL-6 at 48 hours and IL-8 at 24 hours. There was no significant change in secretion of these cytokines by BEAS-2B cells with IL-17 in the absence of RSV infection. The cytokine IL-13 demonstrated no effect on the expression of either of these cytokines in the presence or absence of RSV. These findings suggest that IL-17 and IL-13 do not act in a synergistic way to induce expression of IL-6 and IL-8 in an airway epithelial cell model.

4.4.1 – IL-6 expression of BEAS-2Bs in response to stimulation

In the absence of RSV infection, IL-6 production by BEAS-2B cells was not significantly altered upon addition of varying concentrations of IL-17. This result is in contrast to that reported by Jones et al who found that IL-17 caused significantly increased expression of IL-6 by primary human bronchial epithelial cells[221]. Jones et al used primary airway epithelium from human samples whereas we used the immortalised cell line BEAS-2Bs. It may be the case that primary cells demonstrate a greater response to IL-17 stimulation[221]. However Van den Berg et al reported that IL-17 produced no significant increase in IL-6 expression in the absence of other cytokines[223]. This study was also completed in primary airway epithelial cells and only upon addition of TNFα and IL-17 was a response induced [223]. Our results agree with Van den Berg et

al showing IL-17 in isolation has no significant effect on IL-6 expression in the bronchial epithelium.

On addition of RSV-A2 we found a large increase in epithelial cell expression of IL-6. This finding is in keeping with the literature as IL-6 is a well recognised early cytokine in RSV infection and has been found to be elevated in the airways of infants with bronchiolitis, with airway epithelial cells a proven source[225 226]. At 24 hours there were no significant differences in the groups treated with differing cytokine concentrations. There was a possible trend to IL-17 inducing higher levels of IL-6 compared to RSV control, especially at a concentration of 100ng/mL, but this result was not significant. However, at 48 hours significant increases in IL-6 production were seen with IL-17 present at concentrations of 10ng/mL and 100ng/mL, compared to RSV infected control. No group has yet reported the effect of IL-17 on airway epithelium cells in the context of RSV infection. Wiehler et al demonstrated that hRV-16 infection caused significantly elevated expression of inflammatory cytokines if IL-17 was present, including IL-6 expression[227]. Weihler et al went on to suggest that IL-17 had an effect at the post-transcriptional level and may have implications on other inflammatory cytokines and immune cell recruitment[227]. Similarly our results suggest in the presence of IL-17 and RSV the cytokine milieu is conducive to increased IL-6 expression from airway epithelial cells. Our findings appear to be similar to Weihler et al's but are the first reported description of this in RSV infection of epithelial cells. Jones et al reported the effect of IL-17 on primary airway epithelial cells and also demonstrated that IL-6 production was increased of 48 hours compared to 24 hours[221]. With similar methodology Jones et al stimulated primary human bronchial epithelial cells

with IL-17 and found significant increase of IL-6 at 24 hours and even greater at 48 hours[221]. Previous studies have demonstrated IL-17 to have an effect on mRNA at the post-transcriptional level, increasing the concentration of mRNA in epithelial cells when responding to the cytokines TNFα and IL-1β[222 223]. Together these reported findings support the findings of significant increases of IL-6 being seen at 48 hours rather than 24.

A role for IL-6 has been demonstrated in the induction of IL-17 and differentiation of naive T cells to the Th-17 subtype[228]. In an environment with IL-6 present it has been shown naive Th cells differentiate into active Th-17 cells by a STAT3 mediated mechanism[229]. This suggests IL-17 present during RSV infection might further induce IL-6 from the airway epithelium and thereby a possible source of IL-6 causing TH-17 differentiation. If this were to cause a positive feedback response, raised IL-17 would be likely to cause rapid neutrophil recruitment and airway neutrophilia, similar to that witnessed in over 50% of asthma exacerbations[213].

Our results show IL-6 was not raised when airway epithelial cells were stimulated with IL-13, suggesting IL-13 may have a minor role in acute inflammation of the airway epithelium. Previous literature has described IL-13 to be involved in eosinophilia of the airway, a feature associated with chronic asthma as opposed to severe asthma exacerbations[212 230]. Our results suggest IL-13 may be effect a cell type other than airway epithelial cells. IL-13 causing IL-6 expression in airway epithelial cells has not been described previously.

