THE IMPACT OF DETECTION OF RESPIRATORY VIRUSES ON AT RISK PATIENT POPULATIONS

Alicia Mitchell
B.Med.Sci (Hons)

A thesis submitted in the fulfilment of the requirements
for the degree of
Doctor of Philosophy

School of Life Sciences
Faculty of Science

University of Technology Sydney
2018

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Alicia Mitchell, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This research is supported by an Australian Government Research Training Program Scholarship.

Production Note:

Signature: Signature removed prior to publication.

Date: 6/9/2018

ACKNOWLEDGMENTS

To my Dad, I wish you could have been here to witness this achievement, and to celebrate this with me. I miss you more every day.

The last 3 years have been a journey, one of happiness, sadness and a lot of self-discovery. There are so many people that I am so grateful to for their advice, support and assistance during this time.

To my Supervisor, A/Prof Brian Oliver; thank you for your faith in me, allowing me to run with my ideas but being a sounding board to make sure they could work, and for reassuring me when things seemed a bit overwhelming. You believed in me from the start.

And my co-supervisor, A/Prof Lucy Morgan; thank you for your assistance and support over the last few years. Thank you for welcoming me into Concord with open arms, and for being a friend to me.

To Prof Allan Glanville; thank you for all your support in all aspects of my research and PhD. You welcomed me into SVH and made me feel like a part of the department and helped me whenever things were difficult. You motivated me, encouraged me and gave me opportunities that allowed me to prove to myself what I was capable of.

To A/Prof Benjamin Tang, thank you for collaborating with me on the ICU project. For your positivity, support, ideas, assistance and insights along the way, I am extremely appreciative.

Thank you to everyone at Concord, for allowing me to be part of the Concord family. All the amazing lab staff, the registrars and consultants who helped me with sample

collection, the nurses who welcomed me into the bronchoscopy suite, and especially Lachlan for being such a great friend to me and helping me out where-ever possible.

Thank you to the registrars at SVH who collected and stored all my BAL samples for me. You made my job a lot easier.

To all the Nepean ICU doctors and nurses, and lab staff involved with the viral project, thank you for your help in collecting samples and patient information.

Baz, when life seemed too difficult and motivation levels were extremely low after my Dad died, you helped me to keep going. Your assistance in the lab, your problem-solving skills and your belief in my abilities were invaluable in making this thesis a reality.

To my research group at the Woolcock, thank you for the friendships along the way and the fun we had.

Thank you to my parents for allowing me to stay at home, and for putting up with me. For being excited for me along the way, and for all your support in allowing me to pursue my dreams. Thank you to my brother and sister, who have believed in me and been proud of me every day. To my beautiful cousin, Shannon, you have always been there for me and have giving me strength when I was struggling.

The biggest thank you to my nana, who made the impossible possible. Who did everything in her power to give me the best opportunity to succeed and for being my best friend.

To my amazing friends, I don't know what I would do without you. Steph F, Annalee, Lucy, Sevanne, Matt, Jackie, Radeesha, Emma and Georgia. I don't know what I would do without you in my life. You have supported me every step of the way, been there when I needed time out, fun and support, and have never failed to have faith in me.

To my best friend. What a ride, the last 3 years have been, some of the lowest points in my life and some of the highest. You taught me to believe in myself, to keep going when things were tough, to enjoy life and to make the most of moments of happiness. This PhD has been a huge opportunity for growth, and I wouldn't be where I am without you.

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PUBLICATIONS, PRESENTATIONS, POSTERS AND PRIZES ARISING FROM THIS WORK

PRIZES AND AWARDS

2018 ATS Public Advisory Roundtable Abstract Award – ATS International Conference, San Diego, California, USA

2018 APSR Travel Award to attend the American Thoracic Society Conference, San Diego, California, USA

2018 Finalist in the Early Career Scientist in Transplantation Award – International Society for Heart and Lung Transplant Conference, Nice, France

2017 St Vincent's Clinic Foundation Clinical Excellence Award – Emerging Researcher

2017 ASPR Travel Grant for Respiratory Infections (Non-TB) – APSR Congress Sydney

2017 ERS Young Scientist Sponsorship – European Respiratory Society Congress, Milan, Italy

2017 Finalist in the Early Career Scientist in Transplantation Award – International Society for Heart and Lung Transplant Conference, San Diego, USA

2017 UTS HDR Student Conference Travel Grant – ATS, Washington DC, USA

2016 Lung Foundation Australia and Menarini APSR Travel Grant – Bangkok, Thailand

2015 The AstraZeneca Clinical Research Poster Prize – Newcastle Asthma Meeting

2014 TSANZ NSW Branch Annual Scientific Meeting Highly Commended Oral Presentation

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Mitchell, A. B., Oliver, B. G. and Glanville, A. R. (2016) "Translational Aspects of the Human Respiratory Virome." *Am J Respir Crit Care Med*. 194(12), pp.1458-1464.

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INVITED INTERNATIONAL PRESENTATIONS

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The Translational and Clinical Impacts of the Respiratory Virome

2017 ***University Hospital, UT Health Grand Rounds (San Antonio, Texas)***

Impact of the Respiratory Virome in Lung Transplantation

2016 ***Stanford University Medical Centre Grand Rounds (Palo Alto, California)***

The Human Respiratory Virome

2016 ***Hôpital Bichat Respiratory and Lung Transplant Department (Paris, France)***

Translational aspects of the Respiratory Virome

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LIST OF ABBREVIATIONS

ATS – American Thoracic Society
BAL – Bronchoalveolar lavage
BOS – Bronchiolitis obliterans syndrome
CAP – Community acquired pneumonia
CARV – Community acquired respiratory viruses
CF – Cystic fibrosis
CFTR – Cystic fibrosis transmembrane conductor gene
CI –Confidence interval
CLAD – Chronic lung allograft dysfunction
COAST – Childhood origins of asthma study
COPD – Chronic obstructive pulmonary disease
CRM1 – chromosomal maintenance 1
CT – Cycle threshold
DNA – Deoxyribonucleic acid
ED – Emergency Department
FEV_1 – Forced expiratory volume in 1 second
HA – Hemagglutinin protein
HBEC – Human bronchial epithelial cell
HMPV – Human metapneumovirus
HRCT – High resolution computerised tomography
HRV – Human rhinovirus
ICAM-1 – Intracellular adhesion molecule
ICU – Intensive care unit
IgE – Immunoglobulin E
ILI – Influenza-like illness
LDLR – Low-density lipoprotein receptor
LRTI – Lower respiratory tract infection
M2 – Matrix-2 protein
MERS – Middle-East respiratory syndrome
mRNA – messenger RNA
NA – Neuraminidase protein
NPA – Nasopharyngeal aspirate
NPS – Nasopharyngeal swab
OR – Odds ratio
PCD – Primary ciliary dyskinesia
PCR – Polymerase chain reaction
PIV – Parainfluenza virus
qPCR – Quantitative PCR
RNA – Ribonucleic acid
RSV – Respiratory syncytial virus
RT-PCR – Reverse-transcriptase polymerase chain reaction
SARS – Severe acute respiratory syndrome
siRNA – Small inhibiting RNA
TB – Tuberculosis
vRNP – Viral ribonucleoprotein

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ABSTRACT

Respiratory viruses are ubiquitous and are known to cause acute disease in otherwise healthy individuals and may be associated with severe morbidity and mortality in those with underlying chronic diseases. However, respiratory viruses do not always result in disease. The frequency and implications of asymptomatic carriage within at-risk patient populations remain poorly understood.

This thesis explores the development of a novel methodology for detection of respiratory viruses utilising exhaled breath captured in electret filter, using PCR based detection. Evaluation of this methodology in different clinical cohorts was carried out, specifically exploring rates of asymptomatic detection in patients with bronchiectasis during stable phases and in patients intubated in the ICU, looking within the filter contained in the ventilator circuit. Finally, demonstration of the dynamics of the human respiratory virome in a cohort of patients undergoing lung transplantation, utilising the previously confirmed methodologies, completed this body of work.

The exhaled breath methodology was tested in the two clinical cohorts described above and demonstrated detection of both upper and lower respiratory tract viruses. Results correlated well with traditional sampling methods. The major finding from these studies was the high rate of detection of respiratory viruses in the absence of symptoms and signs suggestive of acute respiratory infection.
Examination of the prospective lung transplant cohort also confirmed a significant rate of asymptomatic viral carriage and provided new insights into the dynamics of the

respiratory virome. This project provides evidence that viruses are transplanted within the donor lung and remain detectable for many months after transplantation. There was no evidence however, that detection of virus correlated with concurrent acute cellular rejection.

Taken together, these studies have allowed development of a novel clinical viral sampling methodology which may have important clinical and diagnostic ramifications. Ultimately, they have enhanced our understanding of asymptomatic viral infection. As well, the role of community acquired respiratory viruses as transient members of the human respiratory virome has now been revealed for the first time.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Overview of the Impact of Respiratory Viral Infections in the Community

Lower respiratory tract infections are one of the leading causes of morbidity and mortality worldwide, and the number one cause of death in developing countries.[1] The most common etiological agents are respiratory viruses,[2] which are ubiquitous in both community and hospital settings. These infections cause a substantial community burden, with estimates of over US$14 billion dollars per annum attributable to direct healthcare costs in the US, and over US$9 billion in lost income due to missed work days.[3] A systematic review and meta-analysis by Luksic *et al* has identified that respiratory viruses are able to be detected in over 50% of all individuals hospitalized for severe acute lower respiratory tract infection (LRTI) episodes in a cohort of over 50,000 cases.[4]

Respiratory viruses infect individuals of all ages, normally causing a self-limiting cold or flu-like illness but are sometimes associated with more severe infection and poorer outcomes. In children, respiratory viruses are often associated with complications such as otitis media, bronchiolitis, bronchitis and pneumonia.[5] In older adults, respiratory viral infection may lead to LRTI such as pneumonia or a deterioration in their pre-existing pulmonary diseases.[6] Furthermore, even though respiratory viruses may be the putative cause of pneumonia, they may also be an important factor affecting the clinical severity of bacterial pneumonia. Preceding respiratory viral infection has been

associated with development of more severe cases of pneumococcal pneumonia,[7] while influenza has been associated with an increased risk of staphylococcal pneumonia.[8]

The greatest impact of respiratory viral infections is seen in children, the elderly and individuals with chronic respiratory conditions. In 2010, 14.9 million cases of severe respiratory tract infection resulted in hospital admissions for young children worldwide, with 1.4 million children dying as a result of severe acute LRTI.[9] The most commonly identified viruses in early childhood are human rhinovirus (HRV) and respiratory syncytial virus (RSV). However, it has been shown that the global burden of influenza in healthy young children is substantial.[10] Influenza presents at a similar frequency in children who present to a hospital Emergency Department (ED) for any indication.[11] Furthermore, influenza has a greater community burden than RSV with an increased risk of causing symptomatic illness and therefore, missed school and work days in other members of the family of the child affected.[11] Influenza vaccination is often recommended only for at risk individuals, such as older adults and those with chronic health conditions. However, studies showing the community spread and impact of influenza infection in young children provide evidence for the importance of vaccination in children over 1 year old.[11,12] Esposito *et al* have also shown that influenza vaccination in young children substantially reduces the incidence of influenza-like illness (ILI) in family members, and thus the associated financial burden to the healthcare system and society through missed work days.[13] New guidelines in Australia now recommend influenza vaccination for all children under 5 years of age.[14]

Elderly populations account for a large percentage of the mortality associated with influenza infection. Even though approximately 1 in 4 hospitalisations for influenza infection are in individuals aged 65 years and above, almost 75% of the influenza-related deaths are in this age-group.[15] Influenza, RSV and Parainfluenza viruses have been identified as the most common etiological agents, which have also been associated with the most severe complications in the elderly.[16] Recently, RSV has been emerging as a significant problem causing over 10,000 excess deaths in the US annually in persons over 65 years of age,[17] and appears to have a similar healthcare utilisation burden as Influenza.[18] Respiratory viral infections in individuals with underlying chronic cardio-pulmonary conditions may lead to an increased burden of disease including complications such as exacerbations and progression of the condition.[19] Asthma and chronic obstructive pulmonary disease (COPD) have received the most attention, and have the largest body of evidence describing the impact of respiratory viral infections in these conditions. Viral respiratory tract infections in very early life have been implicated in the development of childhood asthma, and COPD later in life, and as an important trigger of exacerbations in both asthma[20,21] and COPD.[22,23] Exacerbations are defined as a deterioration of symptoms from baseline, and are associated with significant morbidity and financial costs in all respiratory conditions.[24] Exacerbations of COPD are associated with an increased risk of hospital admission, increased mortality risk and increased rate of lung function decline. Even a single exacerbation can lead to irreversible loss in lung function and are associated with risk of further exacerbations.[25]

1.1.1 Impact of Viral Infections in Early Life

Viral respiratory infections in early life play an important role in immune-modulation and immune development, and may be associated with development of atopy and allergic airways disease in later childhood.[26] Wheezing during early childhood is common, and usually transient in nature, however some children appear to be more at risk of persistent wheeze than others.[26] Severe RSV infections resulting in bronchiolitis in infants have shown a clear association with wheezing and asthma in later childhood.[27,28] Wheezy lower respiratory tract infections associated with rhinovirus and/or RSV positivity during the first 2 years of life are significantly associated with persistence of wheeze at age 5, and development of asthma. The risk of asthma development increases with the number of viral-associated wheezy lower respiratory tract infections in early life.[29] The childhood origins of asthma study (COAST) demonstrated that the risk of developing asthma at 6 years is significantly increased after early exposure to rhinovirus (odds ratio [OR], 9.8) compared with RSV (OR, 2.6).[30] It has been suggested that exposure to viruses and allergens in early life lead to airway inflammation during infant lung development, resulting in remodelling and disruption of tissue differentiation which may cause changes in respiratory function that manifest as wheeze and asthma.[31,32] Therefore, although clear delineation is still ongoing, the interplay between a predisposition to atopy, exposure to allergens and viruses, and early wheeze appear to all be important in the development of asthma.

Early interventions to reduce exposure to respiratory viruses in infancy for children considered at high risk of developing asthma, such as those with a strong family

history of atopy, have reduced the community burden of childhood asthma.[33] Vaccination and prophylactic microbial treatment, to prevent secondary bacterial infections in this cohort may also be a useful consideration in controlling the impact of severe respiratory viral infections, and moderating their impact on immune development.[34]

1.1.2 Respiratory Viral infection in Healthy Adults

In healthy adults, most respiratory viruses usually cause a self-limiting illness that resolves without medical intervention. However, respiratory viruses in healthy adults still generate a significant financial burden to the community due to the cost of reduced productivity and lost work days. In the United States alone, there are estimated to be over 500 million episodes of non-influenza related viral disease every year associated with US$40 billion in costs related to primary care physician visits, prescriptions, and absenteeism.[35]

For any given virus and in any given individual there is heterogeneity in the severity and duration of the consequent clinical syndrome. Influenza and RSV can cause febrile illness with an extended duration of up to 6.8 days for influenza and 9.5 days for RSV causing considerable impact on individuals.[36] The same viruses can be relatively asymptomatic, but similarly contagious in immediate close contacts. A range of respiratory viruses has also been implicated in causing community-acquired pneumonia (CAP) in otherwise healthy adults, leading to hospitalisation and other complications.[37]

1.1.3 Relevance of respiratory viruses in patients with chronic respiratory disease

There is a large body of evidence to demonstrate the importance of respiratory viruses in the development, progression and exacerbations of chronic lung conditions. Particularly in Asthma, COPD and Cystic Fibrosis, there are over 1500 studies as of December 2017, regarding the impact of respiratory viruses. However, in some chronic lung diseases, such as bronchiectasis, there is still very limited information available exploring the role of respiratory viruses.