Given that IL-13 in isolation produced no significant effect on IL-6 expression it was perhaps not surprising that a synergistic effect of IL-13 and IL-17 on epithelial cell IL-6 secretion was not observed. IL-6 production by

samples stimulated with IL-17 and IL-13 was significantly increased in the presence of RSV infection at 48 hours. However at these concentrations IL-17 in isolation caused significant increase in IL-6 expression and so it is unlikely IL-13 was causative of this increase. Previous studies which have demonstrated synergy with the cytokines TNF-α and IL-1β, have both shown the respective cytokines to cause expression of IL-6 prior to addition of IL-17[222] [223].

4.4.2 – IL-8 Expression of BEAS-2Bs in response to cytokine stimulation

We found that in the absence of RSV infection there was no significant change in IL-8 protein production in BEAS-2B cells exposed to IL-17. Previous reports have also shown no significant increase in IL-8 production when exposed to IL-17 in isolation[221]. In addition, studies by Dragon et al and Van den Berg et al found that IL-17 in isolation did not induce a significant change in IL-8 production in primary airway cells when treated with similar concentrations of IL-17[222] [223]. Earlier studies demonstrated IL-17 as a potent inducer of IL-8 in peripheral blood mononuclear cells (PBMC) which express an IL-17 RA and IL-17RC receptor[217] [231]. Despite the presence of these receptors in airway epithelial cells our results suggest that IL-17 does not cause expression of IL-8 in BEAS-2B cells.

Upon addition of RSV infection to our samples significant increases in IL-8 protein production were observed. There was no significant increase in IL-8 expression upon addition of RSV. Oh et al has previously demonstrated IL-8 to not be significantly elevated in NPA samples of children with RSV

infection[225]. However Larranaga et al found the converse with IL-8 significantly raised in the NPAs of RSV bronchiolitis children[232]. Other airway cell types have been previously shown to produce IL-8 in RSV infection, such as alveolar macrophages[233]. The reason for this may be that NPA samples collect secretions of expressed cytokines from many different cell types, not just airway epithelial cells.

We found elevated expression of IL-8 in RSV infected BEAS-2Bs when IL-17 was present at 24 hours. Our study is the first to measure the combined effects of IL-17 and RSV infection in an airway epithelial model. IL-8 is a potent neutrophil recruiter and high levels of neutrophils are found in the paediatric airway in both RSV bronchiolitis and acute asthma exacerbations[213 234]. The finding of elevated IL-8 expression at 24 hours and not 48 hours suggests that IL-17 may play a role in increasing IL-8 expression in the acute phase of infection.

These results also demonstrate IL-13 does not induce significant IL-8 expression in airway epithelial cells. This is the first study to examine the effect of IL-13 on expression of IL-8 from airway epithelial cells. This could represent that IL-13 affects a different cell type in the airway, with no IL-13 receptor having been described in airway epithelial cells.

There was no demonstrable synergy when BEAS-2Bs were stimulated with IL-17 and IL-13 simultaneously, infected or not infected with RSV.

4.4.3 – Further Work

This chapter has characterised the cytokine response to IL-17 and IL-13 in the presence and absence of RSV infection. This work has raised a number of areas for further study however.

There appears to be some debate in the literature about primary airway epithelial cells response to IL-17 with different results being reported[221 223]. If these experiments were to repeat in primary cell cultures based on airway epithelial cells obtained from bronchial brushing it would be the first reported study involving RSV infection and IL-17 and would also add to the evidence base on IL-17s role in inflammation in the lung. It would also be possible to examine whether these responses differ in epithelial cells in individuals with other conditions such as asthma or cystic fibrosis.

Our finding of IL-13 causing no expression of either cytokine is important as no other study has reported this finding previously. A suggested reason for this was that no study had characterised an IL-13 receptor on airway epithelial cells and so it may be a further project to analyse airway epithelial cells for the presence of an IL-13 receptor.

4.4.4 – Conclusions

Taken together our results have highlighted IL-17 as an important cytokine in the processes of airway inflammation and neutrophil recruitment during RSV infection. We have shown that in isolation IL-17 appears to have no significant affect of the cytokines we analysed, only once a second stimulation occurs does IL-17 appear to augment the effect. We also found

that IL-13 has no apparent effect on airway epithelial cells to express these cytokines which is important as IL-13 is found in elevated levels in the airways of asthmatics and is a publicised target for treatment of RSV-disease[212].

5 Final discussion and further work

5.1 Overview

Paediatric ARI represents the largest identifiable cause of mortality in children under five years worldwide[153]. This highlights ARI as an important area for research and has been chosen as a primary target of the Millennium Development Goal to reduce childhood mortality[139]. We aimed to assess the prevalence of causative pathogens of ARI as well as characterising the clinical and demographic features of novel respiratory pathogens H1N1 and hRV-C. Finally we aimed to investigate the interaction between IL-17 and IL-13, and RSV infection in airway epithelial cells. By adding to the literature it will enable further work into the causative pathogens of ARI and the immune response that leads to pathology in respiratory infection. This thesis has succeeded in each of these aims and highlighted further areas for research. The overall findings of each chapter are as follows:

5.1.1 Aim 1 – To characterise the viral and atypical bacterial pathogens detected in children <5 years presenting to hospital in Recife, Brazil with ARI. The secondary aim was to characterise the clinical and demographic features of children in whom H1N1 was detected

Full assessment of the causative pathogens of ARI is important in targeting treatments in ARI. Previously much research has been undertaken on bacterial pathogens as they responded well to traditional culturing techniques, with vaccines being developed for the two most common - Hib and pneumococcus. Comparatively less is known about viral pathogens of ARI. The development of PCR as a diagnostic technique has allowed high throughput rapid viral diagnostics in virology centres. New respiratory viruses have been identified in recent years as well as documented viruses undergoing mutations and altering their clinical importance. The most recent influenza virus to mutate, H1N1, spread rapidly reaching the level of global pandemic and paediatric patients were at high risk of severe disease[126].We collected NPAs from children presenting with ARI to IMIP children's hospital, Recife, Brazil between March 2008 and April 2010. In this setting viral diagnostics are not usually available, but samples were transported to Liverpool, UK where multiplex PCR was undertaken for 18 different viral and atypical bacterial pathogens.

We were successful in analysing for the chosen pathogens and demonstrated high detection rates in 88% of the 630 samples. The most common pathogens detected were RSV, AdV, hBoV and hRV (33%, 29%, 25% and 19% respectively). Levels of AdV and hBoV were higher than other reports in the literature and explanations may be the young age of our cohort or the crowded living conditions in which they live[102 169]. We also reported high levels of co-infection with 43% of children having more than one pathogen detected on PCR. This was higher in other literature and may reflect the wide variety of pathogens we tested for or the living conditions of the children we

recruited. The most common pathogens detected as co-infections were AdV, hBoV and RSV (54%, 48% and 37%). Our findings highlight the wide variety of pathogens that are detected in children presenting with ARI.

Our secondary aim was to characterise the clinical and demographic features of novel H1N1 influenza. The timing of our study allowed us to characterise the emergence H1N1 and compare the pre-pandemic year (March 2008-March 2009) to the pandemic year (April 2009- March 2010). We were able to show influenza to be the pathogen with greatest change in prevalence, quintupling from 3% to 15%. H1N1 represented 79% of the flu cases in the pandemic year. Demographically there were no significant differences in those with H1N1 ARI compared to non-H1N1 flu, or non-flu ARI. However those diagnosed with H1N1 were more likely to require hospitalisation for ARI and have more than one pathogen detected on PCR. Overall H1N1 represented a significant pathogen in the pandemic year and during its peak was detected in 30% of ARI samples in a month and all cases of H1N1 presenting as a LRTI. We hypothesised that based on previous literature there would be demonstrable differences in H1N1 ARI and we confirmed higher hospitalisation rates in the H1N1 cohort.

Our findings also raised questions to the role of co-infection in our cohort. Co-infections have been reported in the literature previously and their importance questioned, most recently in relation to H1N1 infection[186]. Our findings highlight the need for further assessment of co-infection in paediatric ARI.