COPD is characterised by persistent inflammation and irreversible airflow limitation. It is a major cause of both morbidity and mortality, worldwide.[38,39] Toxic exposure is an important risk factor for development of COPD, with cigarette smoking being the most common etiological agent. In COPD, accelerated lung function decline is observed, and an increased rate of disease progression has been associated with acute exacerbations.[40] Multiple studies have indicated the prominence of a range of respiratory viruses in COPD exacerbations, with studies showing viral detection in up to 64% of COPD exacerbations.[41-48] A study by Mallia et al has demonstrated a causal relationship between viral infection and COPD exacerbation, using a human model of experimental rhinovirus infection. Participants who were experimentally infected with rhinovirus 16 developed more severe and prolonged lower respiratory tract symptoms and airflow obstruction than the infected healthy control group.[49] Furthermore, viral detection, specifically with RSV, during stable periods has been associated with increased plasma fibrinogen, serum interleukin (IL)-6 and hypercapnia.[25] Therefore, colonisation with asymptomatic respiratory viruses may also play a role in the chronic

inflammation and accelerated disease progression of COPD. Furthermore, respiratory infections during early childhood lead to a lower initial FEV1 and appear to increase the risk of developing COPD in later life independent of smoking.[50]

The American Thoracic Society (ATS) defines asthma as a heterogeneous condition including acute episodic deterioration (exacerbations) against a background of chronic persistent inflammation and/or structural changes that may be associated with persistent symptoms and reduced lung function.[51] Underlying inflammation is an important pathology associated with asthma, which is targeted through both inhaled and oral therapies. Asthma is extremely prevalent, with over 300 million people affected worldwide.[52] As discussed earlier, viral infections with HRV and RSV in early life are associated with the development of asthma persisting throughout childhood and into early adulthood.[27,30,53] However, viruses have also been shown to be the most common cause of asthma exacerbations, with some studies showing detection of respiratory viruses in up to 85% of exacerbations in children, and up to 80% in adults.[21,54,55] Experimental rhinovirus infection in asthmatics has also been shown to be associated with symptoms of exacerbation such as a fall in FEV_1 and increased use of bronchodilators.[56] Furthermore, associated increases in inflammatory and immune markers, such as immunoglobulin E (IgE), eosinophils and neutrophils, were observed.[57,58] In experimental infections, upper respiratory tract viral symptoms were also more severe and persisted for longer in individuals with asthma compared with healthy controls suggesting that the entire airway in asthma is primed or prone to prolonged inflammation when exposed to virus. In contrast to COPD, the evidence is

not as conclusive regarding the association between repeated viral infections and the progression or severity of asthma.[59]

Cystic fibrosis (CF) is a genetic disease caused by mutations in the CF transmembrane conductor gene (CFTR) affecting multiple organs due to abnormalities in epithelial chloride transport. Mucinous impaction and glandular blockage lead to significant effects in multiple organs including the pancreas, gall bladder, intestines, vas deferens (in men) and in particular, the lungs. These deficits in chloride channels situated on the apical membrane in both the airways and in glandular tissue within the lungs, are ultimately involved in the resultant respiratory failure observed in CF, explaining the need for lung transplantation as a treatment strategy. In the early time course, the consequent dehydrated mucus due to decreased chloride permeability leads to significant respiratory effects including chronic inflammation, glandular dysplasia, impaired mucociliary clearance, recurrent lung infections and problems with innate immunity mechanisms.[60-63] there are a range of impairments in innate immunity in cystic fibrosis at many points of the early response, including epithelial dysfunction coupled with impaired pathogen sensing, leukocyte recruitment, and phagocytic ability. Furthermore, mechanisms linking innate and adaptive immunity are deficient. Clear effects of these abnormalities can be observed from birth with early signs including luminal dilatation due to mucus blockage, which severely impact quality of life. Although there is heterogeneity in severity due to differences in gene mutations, the natural history of cystic fibrosis is one of a high baseline symptom burden and frequent exacerbations, often leading to the development of bronchiectasis. Some of the first bacterial and viral studies were done in the area of CF, with viral studies from

as early as 1981 showing high rates of a range of respiratory viruses.[64-66] The importance of respiratory viruses in CF exacerbations has been confirmed since the advent of PCR, with rates of virus detection in up to 72% of children with CF experiencing an exacerbation.[65,67,68] In CF, there is conflicting evidence regarding the impact of virus infection or viral-associated exacerbations on accelerated lung function decline or a long-term decrease in lung function.[69-71] It is currently unknown whether viruses interact with bacterial species within the CF lung, although some studies suggest that viral infection may play a role in the acquisition of *Pseudomonas aeruginosa* and worsening clinical status.[66,72]

Bronchiectasis is characterised by chronic cough associated with dilatation of the bronchi. The current gold standard for diagnosis of bronchiectasis is high resolution chest tomography (HRCT).[73] Bronchiectasis is the end result of a wide variety of insults that lead to airway dilatation, chronic inflammation, and colonisation with pathogenic organisms. It is associated with other causes of obstructed airways such as asthma, COPD, and the presence of a foreign body, immune deficiencies (e.g. secondary B and T cell dysfunction), and as a consequence of severe pneumonia (including tuberculosis (TB)). There is evidence to indicate that bronchiectasis may develop secondary to bronchiolitis after childhood infections with respiratory viruses such as influenza and adenovirus. A subset of studies have reported rates of idiopathic bronchiectasis ranging from 26%-53%.[74,75] Cole suggested a model of bronchiectasis whereby a vicious cycle occurs. Firstly, an infection or some kind of foreign material enters the respiratory tract. When this is unable to be eliminated, the inflammatory response becomes chronic and causes damage to surrounding structural tissue of the airways.

These structural changes lead to mucus stasis and promote chronic infection within the lungs, which propagates this vicious cycle.[76] Over time, this retained sputum and chronic infection can lead to mucus plugging and further structural damage to the airways, causing advanced bronchiectasis which can severely impact quality of life.[77] Clarification of the etiology has some value where there is specific change in management such as where deficiencies can be replaced or an obstruction removed.[75] Colonisation with pathogenic bacterial species appears to be important in the progression of bronchiectasis, where *Haemophilus influenzae, Streptococcus pneumoniae* and *Pseudomonas aeruginosa* have been implicated as important organisms and appear to correlate with declining lung function and worsening clinical features.[78,79] Despite evidence to indicate a critical role of infection in the pathogenesis and progression of bronchiectasis, much emphasis has focused on the role of bacterial species. There has been less focus on the impact of viral species on exacerbations and progression of bronchiectasis,[80,81]

1.1.4 Viruses in the Intensive Care Unit (ICU)

Pneumonia[82], and other causes of acute respiratory failure, including adult respiratory distress syndrome (ARDS), are frequent indications for ICU admissions. Respiratory viral infections are often implicated in these conditions, including community acquired and nosocomial pneumonias, and can cause significant morbidity and mortality. Especially in the case of pandemic influenza, there is a strong need for ICU support evidenced by the 2009 H1N1 influenza virus resulting in 722 patients over the winter

season being admitted to the ICU with confirmed infection across Australia and New Zealand. (ref – the ANZIC influenza investigators)

Emerging respiratory viral infections such as Middle East Respiratory Syndrome (MERS), or pandemic avian H1N1 influenza, carry even higher rates infectivity, and greater morbidity,[83] and have a significant and unpredictable impact on the resources of the ICU. Using multiplex PCR, it has been shown in recent studies that respiratory viruses have been detected in up to 49% of patients with acute respiratory failure or unspecified LRTI.[84-88] Previously, bacterial infection was assumed to be the major cause of severe pneumonia in hospitalised patients, however similar 28-day mortality rates have been demonstrated for both bacterial and viral pneumonias (25.5% vs. 26.5%), indicating that the possibility of a respiratory viral etiology also needs to be an important consideration for patients who develop pneumonia in the ICU.[89]

There are no recommendations concerning the frequency of viral testing in ICU, however infection control and minimisation of transmission both between patients and health care workers is important. A study by Gianella et al, showed that nearly a third of all patients in a cohort of 105 patients in the ICU who were tested over a period of 3 months, were found to be positive for influenza and in almost half of these, influenza infection had not been suspected.[90]

Adequate specimen collection is essential for accurate identification of putative organisms. Nasal swabs have shown limited sensitivity in the detection of respiratory viruses, particularly in patients in the ICU.[91-93] Therefore, recommendations have been

made to use nasopharyngeal aspirates or BAL in these patients.[94] However, these more invasive methods of sampling may not always be appropriate in very sick, and unstable ventilated patients. It is important that appropriate sampling, and rapid diagnosis of viral infection in ICU patients is made to direct treatment and clinical management, including isolation and implementation of contact and airborne precautions.

1.1.5 Impact of respiratory viruses in lung transplantation

The impact of colonising or persistent bacterial, fungal and viral species in lung transplantation will be discussed more fully later in this review, however there have been a range of studies that have investigated the role of respiratory viral infections following lung transplantation and the possible effects on allograft function. In a longitudinal study of lung transplant recipients, Kaiser et al have shown that human rhinovirus can achieve persistence within the lower respiratory tract of lung transplant recipients, with three patients in this study showing rhinovirus positivity for 8 to 15 months.[95] A prospective longitudinal study over a period of 3 years, found that the incidence of infection in their lung transplant cohort was 0.83 per patient-year (95% CI 0.45 to 1.52), and that 10% of patients had asymptomatic viral carriage during the screening periods.[96] A further surveillance study has shown that 51.6% of patients in their cohort had a respiratory virus detected at some point during the 3 year follow up, with the most common virus being rhinovirus. Additionally, signs of acute rejection were seen in 33.3% of patients who had evidence of a respiratory viral infection, compared with only 6.7% of patients who had no previous viral detection within 3

months.[97] Community acquired respiratory viruses (CARV) appear to be commonly present in lung transplant recipients, possibly due to the need for a maintenance immunosuppressive regime, in addition to impaired mucociliary clearance, absent cough reflex and alterations to lymphatic drainage.[98] It has been suggested that the pathogenesis of obliterative bronchiolitis, the underlying pathology of chronic lung allograft dysfunction (CLAD), may be due to the cumulative effect of aberrant repair of acute injury, possibly due to viral infection, leading to epithelial damage over time. Therefore, early viral infection may be important not just in association with acute allograft injury, but in long term damage leading to development of bronchiolitis obliterans syndrome (BOS). A recent pooled analysis of 34 studies, indicated that a recent history of respiratory viral infection was associated with a slightly increased frequency (18% vs 11.6%) of graft dysfunction and BOS after lung transplantation.[99] A study by Billings et al using a proportional hazards regression analysis investigated the relationship between infection with a community acquired respiratory virus, and the risk of development of BOS. They found that if a respiratory viral infection involved both the upper and lower respiratory tract, there was no increased risk of developing BOS. However, in patients with documented lower respiratory tract infection, there appeared to be a greatly increased risk of development or progression of already established BOS.[100] More well-designed, prospective, longitudinal studies are needed to clearly elucidate the relationship between viral infections, the immune response and the development of acute and chronic rejection.

1.2 Common Respiratory Tract Viruses

As previously mentioned, a range of respiratory viruses are responsible for causing symptomatic upper and lower respiratory tract illnesses in humans. Most notably, HRV, RSV, Influenza A and B, Parainfluenza Viruses (PIV) 1, 2, 3 and 4, Human Metapneumovirus (HMPV) and Coronaviruses are the most commonly human pathogens. Previously, Influenza had been considered to be the most virulent virus associated with the most severe infection and highest rates of morbidity.[101] However, the potential importance of HRV, RSV, PIV and HMPV is emerging, with significant morbidity observed in at risk individuals and immunocompromised populations.[18,102-106]

1.2.1 Human Rhinovirus

Human rhinovirus is a member of the Enterovirus genus, which includes a range of viruses that infect the human respiratory tract and gut. Initially, using cell culture methods, many different enteroviruses were grouped together in diagnostics, with extra tests being needed to differentiate between species. However, molecular methods have made it easy to clearly distinguish rhinoviruses and have allowed the discovery of a new subtype, HRV-C.[107] Human rhinoviruses are responsible for more than a half of all coryzal illnesses, and are the most common cause of upper respiratory tract infection. There are 3 distinct subtypes of rhinovirus, groups A, B and C, based on molecular phylogenicity, which differ in virulence and host receptor tropism.[108,109] Due to the increased use of molecular based viral detection methods, it

has now been realised that HRV which were once associated solely with upper respiratory tract infections, also cause lower respiratory tract infections, particularly in the at-risk groups mentioned above.[110,111]

Viral Structure and Replication

HRV is a positive sense, single-stranded RNA virus of approximately 7,200 base pairs (bp), which is a member of the *Picornaviridae* family, part of the *Enterovirus* genus. This simple virus consists of a single gene, which when translated and cleaved, results in 11 proteins responsible for encoding the viral capsid, genome replication and machinery.[108] HRV has an icosahedral structure, with a canyon on one side which acts as a site for attachment to the cell surface receptor intercellular adhesion molecule 1 (ICAM-1) in the majority of HRV serotypes. Once the virus has bound to ICAM-1, a low-density lipoprotein receptor (LDLR), or cadherin-related family member 3, in the case of HRV-C,[112] pH changes within the cell lead to uncoating of the viral protein, and subsequently, host-cell translation of the viral RNA. The genomic material is packaged into a new virion, and the new rhinovirus particles are able to continue to infect other cells through causing the current host cell to lyse and thus, release all newly-produced virions.[108,113] Using *in situ* hybridisation, it has been shown that HRV replication appears to be localised to a small number of cells in the nasal epithelium and nasopharynx. This may be due to the restricted expression of ICAM-1 receptors in the respiratory tract.[114,115] However, in vitro experiments have shown that HRV is able to replicate in and up-regulate membrane-bound ICAM-1 expression in human bronchial epithelial cells (HBECs), which may play a role in the ability of HRV to cause lower

respiratory tract infection.[116] Further evidence by Papadopoulos *et al*[117] has shown that HRV has similar replication capabilities at both 33°C and 37°C, therefore this virus is unlikely to be limited to the upper respiratory tract solely. In a human study, where volunteers were experimentally infected with HRV-16, nasopharyngeal replication was observed, but HRV was also detected in BAL by reverse-transcriptase polymerase chain reaction (RT-PCR), 2-4 days after initial infection.[118] A further study by Papadopoulos *et al* demonstrated rhinovirus *in vivo* in airway epithelial cells and submucosal cells after experimental upper respiratory infection of human volunteers,[111] while immunostaining of lung biopsies after experimental HRV infection by Grunberg *et al* showed increased expression of ICAM-1 receptors on the bronchial epithelium.[119]

Transmission

It has been shown that HRV can survive in an indoor environment, at an ambient temperature for hours to days and on skin for 2 hours under experimental conditions.[120] Therefore, HRV is quite stable outside the human body and this gives further evidence for the importance of fomites and person-to-person contact transmission in rhinovirus spread. Aerosol spread may also be important, as it has been shown that HRV can be transmitted by the aerosol route in a study of 18 participants who played cards for 12 hours with a group of experimentally infected subjects, 56% of whom became HRV positive following this exposure.[121] HRV has been shown to cause infection all year round in a range of climates, including temperate, tropical and subtropical regions.[122] However, peak incidence appears to be in the early autumn and spring seasons.[123,124] Recent evidence suggests that peaks of HRV

infection often occur in children when they return to school after extended holiday periods. This has been attributed to a decrease in immunological tolerance that develops due to lack of exposure to viruses when not at school. A peak in both viral infections and related asthma exacerbations are observed in the early school term period before this immune tolerance can be re-established.[125]

1.2.2 Respiratory Syncytial Virus

Respiratory syncytial virus is one of the most common respiratory pathogens in children less than 5 years old, with clinical sequelae ranging from mild common-cold like illness to life-threatening lower respiratory tract infection. Almost all individuals will have been exposed to and been infected by RSV within the first 3 years of life. Recurrent infections with RSV are common. A study conducted in Houston, Texas, demonstrated that 69% of infants were infected with RSV in the first year of life, and 83% within their second year of life.[126] A further study showed that rates of RSV infection for children attending day care were even higher, with up to 98% of children demonstrating symptomatic RSV infection during each of the first 3 years of life.[127] RSV is also associated with severe lower respiratory tract infection in adults, particularly elderly and immunocompromised individuals.[128]

Viral Structure and Replication

RSV is an enveloped virus with a non-segmented, single-stranded, negative sense RNA genome of approximately 15-kb with variations in size depending on strain.[129] RSV is

part of the *Paramyxoviridae* family of viruses, within the genus *Pneumovirus*. The RSV genome encodes 11 proteins, of which 9 are structural (L, G, F, N, P, M, M2-1, M2-2 and SH) and 2 are non-structural (NS1 and NS2).[130] On the surface of the RSV viruses are two surface glycoproteins (F and G), which play an important role in the pathogenesis of the virus and in induction of the immune response. The F protein is a fusion protein which is responsible for fusing the viral envelope with the host cell surface membrane to allow the virus to enter the human cell via RhoA proteins, while the G protein is responsible to viral attachment to the cell. It has been shown that the F protein of RSV is able to interact with the Toll-like receptor 4 (TLR-4) co-receptor MD-2,[131] however, there is conflicting evidence regarding the importance of this in RSV pathogenesis.[132]

Once the virus has entered the cell, the viral nucleocapsid and genome are released into the cytoplasm as this is the site of RSV gene expression and replication. The M2-2 gene expressed by RSV guides the host cell transition from transcription to production of genomic RNA. Viral polymerase begins transcription of viral genes into mRNAs at the 3' end of the genome, using start-stop-restart synthesis.[133] Due to this, the genes at the 3' end of the genome are produced more abundantly than those downstream. This is useful, as these code for the non-structural proteins NS1 and NS2, which aim to arrest the cellular antiviral response.[134] It is important that this occurs as early as possible after the virus enters the cell to ensure its survival and propagation. This replication process produces a positive-sense RNA genome, the antigenome, which is used as a template for genome synthesis. The M protein regulates assembly of the

virion, which utilises a budding mechanism to exit from the cell allowing an envelope to be acquired during this process.[133]

Transmission

RSV infection demonstrates a distinct seasonality, with peaks of virus activity during the winter months and in early spring in temperate climates. There are two main strains of RSV, group A and B. Both of these species appear to circulate concurrently,[135] however the A group appears to dominate and is associated with more severe infection.[136] Aerosol, contact and fomite transmission events all appear to be effective in allowing the spread of RSV. A recent study has shown that infants with RSV bronchiolitis generate sufficiently small, infectious particles that are able to remain airborne for a significant period of time, more than 2 hours, allowing them to be inhaled. Furthermore, these recovered airborne virions were inoculated onto ciliated respiratory epithelial cells, where it was shown that these particles were capable of achieving infection *in vitro*.[137] Hence, there is likely an important role for aerosol transmission of respiratory syncytial virus. Previous studies have given evidence for both contact and fomite transmission events,[138] showing the importance of barrier precautions and handwashing regimes in hospitals, day care centres and other institutions. RSV has been recovered from the environment up to 6 hours after initial inoculation, and from hands up to 25 minutes after inoculation,[139] indicating a

role for fomite transmission due to the ability of RSV to survive outside the human body.