5.1.2 Aim 2 – To describe the role of novel hRV group, hRV-C, in paediatric ARI

hRV represents a common respiratory virus detected in childhood ARI, we found it to be present in 19% of children with ARI. In the past hRV was regarded as a trivial respiratory infection and limited to the upper airway. However it has since been proven hRV is an important cause of LRTIs as well as playing a role in asthma exacerbations[97]. Recent diagnostic methods have also shown that the previous classification of hRV into two groups was incomplete with novel hRV-C being discovered. Novel hRV-C has shown to be present in severe asthma exacerbations although its role in ARI in unknown[100]. We aimed to characterise the clinical and demographic features of hRV infection and compare novel hRV-C infection to other groups.

We succeeded in characterising the clinical and demographic features of hRV positive ARI. Compared to non-hRV ARI we found no demographic or clinical differences, however levels of co-infection were increased. hRV rarely presented as an URTI (5%) with the most common diagnoses being bronchiolitis (58%) and pneumonia (25%), this was in keeping with our hypothesis that hRV would be present in LRTI presentations.

Further analysis was successfully achieved in majority of samples with 99 (84%) assigned a hRV strain. hRV-A was the most common group (71%) with all other samples being hRV-C (29%), no hRV-B was detected. Demographically there was no significant difference in patients presenting with hRV-A or hRV-C infection. hRV-C more commonly presented with the diagnosis of EVW / asthma (p=0.01). This also correlated with our original

hypothesis and the previous literature on the topic, that hRV-C has a role in EVW/asthma presentations. We found no significant difference in disease severity in the hRV-C group with regards hospitalisation or other clinical demographics.

Our results also highlighted that the literature was lacking in determining prevalence of hRV-C in children without symptoms of ARI. It has been previously shown in studies into ARI epidemiology that hRV is commonly detected in asymptomatic patients[174]. However no study has yet performed this analysis and accounted for separate hRV groups. Future topics should aim to further our understanding of hRV-C in respiratory disease and reason for why it induces EVW/asthma presentations, this could involve looking at the induced cytokines associated with specific strains of hRV.

5.1.3 Aim 3 – To determine the role of IL-17 in RSV infection of airway epithelial cells and measure for synergy with IL-13.

IL-17 is a pro-inflammatory cytokine that has been highlighted as being detected in the airways of asthmatic children[235]. RSV is one of the most common respiratory pathogens to infect infants and is associated with asthma and wheeze in later life[37 38]. IL-17 has shown a synergistic response when combined with the pro-inflammatory cytokines TNF-α and IL-1β[222 223]. We aimed to determine the role of IL-17 in RSV infection of airway epithelial cells and measure for synergy with the Th-2 cytokine IL-13.

By utilisation of ELISA techniques we were able to successfully show that in the presence of IL-17 significantly increased expression of the pro inflammatory cytokines IL-6 at 48 hours and IL-8 at 24 hours was observed. IL-6 is an important cytokine involved in inflammation and IL-8 is involved in neutrophil recruitment to the airway. Our results add to the literature on IL-17 in airway disease which highlights IL-17 to not cause significant inflammatory response in isolation, however when exposed to RSV infection in the presence of IL-17 significant expression of IL-6 and IL-8 are observed in the airway epithelial models. We also characterised IL-13 to cause no expression of these cytokines from airway epithelial cells which is of interest as IL-13 has been studied as an important cytokine in RSV disease and a target for RSV treatment[212].

Overall we have highlighted IL-17 as an important cytokine in RSV disease and to act upon airway epithelial cells to cause expression of pro-inflammatory cytokines. Further work is necessary to describe the role of IL-17 in asthma exacerbation and also determine the reason for the ability of IL-17 to augment immune responses in respiratory infection and inflammation.