1.2.3 Influenza Virus

Influenza virus is responsible for substantial morbidity and mortality in humans of all ages. In general, this virus causes a more severe illness, with increased lower respiratory tract and systemic involvement compared with HRV and RSV. These viruses are able to cause seasonal, endemic symptomatic infections and sporadically, unpredictable pandemics. There is a distinct history of regular pandemics over the course of the last few centuries, with the 1918 H1N1 pandemic highlighting the devastation this virus can cause with up to 50 million deaths worldwide attributable to this outbreak.[140] There are three subtypes of Influenza, A, B and C; with the majority of severe endemics, and pandemics attributable to subtype A. Influenza B and C are less common, and are usually associated with more mild infections.[141] Seasonal, circulating viral strains are associated with reduced mortality in the general population, but at risk populations, including infants, elderly, immunosuppressed individuals and people with chronic cardio-respiratory diseases still suffer from significant morbidity and increased mortality rates with infection with influenza viruses.[142] Influenza A and B are the leading causes of Influenza-like-illness (ILI), and are most common in school-age children.[143] Conversely, healthy, young adults appear to be the group at a greater risk during influenza pandemics.[144]

Viral Structure and Replication

Influenza viruses are part of the Orthomyxoviridae family. They are enveloped negative-sense single-stranded RNA viruses, with a segmented genome comprising eight segments which code for at least 8 discrete proteins in Influenza A and B strains. Each RNA segment forms ribonucleotide-nucleoprotein complexes within the virion.[145] The influenza RNA polymerase has no proofreading ability, therefore resulting in a high rate of point mutations during replication. This accounts for the clear antigenic drift seen with influenza viruses, and may lead to selective advantages if mutations result in alterations in the surface glycoproteins.[146] The main surface glycoproteins are hemagglutinin (HA) and neuraminidase (NA). Antigenic shift may also occur, when reassortment with another influenza A virus results in acquisition of a new HA subtype on the virus surface.[147] This is the process that normally underlies the emergence of a pandemic strain, when reassortment occurs with an influenza strain from a different mammalian species.[148] In the process of reassortment, two different strains of influenza are needed, usually a human strain and a strain from a different mammalian species. If these two strains infect a cell concurrently, genetic reassortment between different sections of the influenza segmental genome can occur, giving rise to a new viral subtype with different surface receptors.[149]

In addition to HA and NA glycoproteins, there are also M2 proteins, which make up a minor component of all surface structures.[145] Influenza attaches to the host cells via sialic acid receptors which interact with the HA surface proteins.[150] This induces receptor-mediated endocytosis, allowing influenza to enter the host cell within an endosome. The pH inside the endosome is low, around 5-6, which induces a

conformational change of the HA protein and leads to fusion of the viral and endosomal membranes. The low pH also causes the M2 channel to open, causing acidification of the viral core and release of the viral ribonucleoproteins (vRNPs) into the cytoplasm.[151] The vRNPs then localise to the nucleus, the site of viral transcription and replication. In the nucleus, the vRNP utilises the host cellular RNA polymerase to transcribe positive sense mRNA.[152] From here, the mRNA can be used as a template for production of negative-sense viral RNA, which is exported out of the nucleus following replication utilising the CRM1 (chromosomal maintenance 1)-dependent pathway through nuclear pores.[153-155] These viral components travel to the apical membrane of the host cell, where the virion is produced through budding off of the plasma membrane.[156] Finally, the NA glycoprotein cleaves sialic acid residues allowing the new viral particles to be released from the plasma membrane, and thereby continue infecting other nearby cells.[157]

Transmission

Influenza A viruses have a large host range, with the ability to infect a range of mammals. Avian influenza viruses serve as a reservoir for many known circulating influenza viral strains, although recent pandemic strains have been traced to both swine and avian reservoirs.[158] Influenza viruses are identified and named by the antigenic classification of their surface glycoproteins, HA and NA. Currently, there are 16 known HA and 9 known NA subtypes.[159] From 1977 onwards, H1N1 and H3N2 have been the main influenza A subtypes circulating in humans.[158] Influenza virus epidemics show a strong seasonal pattern, with the majority of outbreaks occurring during the

winter seasons in temperate climates.[160] Pandemics may occur at any time of the year.[144] Aerosol, droplet and contact transmission have been demonstrated for influenza virus. There is evidence to suggest that influenza virus can survive on fomites and hands for extended periods of time, long enough to allow onward transmission.[161-164] Studies looking at survival on fomites have been able to detect the virus for periods ranging between a few hours and a couple of days depending on whether the surface is porous or not.[161,165] There is also evidence to show that influenza virions can be recovered from the air in natural settings, giving evidence for the importance of aerosol transmission events.[166-169]

1.2.4 Parainfluenza Virus

Parainfluenza virus is associated with a range of upper and lower respiratory tract illnesses, and is the major causative pathogen of croup (laryngotracheobronchitis) in children.[170,171] PIV can cause severe and fatal lower respiratory tract infections in the elderly, individuals with chronic diseases and in the immunocompromised.[172-177] PIV appears to be second only to RSV, in causing hospitalisations due to lower respiratory tract infection in children.[178] There are 4 major subtypes of human parainfluenza virus, PIV 1, 2, 3 and 4, which are all responsible for a range of respiratory tract infections.

Viral Structure and Replication

Parainfluenza viruses are part of the *Paramyxoviridae* family, which include *Respirovirus* (PIV-1, PIV-3) and *Rubulavirus* (PIV-2, PIV-4) genera.[179] PIV is an enveloped

virus, with a single-stranded, negative-sense RNA genome of approximately 15-kb.[180,181] The PIV genome encodes for at least six structural proteins including two surface proteins, hemagglutinin-neuraminidase (HN) and the fusion protein (F).[182] Parainfluenza virus also utilises host sialic acid receptors to bind to cells, and the fusion protein plays an integral role in causing the viral and host cell membranes to fuse, allowing the viral nucleocapsid to get inside the host cell. Once the viral nucleic acids are in the host cell cytoplasm, transcription occurs utilising the virus-specific RNA-dependent RNA polymerase. The viral mRNAs are translated into viral proteins using cellular ribosomal machinery. As PIV has a negative-sense genome also, the positive-sense RNA is first produced followed by the production of the complementary negative-sense strand. This replicated RNA is encapsidated and packaged for export from the cell.[179]

Transmission

Parainfluenza virus peak activity occurs biennially during the autumn seasons in both hemispheres, usually in odd-numbered years.[171,183,184] There is limited data regarding spread of human PIV, but a single study that investigated airborne transmission by sampling the air around infected children, found that PIV could only be detected from 2 of 40 children at a distance of 60cm.[185] Therefore, it has been concluded that transmission by small-particle aerosol is unlikely for PIV. Fomite transmission is likely the most common mode of transmission, as studies have shown that PIV is able to be detected on non-porous surfaces for up to 10 hours after inoculation.[186] This is not the case with virus survival on hands however, a study has shown that approximately 90%

of the inoculated viral load is undetectable after 10 minutes on a person's hands. Furthermore, this study found no evidence for spread to from the hand of one individual to another.[187]

1.2.5 Human Metapneumovirus

Human metapneumovirus (HMPV) was only recently discovered in 2001, in the Netherlands, where it was first isolated from a paediatric patient exhibiting RSV-like symptoms.[188] HMPV is part of the same viral family as RSV and exhibits similar effects in terms of at risk populations, symptomatology, morbidity and mortality. Seroprevalence studies have shown that the majority of children (90-100%) have antibodies against HMPV, and likely have been infected by the time they are 10 years of age.[188] HMPV can cause severe LRTI in children, manifesting as bronchiolitis, bronchitis and pneumonia. It is normally associated with mild flu-like symptoms in healthy adults, but can also cause significant complications and severe illness in the same at risk populations as the other viruses discussed.[189] HMPV is now recognised as a very common causative pathogen in acute respiratory tract infections, with detection in up to 16% of cases.[190-192]

Viral Structure and Replication

HMPV is a member of the *Paramyxoviridae* family, under the genus *Metapneumovirus*. Two genotypes, A and B, have been identified using whole genome analysis.[193,194] The HMPV genome is also composed of single-stranded, negative-sense RNA. There are 8

genes present which code for 9 proteins. On the surface of the HMPV enveloped virion, there are three surface glycoproteins, F, SH and G, which appear as spikes by electron microscopy.[195,196] The fusion protein (F) and the attachment protein (G) are responsible for attaching to and fusing with the host cell via heparin sulphate receptors on the host cell surface. This allows the viral nucleocapsid to enter the host cell, where replication occurs in the cytoplasm. Once the genomic material has been replicated, the viral P, N, L and M2 proteins are assembled to create the new virion and through the budding mechanism, the new virions are able to leave the cell. HMPV interferes with the host immune response by regulating pattern recognition receptors, modifying dendritic cell activity and reducing antigen-specific T cell activation.[197,198]

Transmission

HMPV also has a seasonal distribution similar to RSV, and influenza, with peak activity seen in the winter months in temperate climates.[199,200] Studies have shown that both HMPV serotypes (A and B) co-circulate during the respiratory virus season.[198,201] As there are a range of HMPV genotypes, re-infection with different strains may occur over a lifetime.[202] There is evidence for aerosol transmission of HMPV[203], and some evidence for large droplet transmission.[204,205] However, there are no studies clearly exploring the role of fomites in HMPV transmission and it is not known how long this virus is able to survive on surfaces.

1.2.6 Clinical Precautions to Reduce Viral Spread

A systematic review by Lessler *et al* demonstrated that there are significant differences in the incubation periods for each respiratory virus. Influenza B demonstrated the shortest incubation period, from time of infection to the time when the first associated clinical symptoms were observed, was a median of only 0·6 days (95% CI 0·5–0·6). The common respiratory virus with the longest observed median incubation time was Adenovirus, at 5·6 days (95% CI 4·8–6·3).[206] During this time, infected but asymptomatic individuals are shedding virus and there is an arguably greater risk of infecting others during this period as the infected individual will not yet be aware that they are unwell. This makes the case, that especially around at-risk individuals and in hospital settings, healthcare workers and other individuals who are in contact with patients should adopt appropriate universal precautions to reduce the spread of respiratory viruses, independent of symptomatology.

A further review by Jefferson *et al.* evaluated physical interventions for reducing respiratory viral spread in both adults and children, and in a range of settings. This review gave evidence to suggest a strong protective impact of handwashing, barrier protection such as gowns, gloves and masks, and isolation of suspected infected individuals in reducing the risk of respiratory viral spread.[207] Many of the case control studies were carried out during epidemics, such as when the SARS virus was circulating. Overall, there was strong evidence from all these studies to suggest a protective benefit from handwashing, and wearing the protective gear mentioned.[207] Gowns and gloves appeared to be important in reducing contact and fomite spread,

while masks helped to reduce any effect of airborne transmission which appears to be especially important in the spread of influenza virus.[208]

1.2.7 Seasonality of Respiratory Viruses

As discussed previously, many of the common respiratory viruses demonstrate a clear peak during certain seasons in temperate climates. Seasonality of these viruses has also been observed in tropical, sub-tropical and arid climates, often associated with the rainy season.[209] It has been suggested that this increased rate of certain respiratory viruses, such as RSV, in non-temperate climates is likely due to increased contact transmission. Enveloped viruses may be able to survive for longer on hands and fomites during this time due to the increased humidity.[210] In temperate climates, a range of studies has shown effects of both temperature and humidity on respiratory virus seasonal peaks.[211,212] The effect of these factors may be both direct, in regards to virus survival at different temperatures and absolute humidity levels, and indirect, through behavioural patterns and increased susceptibility to viral entry to the upper respiratory tract due to drying of the nasal passage. Cold air reduces the moisture content of viral aerosol droplets, thus reducing the size, allowing particles to stay airborne for longer and also increasing their ability to reach the lower respiratory tract when inhaled. Furthermore, as the nasal passage is dried out by the cool, dry air, the nasal mucosa may become cracked[213,214] and the immune defence mechanisms are not as effective,[215] which has been shown to be particularly important for rhinovirus infection. During the winter months, many people choose to spend more time indoors

leading to crowding and closer contact with other people, which may facilitate transmission of respiratory viruses.

Another consideration is the effect of the reduced sunlight hours during winter, which may be associated with decreased vitamin D levels in the population. As vitamin D is implicated in modulating both innate and cellular immune responses, this may be a possible contributor to the increased susceptibility to respiratory viruses during winter.[216] To give further evidence to support this hypothesis, two randomised control trials demonstrated the efficacy of vitamin D supplementation in reducing wintertime respiratory viral infection incidence.[217,218] Therefore, the seasonal peaks observed in respiratory viral infections appear to be multifactorial, and include both viral, environmental and host factors. Due to these complex interactions, it has been difficult to develop effective management and precautions to stop viral spread.

1.2.8 Asymptomatic respiratory viral infections

The host immune response is important in determining an individual's symptomatic response to a viral infection. Previously, when culture-based methodologies were the principal means of viral detection a high viral load and perfect replicating conditions were needed for viral detection. Indeed, the new serotype of rhinovirus, HRV-C, was not able to replicate in traditional cell culture techniques used for HRV A and B and therefore, not discovered until molecular methods of viral detection came into regular use.[109]

However, with the introduction of PCR, sensitivity has increased greatly, uncovering a high rate of virus detection without associated symptoms. A study comparing nucleic acid testing such as PCR, with conventional viral detection methods in asymptomatic individuals, has shown that a respiratory virus was only detected in 4% of samples using conventional testing, but increased to 42% when using PCR.[219] Many studies have focused on asymptomatic children to determine viral detection frequency, with rates of 27% - 51%[219-222] of the cohort having a respiratory virus detected without exhibiting viral symptoms. Furthermore, in a cohort of hematopoietic cell transplant recipients, PIV was detected in 17.9% of asymptomatic patients in the first 100 days post-transplant,[223] indicating that asymptomatic viral carriage may be important in an immunocompromised population.

Heinonen et al demonstrated a clear relationship between host immune response and symptomatic viral infection. In this analysis, four clinical groups of participants were included, HRV negative control subjects, HRV positive asymptomatic subjects, HRV positive outpatients and HRV positive inpatients to compare the host immune response to viral infection using transcriptional profiling. In those individuals who developed symptoms in response to the presence of HRV, there was a robust transcriptional signature seen characterised by the over-expression of innate immunity genes, while adaptive immunity genes were under-expressed. Interferon responses were the most strongly activated, and these were shown to be even more pronounced in HRV+ inpatients, compared with outpatients, reflecting more severe disease. In the HRV positive asymptomatic group, there was minimal change in gene expression, which closely matched the transcription profile seen in the HRV negative group.[224] A

number of other studies have compared gene expression profiles for a range of respiratory viruses in children,[225] and for influenza in adults.[226] Similarly, both these studies found divergent gene expression in symptomatic and asymptomatic subjects, where the asymptomatic individual's gene expression profile reflected the viral negative healthy controls. Therefore, being able to characterise the host transcription profiles may help guide clinical management of viral infections, in an age where asymptomatic viral detection is occurring more commonly.

1.2.9 Importance of detection of multiple respiratory viruses

As mentioned for asymptomatic viral infection, the introduction of PCR, but more specifically, the use of multiplex assays has allowed discovery of the common occurrence of viral co-infection with two or more respiratory viruses at any one time. Studies have found reasonably high rates, as shown below, of multiple virus detection in populations sampled for respiratory virus infections, with higher rates seen in children. Meerhoff et al found co-detection of two or more respiratory pathogens (up to 5 pathogens in 1 child) in 48% of infants in their study.[92] In a study of children in Shanghai who presented with acute respiratory tract symptoms, co-infection with two or more viruses was seen in 29.9% of subjects.[227] Another study done in children showed slightly decreased co-detection rates of 15.3% for two viruses, 0.8% for 3 viruses and one subject (0.2%) had 4 viruses detected.[228] A further study of adults collected samples during 63 respiratory outbreaks and found that 14.5% had two viruses detected and 3% had three viruses detected in their sample.[229] Comparatively, Kim et al found that 12.3% of the adult subjects in their study had co-infection with

multiple viruses.[230] A range of studies has found that co-infection with more than one respiratory virus appears to be a common phenomenon, however, larger cohort studies may be needed to determine population-based rates, as all of these studies involve reasonably small groups.

Furthermore, the method of sampling may be an important consideration. Jeong et al found significantly higher rates of multiple virus detection in sputum samples compared with nasopharyngeal swabs (19% vs 6%).[231] In populations with chronic respiratory diseases such as bronchiectasis and CF, their airways are filled with thick, sticky sputum that is difficult to clear, and these individuals are very prone to lower respiratory tract colonisation with pathogenic bacteria. This environment may be more hospitable to viruses and allow persistence within this mucus matrix which is relatively protected from the host immune response. In a recent prospective, longitudinal study of 2 years' duration, Miro-Canis *et al.* showed that viruses were more commonly detected in the respiratory tract of patients with cystic fibrosis, than were bacteria. Viruses were detected in 41.8% of specimens, while bacteria were only detected in 35.9% of paired sputum and nasopharyngeal swab (NPS) samples over this period. The rate of multiple viral detection in this study was low however, with only 5.4% of samples being positive for more than one respiratory virus.[232] However, another study which looked at viral detection during exacerbations of cystic fibrosis found that 34.6% of their group had respiratory viral co-detection.[70] Therefore, in patients with suppurative lung disease, viral infection is an important consideration and use of multiplex PCR may be necessary to determine if more than one respiratory virus is

implicated. Furthermore, choice of sampling methodology in these conditions is equally important to gain the most clinical insight before making treatment choices.

1.2.10 Susceptibility to Respiratory Viruses

Certain groups appear to be at much greater risk of developing severe respiratory viral infections, and of developing severe complications from them. These have been mentioned in the previous sections, but they include young children and infants, those with chronic cardiorespiratory diseases, immunocompromised individuals and the elderly. The dominant cause is impairments in the immune response, either due to immaturity of the immune system, effects of medication such as immunosuppressive agents or age associated immune senescence. In individuals with underlying cardio-respiratory conditions, current evidence shows that there is an impaired immune response to viral infection, which is likely due to the elevated baseline level of inflammation leading to dysregulated antiviral and inflammatory responses.[233] There is clear evidence exhibiting the increased rate of severe and lower respiratory tract infections in patients with chronic respiratory conditions in comparison to healthy controls.[49,234,235] In asthma, it has been demonstrated that IFN-β production by bronchial epithelial cells[236-238] and IFN-α production by peripheral blood mononuclear cells[239-241] are impaired, leading to an diminished anti-viral response. A set of studies has also shown deficient IFN responses in COPD, especially in the presence of cigarette smoke.[49,242,243] The impact of respiratory viral infections in those with chronic respiratory conditions, patients in ICU, and lung transplant patients are a major focus

of this review due to the higher risks associated with infection as discussed above; and have been investigated as part of this thesis.

1.3 Current sampling and detection techniques and limitations

1.3.1 Sampling Methods

There are a range of sampling techniques in current use in both clinical practice and in research studies. A number of these have been evaluated and compared to determine the best technique for detection of respiratory viruses. An important consideration is the balance between the invasiveness of the sampling technique and the diagnostic yield. For sampling the upper respiratory tract, the most commonly utilised methods include nasopharyngeal and oropharyngeal swabs, nasopharyngeal aspirates and nasal washings. To sample the lower respiratory tract, usually a spontaneously expectorated or induced sputum sample or a BAL will be collected.

The most commonly used method in clinical practice is NPS due to the ease of collection.[244] They can be done in almost any environment, quickly and painlessly without the need for any extra equipment. Differences have been observed between results obtained from regular and flocked swabs. The latter demonstrate higher viral detection rates due to the ability to collect significantly more epithelial cells.[245] A nasopharyngeal aspirate (NPA) involves inserting a catheter into the nasopharynx, where suction is applied to collect nasal secretions for viral testing. A nasal washing simply involves instillation of saline into the nasal space, which is then collected.

Comparisons between NPS and NPA have shown that the aspirates are significantly more sensitive (51% vs 100% for RSV) than swabs for the detection of respiratory viruses.[92]

Sputum yields significantly higher respiratory virus detection results compared with nasopharyngeal swabs using RT-PCR.[231,246] This is likely due to respiratory viruses having a propensity to infect the lower respiratory tract, and only some viruses, such as rhinovirus, effectively causing upper respiratory tract infections.[247] This has been demonstrated in multiple studies, where there has been a clear false negative rate for patients with influenza and RSV infections when only a traditional upper respiratory tract sampling method was used.[231,248,249] A limitation of sputum samples is that some patients may not produce sputum or be able to expectorate it, and furthermore, there may be some contamination from the upper respiratory tract. However, the utility of sputum over upper respiratory tract methods such as swabs and aspirates has been shown in cases where sputum can be obtained.

There are no methodological studies published which characterise the impact of upper respiratory tract viral contamination of lower respiratory tract samples thus far. Therefore, the data for bacterial pathogens investigated using different respiratory tract sampling methods will be discussed. Compared with BAL, sputum presents a less invasive method of sampling the lower respiratory tract but has an increased risk of oropharyngeal contamination due to passing directly through the upper respiratory tract when expectorated. However, a range of studies has shown that when used as a sampling technique in diseases such as CF, bronchiectasis and COPD, the biological

signals detected have been significantly and meaningfully associated with multiple measures including severity of illness, airway inflammation, antibiotic use and risk of subsequent exacerbations.[79,250-254] Bronchoscopy is a more invasive option, but it may offer a lower rate of upper respiratory tract contamination considering that samples do not pass directly through the upper respiratory tract. The bronchoscope is passed either through the mouth or nose, and down through the oropharynx to sample the lungs. Once inserted, lavage fluid is collected directly from distal airways. A recent study [255] using serial BAL analysis, demonstrated that sampling the lungs via bronchoscopy was not significantly confounded by the oral microbiome and this is consistent with previous serial bronchoscopy studies.[256] The evidence from these studies supports the understanding that minimal contamination from the upper respiratory tract is present in lower respiratory tract samples when utilising bronchoscopic techniques. Therefore, the use of either bronchoscopy or induced sputum may allow collection of representative lower respiratory tract samples.

1.3.2 Viral testing

Classically, viral identification and enumeration was achieved through *in vitro* cell culture. The introduction of molecular-based viral detection techniques revolutionised the field of viral diagnostics. Where viral identification was once expensive, time-consuming and often, negative, the use of PCR technology has facilitated a much more simple, rapid and accurate undertaking.[257] Importantly the use of PCR technology has introduced a new level of sensitivity to field of viral diagnostics, and allows isolation and identification of a single virion.[258] In the case of certain chronic respiratory diseases,

such as COPD, this may be important, as studies have shown that as little as 10 virions may be sufficient to induce an exacerbation.[49] Furthermore, these molecular methods are advantageous due to the reduced need for special growth conditions and the obligation to keep large stores of cultured cells and media. In early preparation steps, genetic material is extracted from cells which can be stored frozen in small vials for extended periods of time, thus reducing the need for large storage space.

PCR is a method which causes the exponential amplification of a known polynucleotide DNA sequence, which can be directly observed using fluorescence or run using agarose gel electrophoresis to identify the target. PCR requires knowledge of the genetic sequence of the particular viruses which are being targeted, as the reaction utilises sequence specific primers to amplify conserved gene regions of each virus to allow identification. These two oligonucleotide primers flank the genetic region that is to be amplified, and through cycles of heat denaturation of the double stranded (ds) DNA, annealing of the primers to their complementary sequences and extension of these sequences through the use of a heat resistant *Taq* DNA polymerase, the region of DNA is amplified. This is achieved through temperature cycles which allow optimum conditions for each of these processes to occur. The optimal temperature for the primer annealing is specific for each set of primers and will need to be determined experimentally as part of the reaction optimisation steps. Since the resultant strands of DNA are also complementary to the primers, successful cycles of the PCR allow an exponential increase in the amount of DNA product produced.[259]

Real time, quantitative PCR (qPCR) has also been developed which allows information regarding virus identification and viral load to be determined directly from the PCR assay, without the need for gel electrophoresis to visualise results. qPCR is based on the measurement of emitted fluorescence during the PCR reaction, to determine the amount of PCR product generated during each reaction cycle. This is normally due to the introduction of a fluorescently labelled oligonucleotide, which emits a fluorescent signal at certain points during the reaction. As a result, a reaction curve is generated, mapping the exponential phase of the reaction, which is needed to calculate the initial copy number of DNA fragments at commencement of the PCR. The elimination of post-processing steps has reduced the chances for cross-contamination of products, as well as allowing a significant reduction in cost and time for the technologist.[260] Limitations of PCR include the need for specific primers to detect pathogens, leading to the inability to detect any pathogen not directly assayed; and the inability to differentiate live replicating virus from nucleic acid fragments that may be the result of non-replicating or broken down viruses.

1.3.3 Significance of Viral PCR Detection Levels

Since the advent of PCR, the ability to detect viruses at a low viral load has increased significantly. This has led to the discovery of a great number of individuals who are PCR positive for a respiratory virus, but not exhibiting any symptoms or signs of active viral infection. Diagnostic microbiology laboratories worldwide have set limits of detection on the viral PCR assays, with an arbitrary pre-determined cycle threshold (CT) value to determine whether a patient is positive or negative for a viral infection.[261] However,

there is little evidence available to support a certain cut off value which will differ between each individual assay. Individual response plays a large role in whether presence of a virus is sufficient to cause infection, and therefore, different diagnostic and research laboratories may interpret infection levels contrarily.[262] Furthermore, it has been shown that viral load, and the set cut off value, is not sufficient to determine the predominant causative virus in infections where more than more virus has been detected.[263]

Conversely, studies have shown that viral load may be an important determinate of both symptoms and the likelihood of a viral infection reaching the lower respiratory tract. Pirella et al[264] showed that viral load higher than 10^7 RNA copies/mL in nasopharyngeal samples of children (under 5) was associated with lower respiratory tract infection. Further studies have shown that viral load correlates with illness severity scores in older children and adults.[262,265] However, other studies have observed symptomatic viral infection in individuals with a reduced viral load of 10^3 to 10^4 RNA copies/ml.[266] Therefore, it appears that age and other factors, such as underlying conditions, may play an important role in the determination of symptomatic lower respiratory tract infection.

1.4 Treatment Options for Respiratory Viruses

As previously discussed, viruses are intracellular pathogens that utilise host machinery to replicate. Therefore, discovering treatments that specifically target the virus, while sparing the host cells is important. Many antiviral treatments have targeted viral

specific proteins and enzymes, with attachment and fusion proteins receiving much attention. The high rate of genetic change and mutation, especially by RNA viruses, has meant that even when efficacious antiviral drugs and vaccines are discovered, a high rate of resistance may develop with vaccine failure.[267] Furthermore, in the respiratory virus domain, the emergence of new strains or species, such as the SARS-Coronaviruses or pandemic H1N1 2009 swine-recombinant influenza virus, leads to increased challenges in vaccine and antiviral development. There are currently limited antivirals available for respiratory viruses, with the only effective and commonly used vaccines and antivirals available for influenza viruses.

1.4.1 Anti-rhinovirus agents

There are no currently available licensed antivirals for rhinovirus, however this area has seen a lot of activity and there are a range of compounds currently in clinical trials. One of the major obstacles in the development of vaccines and antivirals against human rhinovirus includes the presence of more than 150 genotypes, the highly mutagenic nature of this virus, lack of epidemiological data concerning the exact strains circulating within different seasons, and the limited animal models available to allow determination of the viral pathogenesis and in vivo response to treatment.[268]

The most advanced antivirals in development are the capsid binders, which block virion uncoating.[269] The compounds in phase 2 and 3 trials include pleconaril, vapendavir and pocapavir which have shown modest benefit in rhinovirus infection.[270] The major limitation of capsid-binding agents is the rapid emergence of resistance,

while some naturally occurring strains of virus have also been identified.[271] A protease inhibitor, ruprintrivir, has also been developed, however in naturally infected patients it failed to reduce the severity of disease burden.[272] This lead to a halt in drug development, but other ruprintrivir derivatives are being investigated.[273] Development of antivirals for rhinovirus is lagging behind the advances being made for other respiratory viruses such as RSV and influenza, however as more information is discovered about the rhinovirus structure, serotypes and replication process, more drug targets may become apparent.

1.4.2 Anti-RSV agents

The currently available RSV antivirals include ribavirin, usually used in immunosuppressed populations, due to the high cost and concerns regarding toxicity[274] and palivizumab, a monoclonal antibody directed against the RSV fusion protein which is licenced only for prophylactic treatment in high-risk infants.[275] Ribavirin appears to be effective in ameliorating the severe symptoms and complications associated with RSV, particularly in transplant populations, and with the evaluation of oral and intravenous therapy compared with the aerosolised route previously used, ribavirin has become more affordable and less toxic to administer.[276-278] There are a range of small molecules in development, namely GS-5806 (Gilead, CA, USA) an inhibitor of the RSV fusion (F) protein[279], and AL-8176 (Alios, CA, USA) which is a nucleoside inhibitor of the L-protein (the RNA-dependent RNA polymerase of RSV).[280] Both of these compounds have shown efficacy in clinical trials, where decreased viral load and symptoms were observed alongside being well-tolerated.[281,282] Furthermore,

AL-8176 shows some promise in targeting other paramyxoviruses due to the relative-conservation of the RNA-dependent RNA polymerase utilised by all these viruses.[267] However, lack of venture capital funding has limited the development and testing of these agents.

There are a few other monoclonal antibodies targeted against the RSV fusion protein that are in earlier stages of development but may offer some promise in reducing the global impact of RSV infection. There is ongoing development and testing of the ALN-RSV01 small interfering RNA (siRNA) molecule from Alnylam (Cambridge, MA, USA). The siRNA targets a conserved epitope on the N-protein to arrest RSV replication. This molecule has undergone a phase IIb trial in lung transplant recipients, where it was shown that ALN-RSV01 reduces the risk of development of bronchiolitis obliterans syndrome (BOS) after RSV infection in this cohort.[283] The high level of activity in the anti-RSV area provides hope that novel agents may be available on the market in the near future.

1.4.3 Anti-influenza agents

Influenza vaccination is the most important tool we have in preventing and limiting the spread of influenza virus, however rates of vaccination in the community remain sub-optimal, some individuals do not mount a sufficient response to the vaccine, and break-through infections still occur. There are two main types of influenza vaccine currently available; live attenuated, and inactivated vaccines.[284] Antigenic drift and RNA mutations lead to inefficacy of vaccine over time, therefore new vaccines are

developed yearly for commonly circulating strains and annual re-vaccination is recommended.[285] These vaccines are normally based on the common strains present during the winter season in the opposing hemisphere, and therefore inefficient matching of the vaccine may occur if unexpected strains emerge.[286] Furthermore, there is currently little data regarding the efficacy of the influenza vaccine in older adults (>65years),[284] which is an at-risk population where influenza vaccination is specifically recommended. Therefore, more studies in this population are required to clearly demonstrate protection from influenza infection. There is ongoing research in this area, to enhance immune protection from both seasonal and pandemic influenza strains, and to speed up the production of vaccines, in preparation for pandemics.[287]

The most common influenza antivirals currently in use are neuraminidase inhibitors, including oseltamivir and zanamivir, and M2 ion channel blockers, including amantadine and rimantadine. These drugs are licenced for use in all populations, and have shown efficacy for both treatment of influenza infection and for prophylactic use.[288] However, there are evolving problems with resistance for all current antivirals,[289] with widespread resistance observed for the M2 ion channel inhibitors leading to a recommendation that this class should no longer be used for therapy.[290] Therefore, much ongoing research is focused on developing antivirals that inhibit components of the specialised influenza polymerase complex, which has 3 sub-units, the PA, PB1 and PB2 proteins. The full structure of the influenza heterotrimeric polymerase complex has been uncovered recently, using x-ray crystallography.[291,292] This presents a captivating target due to more opportunities for intervention within this replication mechanism, and also presents a reduced likelihood for resistance

development. Development of compounds targeting each of the polymerase subunits are underway.

One of the most advanced of these compounds is Favipiravir, a nucleoside analog which is believed to be incorporated into the viral RNA by PB1 (influenza's RNA-dependent RNA polymerase), to inhibit replication. This compound has already been approved for use against pandemic flu in Japan.[293] In recent years, there has also been development of the first inhibitor of PB2 (influenza's cap-snatching subunit) by Vertex (Boston, MA, USA) and Janssen (Beerse, Belgium). JNJ-872 has been shown to significantly reduce viral load and symptom duration, if given early after virus exposure. However, during development, it has been shown that this compound is only effective against Flu A strains, as the binding motif on the PB2 subunit differs significantly between influenza A and B.[291,294] Similarly, there are two compounds in phase I clinical trials directed against the PA protein (endonuclease),[267] which may prove to have greater target range due to the vital role that the endonuclease plays as part of the polymerase complex, and due to the relatively conserved endonuclease active site between influenza A and B. Influenza viruses have received a lot of attention, and have the most advanced treatments available currently. The influenza vaccines are the only vaccinations available for any respiratory virus, and they offer increased protection for a number of at risk groups. This is an exciting area of research, with many new compounds in all stages of development, providing optimism for the availability of new treatments in the near future.

1.4.4 Ongoing development of antivirals against parainfluenza virus and human metapneumovirus

Respiratory viruses such as parainfluenza virus and human metapneumovirus are part of the paramyxoviridae family, similar to RSV. Therefore, there is interest in developing antivirals with a greater range that are active against all members of this viral family. The agent favipiravir, has shown antiviral activity against a broad range of paramyxoviruses in animal models.[295] Ribavirin has also been used to treat PIV infections in immunocompromised patients,[296] but there is currently limited evidence to support its use. There is currently little data regarding specific treatments for these viruses, even though both PIV and HMPV have the ability to cause severe infections in at risk populations. Future research may lead to the development of safe and efficacious antiviral treatments and vaccines and may change the way we approach respiratory viral infections.

1.4.5 Limitations of current treatments

Given the large burden of respiratory viral infections in healthy adults and children, as well as in the at-risk groups discussed, there is a clear unmet need in terms of available therapeutics. As can be seen, there are very limited therapeutic options available and only for specific viruses. Furthermore, there are great limitations of the effectiveness of current antivirals. For example, neuraminidase inhibitors for influenza are only effective if given within 48 hours of symptom onset.[297] Ribavirin which may be aerosolised or given intravenously is very costly, and there are pertinent safety

considerations when aerosolised due to the toxicity of the compound.[298] Oral therapy seems as effective. Therefore, the range of antivirals which are currently being developed or are in clinical trials may revolutionise our approach to respiratory viral infections. In the future, it is hoped that broad-spectrum antivirals may be available which can be used against a range of respiratory viruses by targeting conserved structures or functions, and that a broader range of viral vaccines may be developed.

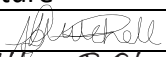
1.5 Are the lungs really sterile? Exploring the role of the Respiratory Virome as part of the Human Respiratory Microbiome and its relevance in lung transplantation

As little is currently known about the virome, the role of the bacterial microbiome in lung transplantation, will be explored in this section. The knowledge we have regarding the bacterial component of the microbiome may act as a model for the viral component.

Mitchell, A. B., and Glanville A. R. (2018) The Human Respiratory Microbiome: Implications and Impact. “Lung Transplantation: Controversies and Evolving Concepts” *Semin Respir Crit Care Med* (Thieme, N.Y.). Vol 39: 199-212.

1.5.1 Statement of Contribution

All research and preparation of the manuscript was done by AB Mitchell. Editing and final preparation of the manuscript was carried out by AR Glanville. All authors agree with these statements.

Name	Signature	Date
AB Mitchell		4/6/18
AR Glanville	Allan R Glanville	04/06/2018

The Human Respiratory Microbiome: Implications and Impact

Alicia B. Mitchell, BMedSci (Hons)[1,2,3] Allan R. Glanville, MBBS, MD, FRACP[3,4]

[1] School of Molecular Biosciences, University of Technology Sydney, Sydney, New South Wales, Australia
[2] Woolcock Institute of Medical Research, Glebe, Sydney, New South Wales, Australia
[3] Department of Thoracic Medicine, St. Vincent's Hospital, Darlinghurst, New South Wales, Australia
[4] Department of Medicine, University of New South Wales, Sydney, New South Wales, Australia

Address for correspondence Allan R. Glanville, MBBS, MD, FRACP, Department of Thoracic Medicine, St. Vincent's Hospital, Darlinghurst, New South Wales 2010, Australia (e-mail: Allan.Glanville@svha.org.au).

Semin Respir Crit Care Med 2018;39:199–212.

Abstract

Once considered a sterile site below the larynx, the tracheobronchial tree and parenchyma of the lungs are now known to harbor a rich diversity of microbial species including bacteria, viruses, fungi, and archaea. Many of these organisms, particularly the viruses which comprise the human respiratory virome, have not been identified, so their true role is unknown. It seems logical to conclude that a "healthy" respiratory microbiome exists which may be modified in disease states and perhaps by therapies such as antibiotics, antifungals, and antiviral treatments. It is likely that there is a critical relationship or equilibrium between components of the microbiome until such time as perturbations occur which lead to a state of dysbiosis or an "unhealthy" microbiome. The act of lung transplantation provides an extreme change to an individual's respiratory microbiome as, in effect, the donor respiratory microbiome is transplanted into the recipient. The mandatory ex-vivo period of the donor lungs appears to be associated with blooms of resident viral species in particular. Subsequently, allograft injury, rejection, and immune suppressive therapy all combine to create periods of dysbiosis which when combined with transient infections such as community acquired respiratory viruses may facilitate the development of chronic allograft dysfunction in predisposed individuals. As our understanding of the respiratory microbiome is rapidly expanding, based on the use of new-generation sequencing tools in particular, it is to be hoped that insights gained into the subtle relationship between the microbiome and the lung allograft will facilitate improved outcomes by directing novel therapeutic endeavors.

Keywords
- microbiome
- lung transplant
- lung allograft
- respiratory tract
- virome

The microbiome consists of all microorganisms and their products that occupy surfaces within the human body. Each major compartment of the human body appears to have a unique microbiome with species which are specific for that environment. The microbiome encompasses bacteria, fungi, viruses (including bacteriophages), and archaea. These microorganisms are an integral part of the functional human unit. The human body hosts more than a trillion microbial cells and microbiome-associated genes outnumber human-coded genes 100-fold.[1] Humans and microbes have co-evolved over millions of years, and subsequently, the human immune system and the microbiome demonstrate complex interactions. The development of the microbiome is integral in shaping the immune response, while the immune system is required to maintain this large, and highly diverse set of microbes. Thus, a symbiotic interface has been established within the human body.[2]

Issue Theme Lung Transplantation: Controversies and Evolving Concepts; Guest Editors: John A. Belperio, MD, and Allan R. Glanville, MB, BS, MD, FRACP

DOI https://doi.org/10.1055/s-0037-1617441.
ISSN 1069-3424.