6 Appendix

6.1 A.1: RT-PCR primers

The following primer sets were used for pathogen detection by RT-PCR. All primer probes were supplied by Dr M Hopkins. Work has been previously published on these probes and coding from oligonucleotide probes is extracted directly from references[167 177]:

From Bezerra et al[167]: (References in table – 32[236] 27[237] 33[238])

"**Table 1.** Additional oligonucleotide primers and probes used in this study. "[167]

Assay	Oligonucleotide	Sequence (5' to 3')	Concentration (μM)	Citation
CoV-4plx	OC43 F	CGATGAGGCTATTCCGACTAGGT	0.4	[32]
	OC43 R	CCTTCCTGAGCCTTCAATATAGTAACC	0.4	
	OC43	(Cyan500)– TCCGCCTGGCACGGTACTCCCT–(BHQ1)	0.16	
	NL63 F	ACGTACTTCTATTATGAAGCATGATATTAA	0.4	[32]
	NL63 R	AGCAGATCTAATGTTATACTTAAAACTACG	0.4	
	NL63	(FAM)– ATTGCCAAGGCTCCTAAACGTACAGGTGTT –(BHQ1)	0.16	
	229E F	CAGTCAAATGGGCTGATGCA	0.4	[32]
	229E R	AAAGGGCTATAAAGAGAATAAGGTATTCT	0.4	
	229E	(HEX)– CCCTGACGACCACGTTGTGGTTCA –(BHQ1)	0.16	
	HKU1 F	TTACTTTCCACACTTTTCATCTCTCTG	0.4	
	HKU1 R	CGGAAGCAGCCAACGAAATTC	0.4	
	HKU1	(LC640)– CGCCCACTTGAAGCCGAGACCG –(BHQ2)	0.16	
CMA-3plx	Cpp F	CAAGGGCTATAAAGGCGTTGCT	0.2	[27]
	Cpp R	ATGGTCGCAGACTTTGTTCCA	0.2	
	Cpp	(LC670)– TCCCCTTGCCAACAGACGCTGG –(BHQ2)	0.1	
	Mpp F	GGAATCCCAATGCACAAGAACA	0.4	[27]
	Mpp R	GCTTTGGTCAACACATCAACCTT	0.4	
	Mpp	(LC610)– GCCTTGAAGGCTGGGTTTGCGCTA –(BHQ2)	0.1	
	Adv F	GCC ACG GTG GGG TTT CTA AAC TT	0.4	[33]
	Adv R	GCC CCA GTG GTC TTA CAT GCA CAT C	0.4	
	Adv	(FAM)– TGCACCAGACCCGGGCTCAGGTACTCCGA –(BHQ1)	0.2	
hBoV 2plx	hBoV NS F	CTTGGGCGGGACAGAATGC	0.4	
	hBoV NS R	AACAGAATTGCCACCAACAACC	0.4	
	hBoV NS	(FAM)– TCAAGCATAGAGACAGT –(MGB)	0.2	
	hBoV NP F	GCTCGGGCTCATATCATCAGG	0.4	
	hBoV NP R	CTCCCTCGTCTTCATCACTTGG	0.4	
	hBoV NP	(VIC)– AAT CAG CCA CCT ATC –(MGB)	0.2	

All oligonucleotides were purchased from Metabion Ltd, Germany, except MGB probes which were obtained from Applied Biosystems, Warrington, UK.
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From Hopkins et al[177]: (References in table – 17[6237 239] 18[4] 19[240] 7[236] 20[241])