Mitchell, A. B., Oliver, B. G. and Glanville, A. R. (2016). "Translational Aspects of the Human Respiratory Virome." *Am J Respir Crit Care Med*. 194(12), pp.1458-1464.

1.5.2 Statement of Contribution

All research and preparation of the manuscript was done by AB Mitchell. Editing and final preparation of the manuscript was carried out by AR Glanville. All authors agree with these statements.

Name	Signature	Date
AB Mitchell		4/6/18
AR Glanville	Allan R Glanville	04/06/2018

CONCISE CLINICAL REVIEW

Translational Aspects of the Human Respiratory Virome

Alicia B. Mitchell[1,2], Brian G. G. Oliver[1], and Allan R. Glanville[2]

[1]The Woolcock Institute of Medical Research, Sydney, New South Wales, Australia; and [2]The Lung Transplant Unit, St. Vincent's Hospital, Sydney, New South Wales, Australia

Abstract

Despite the dominant role of community-acquired respiratory viruses as etiological agents of disease, there has been little focus to date on the translation of rapidly developing diagnostic modalities, such as next-generation sequencing techniques in the examination of lower respiratory tract samples. When applied, these techniques should inform strategies to both understand the nexus between health and disease states of the respiratory virome, and drive a paradigm shift in how the practicing pulmonologist views the conceptual framework of respiratory infections. The lower respiratory tract was once thought to be a sanctuary site from microbiological colonization owing to the efficacy of upper airway–protective mechanisms and the host mucosal barrier function of the lower airways, combined with both innate and adaptive immune responses. As a small number of recent studies confirm, this is a naive vision of the lung, the viral component of which parallels recent revelations from respiratory microbiome studies. Hence, it is now timely to revise our thinking regarding the constituents, diversity, and changing nature of the respiratory virome in health and disease. One area worthy of focus is the interface between community-acquired respiratory viruses and the respiratory virome to better understand the dynamics in acute infection, as well as the factors that may lead to viral persistence and chronic disease. Given recent advances in metagenomics, the tools are now at hand to accomplish these goals.

Keywords: microbiota; metagenome; virome; community-acquired respiratory viruses

(Received in original form June 26, 2016; accepted in final form September 20, 2016)

Author Contributions: Conception and design—A.B.M. and A.R.G.; analysis and interpretation—A.B.M., B.G.G.O., and A.R.G.; drafting the manuscript for important intellectual content—A.B.M., B.G.G.O., and A.R.G.

Correspondence and requests for reprints should be addressed to Allan R. Glanville, M.B. B.S., M.D., The Lung Transplant Unit, Xavier 4, St. Vincent's Hospital, Victoria Street, Darlinghurst, NSW 2010, Australia. E-mail: allan.glanville@svha.org.au

CME will be available for this article at www.atsjournals.org

Am J Respir Crit Care Med Vol 194, Iss 12, pp 1458–1464, Dec 15, 2016

Originally Published in Press as DOI: 10.1164/rccm.201606-1278CI on September 21, 2016
Internet address: www.atsjournals.org

1.6 Outline of Chapters

As mentioned, there is a broad literature surrounding the role of respiratory viral infections. However, there is still a great deal of ongoing discussion regarding our understanding of their impact in at risk patient populations, including in bronchiectasis. Considering that the majority of research has focused on bacterial infections, parallel work needs to elucidate the risk of airborne viruses in an intensive care unit where very unwell and often ventilated patients are present and the role of asymptomatic community acquired respiratory viruses in acute and chronic rejection events for patients who have undergone lung transplantation.

This thesis has firstly investigated a novel viral sampling methodology utilising exhaled breath, followed by characterising the use of adaptations of this methodology in patient cohorts, and investigations of the prevalence of respiratory viruses in at risk patient populations. In the first chapter, the development of a non-invasive viral sampling methodology will be explored in an attempt to overcome many of the limitations that exist with the current respiratory virus sampling methods. These include sampling only the upper respiratory tract, and the discomfort involved with swabs and nasopharyngeal aspirates; the invasiveness of bronchoalveolar lavage of the lower respiratory tract; and the select patient group that are able to expectorate sputum.

The next chapter evaluates the use of this new viral sampling technique in patients with bronchiectasis, to collect exhaled respiratory viruses. This technique was

compared with sampling using spontaneously expectorated sputum. The incidence of viral infections during stable state bronchiectasis was investigated to determine whether viruses were common, and possibly important, in the progression of bronchiectasis in comparison to the current literature which has focused on colonising bacterial species.

A further study was completed in an ICU cohort, where an adaptation of the original exhaled breath sampling methodology was used as part of the ventilator circuits to detect viruses in ICU patients. There has been limited research into the role of airborne viruses in ICU, however this may be particularly important during respiratory virus outbreaks and pandemics, such as the 2009 H1N1 pandemic. A previous study has shown that ventilator filters are efficacious in filtering out viral particles,[418] therefore this chapter focused on determining if these same filters could be used for detecting respiratory viruses from within the intubated patient's lungs.

The final study was done in a cohort of lung transplant recipients. There is little information regarding the impact of asymptomatic respiratory viruses on lung transplant recipients. This prospective, longitudinal study collected multiple respiratory tract samples from patients before, at the time of transplant and after transplantation to determine the incidence and persistence of CARV. An analysis of clinical outcomes, and acute rejection events in relation to detection of CARV was also carried out, as there is very limited literature investigating the impact of respiratory viral presence within the transplanted lungs on early transplant outcomes.

1.6.1 Aims and Hypotheses

Chapter 2:

Spirometry filters can be used to detect exhaled respiratory viruses

Alicia B. Mitchell, Bassel Mourad, Euan Tovey, Lachlan Buddle, Matthew Peters, Lucy Morgan and Brian G Oliver

J Breath Research – September 2016

doi:10.1088/1752-7155/10/4/046002

Aims: To investigate whether disposable spirometry filters, collected as part of routine lung function measurements, could be used to capture and identify exhaled viruses and to compare the detection sensitivity of this novel method with conventional methods of respiratory tract sampling. The results of exhaled viruses will be compared with BAL to validate whether the viruses detected were present in the lower respiratory tract.

Hypotheses: That the spirometry filters would be able to capture exhaled virus, and as patients are performing forced expiratory manoeuvres, viruses from both the upper and lower respiratory tract will be disrupted due to the turbulent airflow and will be exhaled into the filter material. This filter material will allow non-invasive collection and detection of respiratory viruses with similar accuracy to sampling respiratory secretions from patients.

Chapter 3:

Viruses in bronchiectasis: A pilot study to explore the presence of community acquired respiratory viruses in stable patients and during acute exacerbations.

Alicia B. Mitchell, Bassel Mourad, Lachlan Buddle, Matthew J. Peters, Brian G.G. Oliver, and Lucy C. Morgan

BMC Pulmonary Medicine – May 2018

https://doi.org/10.1186/s12890-018-0636-2

Aims: This was a pilot study aimed at determining 1) the incidence of respiratory virus testing ordered by physicians within a cohort of bronchiectasis patients with acute exacerbations at a teaching hospital and 2) separately, to determine the incidence of viral detection within a group of patients with stable bronchiectasis to establish baseline viral prevalence.

Furthermore, to evaluate the incidence of symptomatic viral infections and rate of exacerbations in this cohort.

Hypotheses: 1) That viral testing would be an under-utilised resource in evaluating inpatients with bronchiectasis exacerbations as bacteria are assumed to be a main pathogen in this condition.

2) Viruses will be regularly detected in the stable state of bronchiectasis, and may be achieve a state of colonisation, similar to bacterial species, in the lower respiratory tract. Therefore, detection of viruses in exhaled breath and sputum samples will not be associated with exacerbation frequency.

Chapter 4:

A novel sampling method to detect airborne influenza and other respiratory viruses in mechanically ventilated patients in intensive care unit: A feasibility study

Alicia B. Mitchell, Benjamin Tang, Maryam Shojaei, Lachlan S. Barnes, Marek Nalos, Brian G.G Oliver, and Anthony S. McLean

Ann Intensive Care – April 2018

https://doi.org/10.1186/s13613-018-0396-4

Aims: To evaluate the feasibility of detecting airborne respiratory viruses in mechanically ventilated patients using a novel approach in which we measured viruses trapped in the ventilator filters of mechanically ventilated patients in an intensive care unit.

Hypotheses: The ventilator filters have a large volume of inspired/expired air circulating through them each day (8,640,000 L/day), therefore, these filters should capture and allow sampling of airborne viruses. When the viral load is sufficient, expired air should allow detection of respiratory viruses from the lower respiratory tract in mechanically ventilated patients.

Chapter 5:

Transplanting the Pulmonary Virome: Dynamics of Transient Populations

Alicia B. Mitchell, Bassel Mourad, Lucy C. Morgan, Brian G.G. Oliver, Allan R. Glanville

J Heart Lung Transplant – June 2018

Aims: To analyse the relationship between transient members of the pulmonary virome, predominantly community acquired respiratory viruses (CARV), and early outcomes after lung transplant.

Hypotheses: CARV will be common in the lungs of transplant recipients and will persist due to the high levels of immunosuppression and lack of mucociliary clearance and cough reflex following transplantation. Asymptomatic viruses that do not induce an immune response and result in lung injury, will not be associated with rejection events.

CHAPTER 2

SPIROMETRY FILTERS CAN BE USED TO DETECT EXHALED RESPIRATORY VIRUSES

2.1 Statement of Contribution

Patient recruitment, sample collection, and experiments were conceived and carried out; results were analysed and manuscript was prepared for publication by AB Mitchell. Sample preparation and aspects of experimental work were carried out; and the manuscript was reviewed by B Mourad. Experimental protocols were conceived; and the manuscript was reviewed by E Tovey. Patient recruitment, sample collection and manuscript revision were done by L Buddle. Ethics application preparation, study oversight and manuscript revision were carried out by M Peters. L Morgan and BG Oliver conceived and designed the study, were involved in troubleshooting the experiments, discussed the data, and reviewed the manuscript. All authors agree with these statements.

Name	Signature	Date
AB Mitchell		9/5/18
B Mourad		18/5/18
E Tovey		20/5/18
L Buddle		10/5/2018
M Peters		11/5/18.
L Morgan		10/5/2018
BG Oliver		16/5/2018

Journal of Breath Research

PAPER

Spirometry filters can be used to detect exhaled respiratory viruses

RECEIVED
7 August 2016

ACCEPTED FOR PUBLICATION
26 August 2016

PUBLISHED
26 September 2016

Alicia B Mitchell[1,2,4,7], Bassel Mourad[1,4], Euan Tovey[1], Lachlan Buddle[2], Matthew Peters[2,3], Lucy Morgan[2,3] and Brian G Oliver[1,4,5,6]

[1] Respiratory Cellular and Molecular Biology, Woolcock Institute of Medical Research, The University of Sydney, NSW 2006, Australia
[2] Department of Respiratory Medicine, Concord Repatriation General Hospital, Concord, NSW 2139, Australia
[3] Concord Clinical School, University of Sydney, NSW 2006, Australia
[4] Molecular Biosciences, School of Life Sciences, University of Technology Sydney, NSW 2007, Australia
[5] Centre for Health Technologies, University of Technology Sydney, NSW 2007, Australia
[6] Emphysema Centre, Woolcock Institute of Medical Research, The University of Sydney, NSW 2006, Australia
[7] Author to whom any correspondence should be addressed. University of Technology Sydney, Building 4, 15 Broadway, Ultimo, NSW 2007, Australia.

E-mail: Alicia.B.Mitchell@student.uts.edu.au

Keywords: asthma, bronchiectasis, influenza, respiratory function tests, viral infection, COPD

Abstract

Respiratory viruses are very common in the community and contribute to the burden of illness for patients with chronic respiratory diseases, including acute exacerbations. Traditional sampling methods are invasive and problematic to repeat. Accordingly, we explored whether respiratory viruses could be isolated from disposable spirometry filters and whether detection of viruses in this context represented presence in the upper or lower respiratory tract.

Discovery ($n = 53$) and validation ($n = 49$) cohorts were recruited from a hospital outpatient department during two different time periods. Spirometry mouthpiece filters were collected from all participants. Respiratory secretions were sampled from the upper and lower respiratory tract by nasal washing (NW), sputum, and bronchoalveolar lavage (BAL). All samples were examined using RT-PCR to identify a panel of respiratory viruses (rhinovirus, respiratory syncytial virus, influenza A, influenza B, parainfluenza virus 1, 2 & 3, and human metapneumovirus). Rhinovirus was quantified using qPCR.

Paired filter-NW samples ($n = 29$), filter-sputum samples ($n = 24$), filter-BAL samples ($n = 39$) and filter-NW-BAL samples ($n = 10$) provided a range of comparisons. At least one virus was detected in any sample in 85% of participants in the discovery cohort versus 45% in the validation cohort. Overall, 72% of viruses identified in the paired comparator method matched those detected in spirometry filters.

There was a high correlation between viruses identified in spirometry filters compared with viruses identified in both the upper and lower respiratory tract using traditional sampling methods. Our results suggest that examination of spirometry filters may be a novel and inexpensive sampling method for the presence of respiratory viruses in exhaled breath.

CHAPTER 3

VIRUSES IN BRONCHIECTASIS: A PILOT STUDY TO EXPLORE THE PRESENCE OF COMMUNITY ACQUIRED RESPIRATORY VIRUSES IN STABLE PATIENTS AND DURING ACUTE EXACERBATIONS

3.1 Statement of Contribution

Patient recruitment, sample collection, and experiments were conceived and carried out; retrospective data and results were analysed and manuscript was prepared for publication by AB Mitchell. Sample preparation and aspects of experimental work were carried out; and the manuscript was reviewed by B Mourad. Patient recruitment, sample collection and manuscript revision were done by L Buddle. Ethics application preparation, study oversight and manuscript revision were carried out by M Peters. BG Oliver and L Morgan conceived and designed the study, aided in patient recruitment, were involved in troubleshooting the experiments, discussed the data, and reviewed the manuscript. All authors agree with these statements.

Name	Signature	Date
AB Mitchell		9/5/18
B Mourad		18/5/18
L Buddle		10/5/2018
M Peters		11/5/18
BG Oliver		16/5/2018
L Morgan		10/5/2018

Mitchell et al. BMC Pulmonary Medicine (2018) 18:84
https://doi.org/10.1186/s12890-018-0636-2

BMC Pulmonary Medicine

RESEARCH ARTICLE Open Access

Viruses in bronchiectasis: a pilot study to explore the presence of community acquired respiratory viruses in stable patients and during acute exacerbations

Alicia B. Mitchell[1,2,4*], Bassel Mourad[1,4], Lachlan Buddle[2], Matthew J. Peters[2,3], Brian G. G. Oliver[1,4,5,6] and Lucy C. Morgan[2,3]

Abstract

Background: Bronchiectasis is a chronic respiratory condition. Persistent bacterial colonisation in the stable state with increased and sometimes altered bacterial burden during exacerbations are accepted as key features in the pathophysiology. The extent to which respiratory viruses are present during stable periods and in exacerbations is less well understood.

Methods: This study aimed to determine the incidence of respiratory viruses within a cohort of bronchiectasis patients with acute exacerbations at a teaching hospital and, separately, in a group of patients with stable bronchiectasis. In the group of stable patients, a panel of respiratory viruses were assayed for using real time quantitative PCR in respiratory secretions and exhaled breath. The Impact of virus detection on exacerbation rates and development of symptomatic infection was evaluated.

Results: Routine hospital-based viral PCR testing was only requested in 28% of admissions for an exacerbation. In our cohort of stable bronchiectasis patients, viruses were detected in 92% of patients during the winter season, and 33% of patients during the summer season. In the 2-month follow up period, 2 of 27 patients presented with an exacerbation.

Conclusions: This pilot study demonstrated that respiratory viruses are commonly detected in patients with stable bronchiectasis. They are frequently detected during asymptomatic viral periods, and multiple viruses are often present concurrently.

Keywords: Bronchiectasis, Respiratory viruses, Viral infection, Influenza

Background

Bronchiectasis is a progressive disease characterised by permanent dilatation of bronchi, impairment of mucociliary clearance, and retention of secretions. Recurrent respiratory infections are a key feature of bronchiectasis, with the majority of research focusing on the role of bacteria in stable patients, during acute exacerbations and particularly in disease progression [1, 2]. Despite significant advances in diagnostic immunology and radiology, and a growing global awareness of bronchiectasis as a significant twenty-first century clinical problem, the underlying cause of bronchiectasis in a given patient is not always clear. Approximately 40% of cases remain idiopathic [3], after the most common causes (immunodeficiencies, cystic fibrosis (CF), primary ciliary dysfunction (PCD), allergic bronchopulmonary aspergillosis (ABPA), connective tissue disorders, chronic obstructive pulmonary disease (COPD)-related, or asthma-related) have been excluded [4, 5].

Respiratory infections in early childhood are an important cause of airway damage with the potential to

* Correspondence: amit9422@uni.sydney.edu.au
[1]Respiratory Cellular and Molecular Biology, Woolcock Institute of Medical Research, The University of Sydney, Sydney, NSW 2006, Australia
[2]Department of Respiratory Medicine, Concord Repatriation General Hospital, Concord, NSW 2139, Australia
Full list of author information is available at the end of the article

initiate the vicious cycle of epithelial damage, airway dilatation, mucostasis, and bacterial colonisation [6]. Prior to widespread vaccination in the mid twentieth century, measles and pertussis played a major role in post-infectious damage leading to bronchiectasis [7]. The incidence and mortality of pneumonia associated with influenza and pneumococcal infection has also been reduced in both paediatric [8–10] and adult populations with access to vaccination programs [11, 12]. Pneumonia in childhood caused by common respiratory viruses has been associated with significant early airway damage and these viruses are emerging as major factors in the subsequent development of bronchiectasis [4, 13]. While research has focused on defining the aetiology of bronchiectasis due to its implications in individualised treatment and management of the disease, little work has been done to define the role of respiratory viruses in stable and acute bronchiectasis.

As there is considerable phenotypic overlap between bronchiectasis, CF and COPD, the basic understanding gained from investigating the role of viruses in exacerbations and asymptomatic viral detection during stable phases in these diseases may guide our knowledge regarding bronchiectasis.

The association between viral infection and bacterial superinfection is well described in the literature, and more recently, with changes in the microbiome. In COPD and CF, respiratory viruses precipate exacerbations, which in turn, are associated with accelerated disease progression [14]. Mallia et al. [15] demonstrated that experimental rhinovirus infection in patients with COPD could induce symptoms associated with exacerbations, and induce changes in the microbiota. These findings in COPD have been further confirmed by a serial analysis of the lung microbiome following rhinovirus infection [16]. In patients with CF, significantly higher levels of respiratory viruses were detected during exacerbations (46%) compared to stable phases (17%) [17]. The detection of viruses during exacerbation has also been associated with an increase in colony counts of Pseudomonas aeruginosa, suggesting that viruses may also affect the stability of the microbiome in cystic fibrosis [18]. In these diseases, increased viral presence was often observed during exacerbations which also lead to changes in the resident microbial communities. Bacterial colonisation is a common and key feature of the pathophysiology of bronchiectasis. Less is known about the role of viruses in stable state bronchiectasis, or the effect of viruses on the equilibrium between symbiotic and pathogenic bacterial species.

Therefore, this pilot study aimed to determine the incidence of respiratory virus testing ordered by physicians within a cohort of bronchiectasis patients with acute exacerbations at a teaching hospital and separately, to determine the incidence of viral detection within a group of patients with stable bronchiectasis to establish baseline viral prevalence. The incidence of symptomatic viral infections and rates of exacerbations in this cohort was also evaluated.

Methods

Part 1

A retrospective clinical audit was undertaken to determine the rate of testing for respiratory viruses for patients admitted to Concord Repatriation General Hospital July 2011 to June 2016 with an acute exacerbation of bronchiectasis. Patient data regarding exacerbation frequency, previous lung function and hospital admissions were collected from the Australian Bronchiectasis Registry.

Part 2

Clinical measures

Two cohorts of patients were recruited from an outpatient clinic whilst clinically stable. All patients attending the specialised bronchiectasis clinic during the recruitment months were asked to participate. Bronchiectasis was deemed to be clinically stable from the point of view by the consultant physician in clinic based on the patient's history, and no deterioration in clinical symptoms in the month prior to their clinic visit. A history of viral-related symptoms was not an exclusion criteria. One cohort was recruited during the winter months in Australia (May – September), while the other was recruited during the summer months (January – March). Samples were collected from each patient during their clinic visit, to determine if viruses were present within the lungs of bronchiectasis patients when clinically stable, similar to resident bacterial species. This is a tertiary referral centre for PCD, where the diagnosis of PCD was made based on ciliary motility studies and electron microscopy. Patients provided a basic medical history and filled out a common cold questionnaire at the time of recruitment [19]. The common cold questionnaire (CCQ) assesses viral symptoms on an 11-point scale. Based on the presence or absence of these symptoms, the questionnaire predicts the likelihood of a viral infection. Results are classified into three categories; 'no virus', 'possible virus' or 'probable virus' depending on how many symptoms are reported [19]. The results of the questionnaires were considered at the time of analysis in conjunction with the viral PCR results, and were not used as inclusion or exclusion criteria.

Spirometry was performed at the time of sample collection (according to ATS/ERS guidelines) [20] and compared to previous results to ensure that patients were at baseline. FEV_1 was used as a surrogate measure of severity in this cohort of patients. The filters from the

spirometer mouthpieces were frozen during storage, then processed for RNA extraction from exhaled breath using a methodology described previously [21], and spontaneously expectorated sputum samples were also collected. All patients were reviewed by the physiotherapist in clinic if sputum was not easily spontaneously expectorated.

To investigate if asymptomatic infections could develop into acute exacerbations, information regarding exacerbations and hospitalisations in the following 2 months were collected for all patients. Other patient outcomes, including lung function, acute viral or bacterial infections, were also collected.

Sample molecular processing

Filters and sputum samples were analysed for a panel of respiratory viruses using PCR. Virus RNA was extracted from the exhaled breath captured in spirometry filters using a methodology published previously [21]. Filters were first removed from the spirometry mouthpieces and 1 ml of Bioline Lysis Buffer RLY (Bioline, Alexandria, Australia) was then added. This was centrifuged for 2 min at 10 000 rpm. The eluate was collected after the final spin and stored at − 20 °C until RNA extraction. Sputum samples were homogenised by mixing the secretion with 1 ml of 1% B-ME Lysis Buffer RLY to achieve a final volume of 1.5 ml, which was then stored at − 20 °C. Following this, RNA was purified using the Isolate II RNA Mini Kit (Bioline, Alexandria, Australia) before conversion to cDNA using the Bioline SensiFAST cDNA Synthesis Kit (Bioline, Alexandria, Australia).

cDNA was assayed by uniplex real time reverse transcription polymerase chain reactions for human rhinovirus (HRV), respiratory syncytial virus (RSV), influenza virus type A and influenza virus type B, parainfluenza virus (PIV) 1, 2 and 3, and human metapneumovirus (HMPV). Real Time quantitative PCR (qPCR) assays utilised the StepOnePlus Real-Time PCR System (Applied Biosystems, ThermoFisher, Massachusetts, USA). All samples were run in triplicate, with 2 μl of cDNA template added to Bioline SensiFAST Probe Hi-ROX Master Mix. PCR primers were sourced from the literature [21–25], and have been previously optimised using clinical samples. Forward and reverse primers were added along with virus specific probe. The qPCR was run for 40 cycles, and the cycle threshold (CT) value was defined for each reaction.

Statistical analysis

T-tests were used to compare parametric data sets, Mann-Whitney tests for non-parametric data, and Fisher's exact test was completed for contingency table analyses using GraphPad Prism version 6.

Results

Part 1

During the study period 47 patients were identified from the Bronchiectasis Registry as having been admitted to Concord Repatriation General Hospital for an exacerbation of bronchiectasis with a total of 83 admissions. The average age for this cohort was 72 ± 14 years, mean ± SD (range 24–88) (male = 19).

Of the 83 total admissions, viral PCR was requested in only 23. In comparison, bacterial and fungal cultures were requested in 73 admissions.

Viral PCR was positive in 9 of 23 cases (39%), with 3 cases of influenza A and 6 cases of HRV.

Bacterial and fungal cultures were positive in 22/73 admissions (30%). The most commonly detected pathogen by culture was *Pseudomonas aeruginosa* in 9 admissions, followed by *Haemophilus influenzae* in 7 cases, *Burkholderia cepacia* in 1, and *Achromobacter xylosoxidans* in 1 case. Fungal species were less common, with *Aspergillus* spp. detected during 3 exacerbations, and *Candida albicans* in 1 case.

Part 2

Winter cohort

Twelve patients with stable bronchiectasis were recruited in the winter cohort. The clinical characteristics of these patients are summarised in Table 1. Four patients were on maintenance therapy with an inhaled corticosteroid (ICS) /long acting beta agonist (LABA) combination inhaler, while the majority had been prescribed a short acting beta agonist (SABA) as needed. Only one patient reported being a past smoker, all other patients had never smoked.

Of the 12 patients with bronchiectasis recruited during the winter period, 9 of these also had a concurrent diagnosis of PCD. The majority of these patients (11/12) had relatively preserved lung function with an FEV_1 greater than the lower limit of normal. One patient had severely reduced lung function, with an FEV_1 of only 21% predicted (0.56 L), and an FEV_1/FVC ratio of 50% based on ATS/ERS guidelines [26].

All patients completed the CCQ on the day of secretion sampling. None reported enough symptoms on the 11-point scale to be categorised as "probable virus". All patients remained stable, with no reported exacerbations or hospital admissions within a month prior to or 2 months after sample collection.

Filters and sputum samples were processed for a panel of respiratory viruses. Nine of 12 patients had respiratory virus RNA identified in filter samples. In the filters, influenza was the most commonly detected respiratory virus (9/12), with 3 patients having influenza A, 3 with influenza B, 2 with concurrent influenza A and B detection and one patient who demonstrated co-detection of

Table 1 Summary of patient characteristics and respiratory virus detection and exacerbation rates in both the winter and summer cohorts

Season	Mean age ± SD	Gender (F/M)	Mean FEV_1 ± SD	Comorbid PCD	Overall viral detection rate	Number of patients with Influenza	Multiple virus detection rate	Exacerbation rate during follow up period
Winter ($n = 12$)	36 ± 12	10/2	77% ± 22%	9/12 (75%)	11/12 (92%)	10/12 (83%)	10/12 (83%)	0/12 (0%)
Summer ($n = 15$)	60 ± 17	12/3	60% ± 33%	3/15 (20%)	5/15 (33%)	5/15 (33%)	0/15 (0%)	1/15 (7%)
Overall ($n = 27$)	49 ± 19	22/5	67% ± 29%	12/27 (44%)	16/27 (59%)	15/27 (56%)	10/27 (37%)	1/27 (4%)

human rhinovirus and influenza A (Table 2). Using qPCR, in the samples where the same virus was detected in the filter as the sputum sample, the CT value was lower (approximately 33 cycles) compared with those viruses found in the sputum alone (approximately 37 cycles).

Table 2 Specific viruses detected in the filter and sputum samples of patients in Summer and Winter cohorts

Patient	Winter	
	Filter positive	Sputum positive
1	Flu A	RV, RSV, Flu A + B
2	Flu B	RV, RSV, Flu A + B
3	Flu B	RV, RSV, Flu A + B
4	Flu B	RV, RSV, Flu A + B
5	Flu A	RV, RSV + Flu A
6		RV + RSV
7		Flu A
8	RV + Flu A	RV + Flu A
9	Flu A + B	RV, RSV, Flu A + B
10	Flu A + B	RV, RSV, Flu A + B
11	Flu A	RV, RSV, Flu A + B
12		
Patient	Summer	
	Filter positive	Sputum positive
13		
14	FluA	FluA
15		
16		
17		
18	FluA	FluA
19		FluA
20		FluA
21		
22		
23		
24		
25		
26	FluA	FluA
27		

In sputum samples, 11 of 12 patients had a respiratory virus identified. A single patient had only influenza A identified. Co-infection was more common with 7 patients showing concurrent detection of HRV, RSV, influenza A and B; 1 patient with HRV, RSV and influenza A; 1 with HRV and RSV; 1 with HRV and influenza A. All 9 subjects with virus detected in exhaled breath also had virus detected in the matched sputum sample. As 11 of 12 patients in the winter cohort were viral positive, it was not possible to correlate virus detection with disease severity based on FEV1. Similarly, a correlation between viral detection and use of SABA, or ICS/LABA combination treatment could not be deduced.

Summer cohort

Fifteen patients were recruited in the summer cohort. Their clinical characteristics are summarised in Table 1. Ten patients were on maintenance therapy with an ICS/LABA combination inhaler, two had additional tiotropium therapy; 12/15 had been prescribed a SABA PRN. All patients reported never smoking.

In this cohort, 3 of 15 patients had a concomitant PCD diagnosis, while 2 of 15 also had a diagnosis of asthma. This was a slightly more severe cohort of bronchiectasis patients based on spirometry when compared with the group recruited during the winter season. The mean FEV_1 in this group was 59% of predicted, however this was not significantly different to the winter group. Only one patient reported symptoms of viral infection at the time of sample collection, the rest of the patients reported feeling well at the time of their clinic visit which was confirmed by responses to the common cold questionnaire. Two patients with severe bronchiectasis (one with comorbid asthma) were subsequently admitted to hospital for an exacerbation within 2 months of sample collection.

During the summer season, respiratory viruses were less commonly detected, with 3 of 15 patients demonstrating influenza A detection in the filters and 5 of 15 samples detecting Influenza A in the sputum sample (Table 2). No other respiratory viruses on our panel were detected in these samples. Furthermore, none of the patients in the summer cohort had a "probable virus" based on the CCQ. In the patients who were viral positive in the summer cohort, the average FEV1 was lower

($p > 0.05$), compared with the viral negative group. However, no associations between medication usage and viral detection were observed.

One patient was admitted to hospital within 2 weeks of the clinic visit with an exacerbation of bronchiectasis, and influenza A was again detected in both exhaled breath and sputum samples. Another patient who experienced an exacerbation 6 weeks after their clinic visit, did not have any viruses detected in either sample.

Comparison of cohorts

There was a significant difference in viral detection between Summer and Winter cohorts ($p < 0.01$), with a greater rate of viral detection observed during the winter months (Fig. 1). These cohorts were not age or severity matched, and there was a significantly higher rate of underlying PCD in the winter cohort ($p < 0.05$).

Discussion

Our small retrospective audit of admissions for exacerbation of bronchiectasis revealed how infrequently viral PCR testing was requested in a large teaching hospital with ready access to on site rapid respiratory viral PCR. Reflecting the current state of the literature, bacterial and fungal species were more frequently assumed to be the etiological agents and therefore, tested for in the majority of patients presenting with an exacerbation. Viral PCR testing was only requested in 28% of the bronchiectasis exacerbations included in the audit, compared with 88% of admissions where bacterial and fungal culture were requested.

However, despite the greater frequency of request for bacterial and fungal culture, viruses were still detected in 39% of the samples when viral PCR was requested, compared with bacterial or fungal pathogen detection in 30% of samples sent for testing. It is important that sampling rates increase, and prospective, longitudinal studies of both bacterial and viral pathogens in stable and exacerbating bronchiectasis are undertaken, if we are to understand with more precision, the role of viruses in exacerbations and their seasonality.

Due to the low rate of viral testing in acute exacerbations of bronchiectasis at our centre, we designed a study to determine the incidence of respiratory virus detection during stable periods and whether this was associated with an increased risk of exacerbation or developing a symptomatic viral infection. Studying stable patients with bronchiectasis provides information regarding the background level of viruses to inform future analysis of results obtained during acute exacerbations. Our pilot study demonstrated that respiratory viruses are commonly detected in respiratory secretions and the exhaled breath of patients with stable bronchiectasis. They are frequently detected during asymptomatic periods, and multiple viruses are often present concurrently.

In this study, there was a 92% detection rate in the winter cohort, and a 33% detection rate in the summer cohort. Other studies have detection rates of around 20% in stable bronchiectasis rising to around 40–50% in exacerbations in non-CF bronchiectasis in adults [27, 28]. One potential reason for obtaining such high virus detection by PCR is contamination within the PCR reaction. We are confident that high viral detection rates in the winter cohort this is not caused by poor PCR technique or experimental contamination as out negative controls were always negative. Furthermore, whilst the samples were collected during different periods of the year, the PCRs were carried out simultaneously. However, we used a highly sensitive PCR which can detect as few as 5 virions. In our study, even low CT values were classified as viral positive whereas in other studies these might be classified as negative. Whist not a part of this study, we have compared our research lab virus PCR results to virus positivity by PCR obtained from a diagnostic lab. We found almost 100% agreement for all viruses, apart from rhinovirus, where we found our PCR was more sensitive (twice the detection frequency). We think that the most plausible explanation for the high sample detection in our winter cohort happened to be sampled during a year that was recognised to have a heavy burden of influenza infections. Other possible reasons might be the increased severity of bronchiectasis based on FEV_1 values in our cohort, and

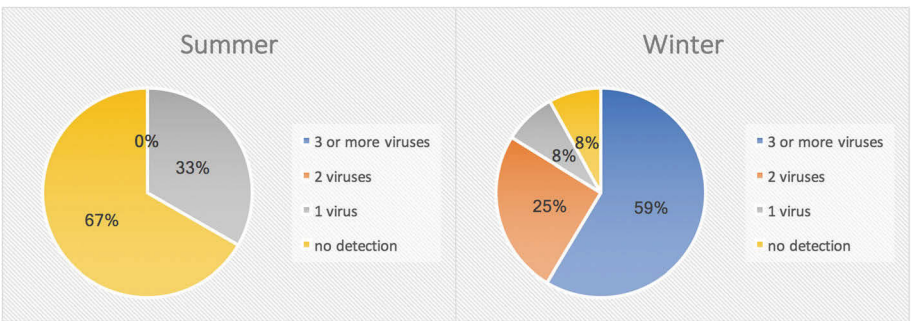


Fig. 1 Rates of single or multiple respiratory virus detection during stable state in the summer and winter cohorts

also, the high incidence of PCD as our clinic is a statewide referral centre for PCD. However, it is unusual to have found such high rates of multiple virus detection, and further studies are needed in PCD to confirm these findings. There is currently little literature regarding the detection and persistence of respiratory viruses in the respiratory tract of individuals with PCD.

Respiratory viruses were more common during winter season, compared with the summer season. This study confirms previously reported seasonality of respiratory viruses [29] for RSV, however rhinovirus has been shown to occur all year round in respiratory specimens which was not observed in this cross-sectional study of bronchiectasis patients. Influenza has also demonstrated peak detection during the winter months in temperate zones, and year-round distribution in tropical areas [30]. In this study, we observed a heavy burden of influenza during the winter season, however influenza A virus was still detected in multiple asymptomatic individuals during the summer months.

Detection of respiratory viruses in the exhaled breath and sputum samples of this bronchiectasis cohort, was not significantly associated with disease severity or risk of exacerbation within the 2 month follow up period. In the one patient who was admitted to hospital with a bronchiectasis exacerbation within 2 weeks of their clinic visit, influenza A was present within both the exhaled breath and sputum sample. However, this was the only case where an exacerbation was associated with virus detection in our study. The short duration of this follow up time may not be adequate, however, to make a clear determination of exacerbation risk in this cohort. A longitudinal study design with regular viral sampling during periods of both stable disease and exacerbation, and more in depth analysis of patient outcomes may be needed to elucidate this risk.

No association was observed between viral detection and treatment with ICS/LABA or SABA alone. In the summer cohort, viruses were more commonly detected in patients with more severe disease as indicated by spirometry, with 80% patients who had influenza A detection demonstrating an FEV_1 below 30% predicted based on the GLI-2012 reference set [31]. In the winter cohort, viral detection had no significant association with spirometry values.

Real time PCR allowed quantification of viral load, with higher viral load detection in the sputum sample predicting detection in the exhaled breath sample collected using the spirometry filters. Huang et al. [32] showed that presence of influenza virus within the respiratory tract is necessary but not sufficient to cause a symptomatic influenza infection. Host immune responses play an important role, and activation of multiple simultaneous pattern recognition receptors to cause antiviral and inflammatory responses are associated with symptomatic infection. Individuals who retain tight control over these responses usually remain asymptomatic, and may explain why asymptomatic infection was so prevalent in our cohort. These patients with bronchiectasis all have chronic bacterial lung colonisation, which may play a role in downregulating immune responses [33].

A surprising finding was that of influenza A detection only during the summer months. Traditionally, influenza A activity peaks during the winter months and viruses such as rhinovirus are more commonly seen in summer and early autumn. The Australian influenza surveillance network showed that there was a higher than normal level of influenza A detected during January to March of 2017, the sampling period of our summer cohort. Likely due to the fact that this group of bronchiectasis patients have an underlying respiratory disease, and impaired muco-ciliary clearance, it is possible that these individuals were more susceptible to acquiring these circulating viruses.