Table 1. Oligonucleotide primers and probes used in the Flu-6plx assay

Assay	Oligonucleotide	Sequence (5′–3′)	Concentration (μm)	Target gene	References
Flu-6plx	hMPV_383F	ACAAAGARGCAAGAAAAACAATGG	0·4		
	hMPV_451R	GGTGTGTCTGGTGCTGArGG	0·4	hMPV NP	Modified
	hMPV_424A	(FAM)– TCATCAGGyAATATyCCACAAAATCAGAG –(BHQ1)	0·2		from 17
	hMPV_424B	(FAM)– TCATCAGGTAACATCCCACAAAACCAGAG –(BHQ1)	0·1		
	RSV_F	GCAAATATGGAAACATACGTGAACA	0·4		
	RSV_R	GCACCCATATTGTWAGTGATGCA	0·4	RSV matrix	18
	RSV_LC610	(LC610)– CTTCACGAAGGCTCCACATACACAGCWG –(BHQ2)	0·1		
	IfB_F	AAATACGGTGGATTAAATAAAAGCAA	0·4		
	IfB_R	CCAGCAATAGCTCCGAAGAAA	0·4	Influenza B HA	18
	IfB_cyan500	(Cyan500)– CACCCATATTGGGCAATTTCCTATGGC –(BHQ1)	0·1		
	IfA_F	AAGACCAATCCTGTCACCTCTGA	0·4		
	IfA_R	CAAAGCGTCTACGCTGCAGTCC	0·4	Influenza A matrix	18
	IfA_VIC	(VIC)– TTTGTGTTCACGCTCACCGT –(MGB-NFQ)	0·1		
	H1_F	ATTGCCGGTTTCATTGAAGG	0·4		
	H1_R	ATGGCATTYTGTGTGCTYTT	0·4	Influenza A HA	19
	Swine_H1_LC640	(LC640)– ATGAGCAGGGGTCAGGATATGCAGCCGACC –(BHQ2)	0·1		
	Human_H1_LC670	(LC670)– ATGAGCAAGGATCTGGCTATGCTGCAGATC –(BHQ2)	0·1		
PIV-6plx	PIV1 HN525 F	GATTTCTGGAGATGTCCCGTAGG	0·4		
	PIV1 HN722 R	TGACTTCCCTATATCTGCACATCC	0·4	PIV type 1 HA-NM	
	PIV-1 HN556	(FAM)– TACTGAGCAACAACCC –(MGB-NFQ)	0·16		
	PIV-2 F	CCATTTACCTAAGTGATGGAA	0·4		
	PIV-2 R	CGTGGCATAATCTTCTTTT	0·4	PIV type 2 HA-NM	7
	PIV2 LC640	(LC640)– AATCGCAAAAGCTGTTCAGTCAC –(BHQ2)	0·16		
	PIV3 NP300 F	CTTTCAGACAAGATGGAACAGTGC	0·4		
	PIV3 NP800 R	AGTTACCAAGCTCTGTTGAGACC	0·4	PIV type 3 NP	
	PIV3 NP766	(LC610)– CCAATCTGATCCACTGTGTCACCGCTCA –(BHQ2)	0·16		
	PIV4 NP271 F	CAGGCCACATCAATGCAGAATC	0·4		
	PIV4 NP407 R	ATGTCATCCCAGCCAGATCTTG	0·4	PIV type 4 NP	
	PIV4 NP298	(LC670)– ATGATTGCTGCCAGAGCCCCAGATGC –(BHQ2)	0·16		
	hRV F	TGG ACA GGG TGT GAA GAG C	0·4		
	hRV R	CAA AGT AGT CGG TCC CAT CC	0·4	Rhinovirus 5′ UTR	7
	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 phage	20
	MS2 Cyan500	(Cyan500)– CACATCGATAGATCAAGGTGCCTACAAGC–(BHQ1)	0·08		

C denotes position of pdC nudeic acid bases. PIV; parainfluenza virus, NP; nudeoprotein, HA; haemagglutinin, NM; neuraminidase, UTR; untranslated region.

All oligonucleotides were purchased from Metabion Ltd, Martinsried, Germany, except MGB probes that were obtained from Applied Biosystems, Warrington, UK.

6.2 A.2: Cycling conditions for pathogen detection PCR

	Temperature (°C)	Duration	Number of Cycles
Flu, H1N1, hMPV, RSV, CoV, PIV, 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	

6.3 A.3: Reagents and manufacturers

More detailed information on reagents and manufacturers utilised in the methodology of chapter 4 is as follows:

- Bronchial epithelial growth medium (BEGM) was made from 500mL volume of BEBM (Lonza Walkerville Inc) to which the following were added: Bovine pituitary extract, hydrocortisone, epidermal growth factor, epinephrine, insulin, transferrin, tri-iodothyronine, retinoic acid and gentamicin.
- Phosphate buffered saline was purchased from Sigma, St Louis, USA.
- The cytokines IL-17 and IL-13 used for stimulation were obtained from R&D systems, Oxford, UK

- RSV-A2 virus was obtained from Dr Angela Fonceca
- Reagents used for reverse transcription in this chapter were all purchased from Promega, UK.
- Buffer RLT was purchased from Qiagen, Crawley, UK as was total RNA extraction kit
- Duoset ELISA kits were purchased from R&D systems, Oxford, UK

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