A large proportion of those recruited during the winter months had PCD as the underlying cause of bronchiectasis, as this study was undertaken at a tertiary referral centre for PCD. Ciliary dysmotility impairs mucociliary clearance and it seems plausible that this might result in persistence of viral nucleic acids within sputum, even if the virus is not actively replicating. Whilst the number of subjects in this pilot is small, it raises the possibility that differences in underlying pathophysiology of bronchiectasis extend to heterogeneity in the pathogenesis of viruses.

Since the introduction of culture-independent techniques, a substantial increase in bacterial detection has been observed [34]. Molecular methods that identify bacterial species based on nucleic acid presence has greatly improved diagnostic accuracy [35, 36], and has allowed discovery of a whole range of bacterial species that are present within the lower respiratory tract. The introduction of these molecular based methods such as PCR, have also allowed the detection of respiratory viral species to become faster and easier [37]. This greatly increased the rate of respiratory infections that were found to be attributable to viruses, as this is a much more sensitive and specific tool. This was an important step in realising the high frequency of respiratory viral infections, and thus their importance in clinical disease. It also allowed a more guided approach to treatment, with a decrease in the use of antibacterial agents in some cases. Characterising the role of viruses in both stable bronchiectasis and during exacerbations may allow a greater understanding of disease pathogenesis.

Conclusions

Our pilot study provides preliminary data supporting the notion that respiratory viruses are an important part of the lung microbiome in patients with bronchiectasis. The high rates of respiratory virus detection in patients with stable bronchiectasis encourages further studies in this area to determine how viruses may impact both chronic and transient bacterial species within the lung, and the role that viruses may have in exacerbations. This is the first study to investigate the potential impact of viruses in bronchiectasis. Many fundamental questions have been raised regarding the role of respiratory viruses in this disease process, and as outlined, recent advances in metagenomic techniques have provided the tools to investigate this area. We are just beginning to understand the role of viruses in many chronic respiratory diseases and it is now timely to apply this work in patients with bronchiectasis.

Abbreviations

ABPA: Allergic bronchopulmonary aspergillosis; ATS: American thoracic society; CCQ: Common cold questionnaire; CF: Cystic fibrosis; COPD: Chronic obstructive pulmonary disease; CT: Cycle threshold; ERS: European respiratory society; HMPV: Human metapneumovirus; HRV: Human rhinovirus; PCD: Primary ciliary dyskinesia; PCR: Polymerase chain reaction; PIV: Parainfluenza virus; qPCR: Quantitative polymerase chain reaction; RSV: Respiratory syncytial virus

Acknowledgements

The authors would like to thank the lab and physiotherapy staff at Concord Repatriation General Hospital for their aid in patient recruitment and sample collection.

Funding

This research is supported by an Australian Government Research Training Program Scholarship.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ABM, BGGO, LCM were involved in the design of this study; ABM, LB were involved in participant recruitment and sample collection; ABM, BM were involved in sample processing and experimentation; ABM, BM, LB, MJP, BGGO, LCM were all involved in manuscript preparation and editing. All authors approved the final manuscript.

Ethics approval and consent to participate

Ethics approval had been granted by the Sydney Local Health District Human Research Ethics Committee – CRGH (HREC/14/CRGH/6) and all subject gave written informed consent to participate.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Respiratory Cellular and Molecular Biology, Woolcock Institute of Medical Research, The University of Sydney, Sydney, NSW 2006, Australia. [2]Department of Respiratory Medicine, Concord Repatriation General Hospital, Concord, NSW 2139, Australia. [3]Concord Clinical School, University of Sydney, Sydney, NSW 2006, Australia. [4]Molecular Biosciences, School of Life Sciences, University of Technology Sydney, Building 4, 15 Broadway, Ultimo, NSW 2007, Australia. [5]Centre for Health Technologies, University of Technology Sydney, Sydney, NSW 2007, Australia. [6]Emphysema Centre, Woolcock Institute of Medical Research, The University of Sydney, Sydney, NSW 2006, Australia.

Received: 13 August 2017 Accepted: 25 April 2018
Published online: 22 May 2018

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CHAPTER 4

A NOVEL SAMPLING METHOD TO DETECT AIRBORNE INFLUENZA AND OTHER RESPIRATORY VIRUSES IN MECHANICALLY VENTILATED PATIENTS IN INTENSIVE CARE UNIT: A FEASIBILITY STUDY

4.1 Statement of Contribution

Experiments were conceived and carried out; results were analysed and manuscript was prepared for publication by AB Mitchell. AB Mitchell, B Tang and BGG Oliver were involved in the design of this study; B Tang, LS Barnes were involved in participant recruitment and sample collection; AB Mitchell, LS Barnes, M Shojaei, M Nalos were involved in sample processing and experimentation; AB Mitchell, B Tang, M Shojaei, LS Barnes, M Nalos, BGG Oliver, A McLean were all involved in manuscript preparation and editing. All authors approved the final manuscript; and agree with these statements.

Name	Signature	Date
AB Mitchell		16/5/18
B Tang		3/5/18
LS Barnes		3/5/18
M Shojaei		08/05/2018
M Nalos		3.5.18
BGG Oliver		8/5/18
A McLean		3-5-18

Mitchell *et al. Ann. Intensive Care (2018) 8:45*
https://doi.org/10.1186/s13613-018-0396-4

Annals of Intensive Care

RESEARCH Open Access

A novel sampling method to detect airborne influenza and other respiratory viruses in mechanically ventilated patients: a feasibility study

Alicia B. Mitchell[1*], Benjamin Tang[2,3,4], Maryam Shojaei[2,3], Lachlan S. Barnes[5], Marek Nalos[2], Brian G. Oliver[1] and Anthony S. McLean[2]

Abstract

Background: Respiratory viruses circulate constantly in the ambient air. The risk of opportunistic infection from these viruses can be increased in mechanically ventilated patients. The present study evaluates the feasibility of detecting airborne respiratory viruses in mechanically ventilated patients using a novel sample collection method involving ventilator filters.

Methods: We collected inspiratory and expiratory filters from the ventilator circuits of mechanically ventilated patients in an intensive care unit over a 14-month period. To evaluate whether we could detect respiratory viruses collected in these filters, we performed a reverse transcription polymerase chain reaction on the extracted filter membrane with primers specific for rhinovirus, respiratory syncytial virus, influenza virus A and B, parainfluenza virus (type 1, 2 and 3) and human metapneumovirus. For each patient, we also performed a full virology screen (virus particles, antibody titres and virus-induced biomarkers) on respiratory samples (nasopharyngeal swab, tracheal aspirate or bronchoalveolar fluid) and blood samples.

Results: Respiratory viruses were detected in the ventilator filters of nearly half the patients in the study cohort ($n = 33/70$). The most common virus detected was influenza A virus ($n = 29$). There were more viruses detected in the inspiratory filters ($n = 18$) than in the expiratory filters ($n = 15$). A third of the patients with a positive virus detection in the ventilator filters had a hospital laboratory confirmed viral infection. In the remaining cases, the detected viruses were different from viruses already identified in the same patient, suggesting that these additional viruses come from the ambient air or from cross-contamination (staff or visitors). In patients in whom new viruses were detected in the ventilator filters, there was no evidence of clinical signs of an active viral infection. Additionally, the levels of virus-induced biomarker in these patients were not statistically different from those of non-infected patients ($p = 0.33$).

Conclusions: Respiratory viruses were present within the ventilator circuits of patients receiving mechanical ventilation. Although no adverse clinical effect was evident in these patients, further studies are warranted, given the small sample size of the study and the recognition that ventilated patients are potentially susceptible to opportunistic infection from airborne respiratory viruses.

Keywords: Virus, Intensive care unit, Airborne, Influenza, Ventilator

*Correspondence: amit9422@uni.sydney.edu.au
[1] School of Life Sciences, University of Technology Sydney, Sydney, NSW 2007, Australia
Full list of author information is available at the end of the article

Background

Influenza and other respiratory viruses spread via three main transmission routes, namely direct contact, respiratory droplets and airborne transmission. The first two routes (direct contact and respiratory droplets) can be reduced by infection control measures (e.g. hand washing and wearing face masks). The third route, airborne transmission, is difficult to prevent since respiratory viruses are ubiquitous in the environment and virus particles constantly circulate in the air [1]. The concentration of airborne viruses is usually low and insufficient to cause disease in humans; however, in those with a compromised immune system (e.g. critically ill patients), the risk of infection increases dramatically [2].

The risk of infection from circulating respiratory viruses is higher in mechanically ventilated patients compared to non-ventilated patients. These patients have an exposed lower airway (the endotracheal tube bypasses the upper airway defence which normally acts as a physical barrier to airborne viruses). In addition, they have multiple risk factors that may further compromise their host defence system, including local trauma (due to intubation and airway manipulation), a weakened local defence (from a loss of mucociliary clearance and cough reflex) and a diminished immune response (e.g. reduced alveolar macrophages in the lungs). Despite this increased infection risk, the air in the ICU is not routinely sampled for the presence of respiratory viruses because no method is currently available for measuring airborne viruses.

The aim of the present study was to evaluate the effectiveness of a novel sampling method that collected inspired/expired air within the ventilator circuit to allow for the measuring of airborne viruses. Detecting airborne viruses in these patients is technically challenging as the concentration of viruses in the inspired/expired air is usually very low; a substantially large volume of air per sample is required for detection. To address this challenge, we applied a novel approach in which we measured viruses trapped in the ventilator filters of mechanically ventilated patients. The ventilator filters have a large volume of inspired/expired air circulating through them each day, thus making them an ideal medium for sampling airborne viruses. Here, we report the findings of a feasibility study using ventilator filters to detect airborne viruses in mechanically ventilated patients admitted to an intensive care unit.

Methods

Patient recruitment

We recruited mechanically ventilated patients in an intensive care unit over a 14-month period. Eligible patients included those (1) over 18 years old; (2) suspected of having pneumonia with a viral aetiology ("flu-like" illness in the preceding 7 days); and (3) mechanically ventilated for at least 24 h. Pneumonia was defined as a new lung infiltrate on chest radiography at hospital admission with symptoms and signs of lower respiratory tract infection. "Flu-like" illness was defined as having at least one symptom from two or more symptoms categories. The symptom categories were as follows: (1) fever, (2) constitutional symptoms (e.g. chill, headache, muscle ache) and (3) respiratory symptoms (e.g. cough, sore throat, nasal congestion). Informed consent was obtained from relatives or the legal guardian of the patient. The study was approved by the human ethics committee of our institution.

Filter collection

To evaluate whether we could detect respiratory viruses in the inspired/expired air, we sampled both the inspiratory and expiratory filters from the ventilator circuits (Fig. 1). After the first 24 h of mechanical ventilation, ventilator filters were collected, placed in pre-prepared sample bags and stored in −80 °C for later processing (see below).

Processing

Prior to the processing of the filters, care was taken to ensure that the filters were not exposed to ambient air during transportation. During processing, filters were first dismantled to allow the filter membrane to be extracted. 1 ml of Bioline Lysis Buffer RLY (Bioline, Alexandria, Australia) was then added to the filter membrane in a tube, followed by centrifugation for 2 min at 2000 rpm. The full 1 mL of eluate was collected after the final spin and stored at −20 °C until RNA extraction. Viral RNA in the eluate was extracted using the Isolate II RNA Mini Kit (Bioline, Alexandria, Australia) as per manufacturer's instructions before conversion to cDNA.

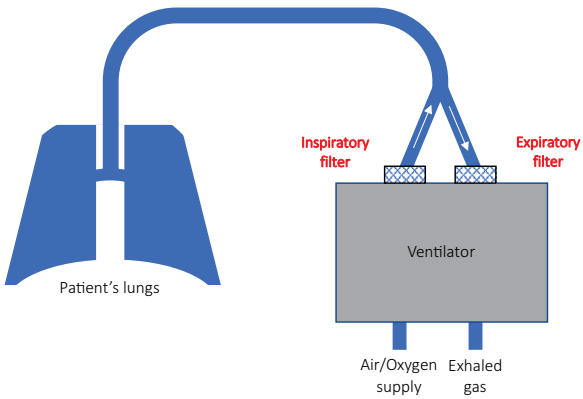

Fig. 1 Ventilator circuit with the position of filters shown. A simplified schematic drawing showing the position of the inspiratory and expiratory filters (highlighted in red). Arrows inside each arm of the ventilator circuit indicate the direction of air flow

Virus detection in filters

The reverse transcription polymerase chain reaction (RT-PCR) (Bioline, Alexandria, Australia) was performed on the extracted filter membrane to detect rhinovirus, respiratory syncytial virus, influenza virus A and B, parainfluenza virus (type 1, 2 and 3) and human metapneumovirus. As an internal control, positive viral cDNA was included in each PCR assay. All primer sequences are provided in the (Additional file 1: Table S1).

Extracted RNA was converted to cDNA using the Bioline SensiFAST cDNA Synthesis Kit (Bioline, Alexandria, Australia) as per manufacturer's instructions, with 8 µl of extracted RNA, 7 µl of DPEC water, 4 µl reaction buffer, 1 µl of reverse transcriptase added to each reaction to make a total volume of 20 µl. The PCR assay was performed as follows: all samples were run in triplicate, with 2 µl of cDNA template added to Bioline SensiFAST Probe Hi-ROX Master Mix. Specific primers and probes (Table 1) for each virus were added to the PCR assay along with DEPC water. The dual-labelled probes utilised the FAM fluorophore and BHQ-1 quencher. These samples were run on the StepOnePlus Real-Time PCR System (Applied Biosystems, California, USA) for 40 cycles. The threshold was automatically detected based on amplification. Positive viral samples and negative controls were run individually for each assay.

Additional laboratory tests

In addition to ventilator filters, clinical respiratory samples were collected from each patient, including a nasopharyngeal swab, tracheal aspirate and/or bronchoalveolar fluid. Multiplex viral PCR (BioFire FilmArray, Salt Lake City, USA) was performed to detect the presence of rhinovirus, respiratory syncytial virus, influenza virus A and B, parainfluenza virus (type 1, 2 and 3) and human metapneumovirus in these samples. Assay characteristics and methodology of this multiplex viral PCR have been previously published [3–5]. This clinical testing was performed by the hospital laboratory scientists, separate from the researchers who performed the PCR assay on the ventilator filters. The researchers who performed the PCR assay on the ventilator filters were blind to the results of the multiplex viral PCR and vice versa. Tests for bacterial pathogens were also carried for each patient, including both typical and atypical respiratory pathogens.

Table 1 Virus inoculation and subsequent recovery by PCR

Virus	Treatment	Conditions	1 week	2 weeks	4 weeks
Influenza virus	Negative controls[a]		No	No	No
	Virus added	20 °C	Detected	No	No
	Virus added	− 20 °C	Detected	Detected	Detected
Rhinovirus	Negative controls[a]		No	No	No
	Virus added	20 °C	Detected	No	No
	Virus added	− 20 °C	Detected	Detected	Detected

10 uL of viral stock was inoculated onto each ventilator filter. These filters were then stored at either room temperature (20 °C) or low temperature (− 20 °C) for 1, 2 or 4 weeks. Triplicates were stored for each condition

[a] Negative controls did not have any virus particles added to the filter

Serology and host response biomarker

To assess the host response to respiratory viruses, a blood sample was taken from each patient to measure (1) serological changes and (2) biomarker IFI27 changes. For the serological test, a positive seroconversion to influenza virus is defined as a low baseline antibody titre (< 1:10) followed by an increase (> 4 fold) in antibody titre between the two blood samples. For the *IFI27* biomarker, an increased *IFI27* gene expression in peripheral blood indicates an immune response to a specific respiratory virus with the following threshold cut-off values: influenza (> 74 fold change), parainfluenza virus (> 74 fold change), respiratory syncytial virus (> 40 fold change) and human metapneumovirus (> 40 fold change) [6].

Statistical analysis

For continuous variables, comparisons between two groups were made using an unpaired two-tailed Student's *t* test or the nonparametric Mann–Whitney *U* test, where appropriate. For categorical variables, comparisons between two groups were calculated using Fisher's exact test. Statistical significance was defined as $p < 0.05$.

Results

Technical feasibility study

We first assessed the feasibility of detecting respiratory viruses in clean, unused ventilator filters. To this end, we inoculated two different respiratory viruses (influenza A and rhinovirus) using viral stock onto clean ventilator filters. These filters were then stored for 1, 2 and 4 weeks under different temperatures (room temperature or − 20 °C). After the storage period, we extracted the filter membrane from each filter casing and amplified viral nucleic acids using RT-PCR (as described in the Methods section). We detected viral nucleic acids after 1 week at room temperature and up to 4 weeks at − 20 °C (Table 1). Both influenza virus and rhinovirus were recovered in the inoculated filters (Table 1). This finding demonstrates the feasibility of using ventilator filters as a collection device, providing the basis for our sampling approach subsequently used in this study.

Airborne viruses in ventilated patients

Having demonstrated the technical feasibility of our sampling method, we next investigated whether we could detect airborne viruses in mechanically ventilated patients. A total of 35 mechanically ventilated patients were recruited for the study. Full, detailed demographic and clinical features of the patients are provided in Table 2. In brief, 35 patients were admitted to the intensive care unit for the management of respiratory failure. Thirty of these 35 patients had pneumonia. Five patients had no evidence of infection—these patients acted as controls in the study. Infectious agents identified in the patients with pneumonia included viruses ($n=20$), bacteria ($n=18$), fungi ($n=1$) and virus–bacteria co-infection ($n=8$). A full list of identified infectious agents is provided in the (Additional file 2: Table S2). No infectious agents were identified in the control patients after a full microbiological and virology screen on each patient's blood, urine and airway samples.

A total of 70 ventilator filters were collected from the recruited patients, with one expiratory filter and one inspiratory filter collected from each patient. Airborne respiratory viruses were detected in nearly half of the filters ($n=33$) using RT-PCR (Table 3). There were more viruses detected in the inspiratory filters ($n=18$) than in the expiratory filters ($n=15$). The most common virus detected was the influenza A virus ($n=29$).

Inspired air versus expired air

We hypothesised that the expired air reflects the virus ecology inside the patients' lungs. This means the distribution of viruses detected in the expiratory filters would resemble the viruses circulating in the local patient population, which would display seasonal fluctuations related to the onset/end of each flu season. To assess the impact of seasonal changes on virus detection in inspired/expired air, we divided the recruitment period into stages including (1) peak flu seasons and (2) off-peak flu season (Fig. 2). In this analysis, we found that the airborne viruses in the expiratory filters did show a seasonal pattern and matched the seasonal increase/decrease reported in our local institution (data not shown). In contrast, no seasonal fluctuation was observed in the influenza viruses detected in the inspiratory filters (Fig. 2), in keeping with the fact that inspired air came from the main hospital air supply (which is insulated from the viruses circulating in the local population) (Table 3).

Table 2 Patient demographic and clinical characteristics

	Infected patients	Control patients
Number of patients	30	5
Age (years)[a]	58.6 (23–86)	52.6 (21–71)
Gender (male/female)	10/20	3/2
Infection types		
Bacterial	8	0
Viral	11	0
Bacteria–bacteria	2	0
Virus–bacteria	8	0
Virus–fungus	1	0
Severity and outcomes		
APACHE III scores[a]	67 (36–128)	57 (35–83)
Length of ventilation (days)[a]	8.7 (2–28)	3.8 (1–8)
Length of ICU stay (days)[a]	11.5 (2–37)	6 (2–11)
Length of hospital stay (days)[a]	16 (2–45)	8.6 (2–16)
Alive/dead	22/8	5/0

[a] Data are presented as mean and range (minimum–maximum)

Sources of airborne viruses

Having demonstrated the presence of airborne viruses in mechanically ventilated patients, we next sought to identify the possible sources of these airborne viruses. A third of the cases ($n=9$) were found in patients with an established diagnosis of respiratory virus infection, indicating that these patients were actively shedding viruses during the study period and some of these virus particles were detected by our method. In the remaining cases, the detected viruses were different from viruses identified in the respiratory secretions of the same patient, suggesting that these new viruses might come from either the ambient air (from routine change of the patient's ventilator circuit) or from cross-contamination (staff or visitors).

Host response to airborne viruses

We next assessed the acute host response to the presence of airborne viruses in each patient. To this end, we analysed patients' peripheral blood samples to measure the gene expression levels of the biomarker *IFI27*, an established marker of virus-induced immune response [3]. We compared the *IFI27* levels between patients with an established diagnosis of viral infection, patients in whom a new virus was detected (in their ventilator filters) and patients in whom no respiratory virus was found anywhere (in blood, respiratory secretions or ventilator filters). We found that *IFI27* levels were significantly elevated in those with confirmed respiratory viral infection (mean fold change = 483), confirming the presence of an immune response to the viral infection. In patients in whom a new virus was detected in their ventilator filters, the *IFI27* levels were low (mean fold change = 13) and not statistically different to patients who had no evidence of viral infection (Fig. 3); this result suggested an absence of virus-induced immune response in these patients (Table 4).

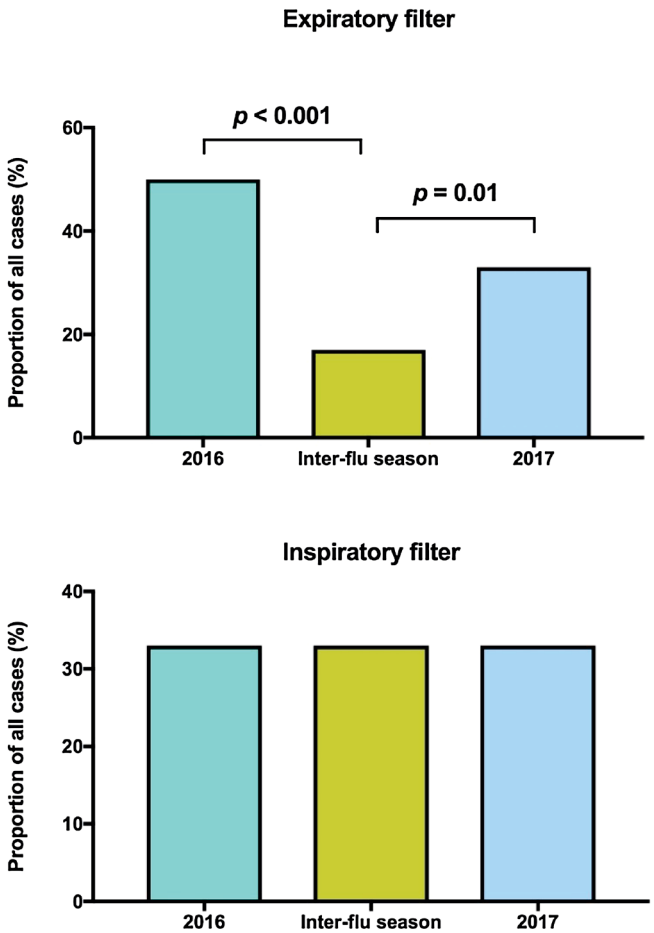

Fig. 2 Seasonal changes in the frequency of detected viruses. The recruitment period covered two flu seasons in the Southern hemisphere one inter-seasonal period. "2016" refers to the first flu season (early July–late October 2016). "2017" refers to the second flu season (late July–mid-October 2017). "Inter-flu season" refers to the period in between the two seasons (November 2016 to early July 2017). *p* values were calculated using Fisher exact test. No difference was detected in the distribution of detected viruses in the inspiratory filters

Discussion

This is the first report to assess the feasibility of using a novel sampling method to detect airborne respiratory viruses in a critically ill patient population. The results show that airborne viruses were present in 44% of the ventilator filters collected from mechanically ventilated patients. The vast majority of the detected airborne viruses (88%) were influenza viruses. In some cases, the airborne viruses detected reflected the carrier status of the patients, with the same virus found in both the ventilator filter and patients' respiratory secretions. In other cases, where a new virus was detected, the clinical significance of these viruses remains unclear, since the affected patients showed no evidence of a virus-induced immune response.

A large number of studies have demonstrated that respiratory viruses (e.g. influenza viruses) are always present in the ambient air [2]. The importance of detecting airborne viruses present in the hospital environment is increasingly being recognised. Several recent studies have provided a direct demonstration that influenza viruses were present in aerosolised droplets from the tidal breathing of infected persons and in the air of the emergency department [7, 8]. During peak flu season, the concentration of airborne viruses in the environment rises to 5800–37,000 virus particles per m^3. At this concentration, breathing air for 1 h is sufficient to cause clinical infection in a previously unexposed person [9]. Thus, monitoring airborne virus concentrations may be important in a high-risk clinical environment such as the intensive care unit, where many patients have immune-compromised status and an increased susceptibility to opportunistic infection. Furthermore, there is significant risk of droplet transmission if visitors or healthcare staff

Table 3 Airborne viruses in patients' ventilator filters

	Virus	Inspiratory filters	Expiratory filters
Infected patients[a]	Influenza	14	9
	Rhinovirus	0	1
	Metapneumovirus	0	1
	Parainfluenza virus	1	1
Non-infected controls	Influenza	3	3
	Rhinovirus	0	0
	Metapneumovirus	0	0
	Parainfluenza virus	0	0
	Total	18	15

[a] Infected patients refer to pneumonia patients in whom a bacterium was identified by culture or a respiratory virus was identified either by PCR assay on respiratory secretions (e.g. nasopharyngeal swap, bronchoalveolar lavage fluid) or by serology on serum samples

are infected with respiratory viruses and are in close contact with these critically ill patients. The method outlined in this study may provide a tool to monitor both airborne and droplet sized viral particles that mechanically ventilated patients are exposed to.

Monitoring airborne viruses requires a different approach than the conventional testing method of respiratory viruses. This difference is due to the fact that the concentration of viruses in the ambient air is much lower than that of the respiratory secretions (e.g. nasopharyngeal swab or bronchoalveolar lavage). Unfortunately, there is a lack of data on detection method specifically developed for airborne viruses. This study represents the first step towards developing a reliable, easy-to-perform method for airborne virus detection. Future studies should investigate whether such methods could increase the diagnostic yield of detecting viral aetiology in patients with community-acquired pneumonia or whether such methods could prevent hospital-acquired viral infection in mechanically ventilated patients.

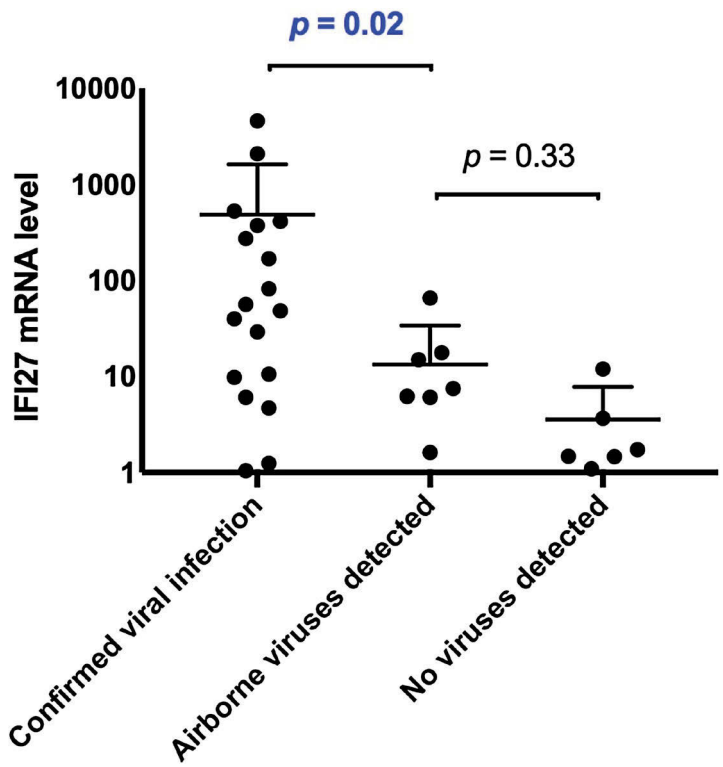

Fig. 3 Host response biomarker and clinical outcomes. "*Confirmed viral infection*" group refers to all patients in whom a respiratory virus was identified in their respiratory secretions (e.g. nasopharyngeal swap, bronchoalveolar lavage fluid) or increased anti-viral titres in their serum as measured by serology. "*Airborne viruses detected*" group refers to patients in whom a new respiratory virus was detected in the inspiratory filter or the expiratory filter. "*No viruses detected*" group refers to patients in whom no respiratory virus was detected in the respiratory secretions, serum or the ventilator filters. The *IFI27 mRNA-expression* was measured by quantitative real-time PCR, and its level is expressed as fold change (relative to GAPDH). The *p* values were calculated using Kruskal–Wallis test (for comparison of multiple groups). The error bars are mean plus standard deviation

Table 4 Virus detected in each patient

Subjects	Status[a]	Airway	Serology	Inspiratory	Expiratory
1	Infected			Influenza	Influenza
2	Infected	RSV		Influenza	Influenza
3	Infected			Influenza	Influenza
4	Infected	Rhinovirus		Influenza	Influenza
5	Control				
6	Infected	Influenza		PIV	Influenza
7	Infected			Influenza	PIV
8	Infected	Rhinovirus			
9	Control			Influenza	Influenza
10	Infected				
11	Infected	Rhinovirus		Influenza	
12	Control			Influenza	
13	Infected	PIV			
14	Control			Influenza	Influenza
15	Infected	Rhinovirus		Influenza	Influenza
16	Infected	RSV		Influenza	
17	Infected				
18	Infected				
19	Infected				
20	Infected				
21	Infected	Influenza		Influenza	
22	Infected	Influenza			
23	Infected		Influenza		
24	Infected	Influenza			
25	Infected	HMPV			HMPV
26	Infected	Influenza			
27	Infected	Influenza		Influenza	
28	Infected	Influenza		Influenza	Influenza
29	Infected	Influenza			Influenza
30	Infected	Influenza	Influenza		Influenza
31	Control				Influenza
32	Infected	Influenza		Influenza	
33	Infected			Influenza	
34	Infected	Influenza		Influenza	Rhinovirus
35	Infected	Influenza	Influenza		

RSV respiratory syncytial virus, *HMPV* human metapneumovirus, *PIV* parainfluenza virus

[a] In this column, "Infected" refers to any of the following status; (1) bacterial infection, (2) viral infection or (3) viral–bacterial co-infection

There are additional challenges with investigating airborne respiratory viruses in critically ill patients due to experimental difficulties in sampling aerosolised virus particles, including the potential inactivation of viruses by current sampling methodology [10]. To overcome this sampling difficulty, we used ventilator filters as a collection device, since these filters collect a large volume of air which potentially increases the yield of detected virus particles. This approach was first tested in the pilot phase of this study, in which we inoculated live viruses into clean, unused filters. Days later, we were able to recover the same viruses in the filters. This finding provides strong support for using this approach in our study. The result is also in keeping with findings from a previous study by Heuer et al., who demonstrated that aerosolised influenza virus particles could be trapped inside ventilator filters [11]. In Heuer's study, three different brands of commercially available filters were tested; all showed that the filters could successfully collect airborne viruses. Whilst the Heuer study was designed as an in vitro study, our study provides real-world data in a clinical setting. Collectively, both the in vitro and the clinical data confirm the technical feasibility of using ventilator filters as a collection device.

We used a RT-PCR assay to detect viruses in the present study, which is the method of choice for airborne viruses suggested by the established literature [1]. We purposefully adopted a more sensitive detection threshold (cycle threshold (Ct) value of 37–38) in order to quantify the lowest background virus level inside the ventilator circuits. This information allowed us assess the baseline risk level of airborne viruses in our clinical environment. It is important to note that the low detection threshold used in this study detects viruses at concentrations less than 500 virus particles per m^3 [12]. Such a low virus concentration is generally insufficient to breach the normal defence barrier of the host's airways and, hence, is unlikely to cause clinical infection in the affected individual. This helps explain the observation that there was no evidence of virus-induced immune response in patients in whom a virus was detected in the filters and the clinical course/outcomes of these patients did not differ from the control patients.

The current study has some limitations, first of which was a small sample size and selected cohort of patients, meaning the generalisability of the findings to other patient populations is limited. As part of this, it was impossible to determine the original size of the viral particles that were collected on ventilator filters making it difficult to delineate whether this was detection of airborne transmission, droplet transmission or a combination of the two. Secondly, we did not quantify the number of virus particles in the collected samples (ventilator filter or respiratory samples). As a result, no information was available regarding the precise viral load in each sample. Thirdly, we did not perform sequencing of the identified viruses, making it difficult to know with certainty the exact source of each virus. Fourthly, we did not assess whether the detected virus particles could replicate in human cells and therefore had no information regarding the viruses' viability or infectivity, both of which are clinically important. A further limitation of this study is

that 24 h was used as a sampling time; it is possible that a longer sampling time (e.g. 48 h, 72 h) may increase the yield of virus detection.

Conclusion

This preliminary study shows that it is technically feasible to detect airborne viruses in the ventilator filters collected from patients receiving mechanical ventilation. Our findings provide important baseline data regarding the presence of airborne viruses in critically ill patients and may help inform the design of future studies in a similar setting.

Additional files

Additional file 1: Table S1. Virus-specific primer and probe sequences for real-time PCR.

Additional file 2: Table S2. Pathogens identified in patient samples$^{\Psi}$ (excluding ventilator filers samples).

Abbreviations

Ct: cycle threshold; RT-PCR: reverse transcriptase polymerase chain reaction.

Authors' contributions

ABM, BT and BGGO were involved in the design of this study; BT, LSB were involved in participant recruitment and sample collection; ABM, LSB, MS, MN were involved in sample processing and experimentation; ABM, BT, MS, LSB, MN, BGGO, AM were all involved in manuscript preparation and editing. All authors read and approved the final manuscript.

Author details

[1] School of Life Sciences, University of Technology Sydney, Sydney, NSW 2007, Australia. [2] Department of Intensive Care Medicine, Nepean Hospital, Sydney, Australia. [3] Centre for Immunology and Allergy Research, Westmead Institute for Medical Research, Sydney, Australia. [4] Respiratory Virus Infection Research, Marie Bashir Institute for Infectious Diseases and Biosecurity, Sydney, Australia. [5] Nepean Clinical School, Faculty of Medicine, University of Sydney, Sydney, Australia.

Acknowledgements

Nepean hospital intensive care unit staff who provided the collection of ventilator filers and patient samples.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Ethics approval had been granted by the Nepean Blue Mountains Local Health District Human Research Ethics Committee—Nepean (HREC/15/NEPEAN/130), and all subjects gave written informed consent to participate.

Funding

This research is supported by an Australian Government Research Training Program Scholarship and the Sydney Medical School Foundation.

Publisher's Note

Received: 15 January 2018 Accepted: 12 April 2018
Published online: 17 April 2018

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CHAPTER 5

TRANSPLANTING THE PULMONARY VIROME: DYNAMICS OF TRANSIENT POPULATIONS

5.1 Statement of Contribution

AB Mitchell was involved in conception and design of the study, sample collection and processing, carrying out experimental work and results analysis, and preparation of the final manuscript. B Mourad carried out sample processing, experimental work, data analysis and review of the final manuscript. LC Morgan and BGG Oliver reviewed the data, were involved with data analysis and reviewed the manuscript. AR Glanville was involved in study conception and design, patient recruitment, sample collection, data analysis and preparation of the final manuscript.

Name	Signature	Date
AB Mitchell		9/5/18
B Mourad		18/5/18
L Morgan		9/5/2018
BG Oliver		16/5/2018
AR Glanville	Allan R Glanville	04/06/2018

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Transplanting the pulmonary virome: Dynamics of transient populations

Alicia B. Mitchell, BMedSc (Hons),[a,b,c] Bassel Mourad, BMedSc,[b,c] Lucy C. Morgan, BMed, PhD,[d] Brian G.G. Oliver, MSc, PhD,[b,c] and Allan R. Glanville, MBBS, MD[a]

From the [a]The Lung Transplant Unit, St. Vincent's Hospital, Sydney, New South Wales, Australia; [b]The Woolcock Institute of Medical Research, Sydney, New South Wales, Australia; [c]School of Medical and Molecular Biosciences, University of Technology Sydney, Sydney, New South Wales, Australia; and the [d]Concord Clinical School, University of Sydney, Sydney, New South Wales, Australia.

KEYWORDS:
respiratory virome;
microbiome;
lung transplantation;
community-acquired respiratory viruses;
chronic lung allograft dysfunction

BACKGROUND: Lung transplantation provides a unique opportunity to investigate the dynamics of the human pulmonary virome that is transplanted within the donor lungs. The pulmonary virome comprises both "resident" and "transient" viruses. In this study we aimed to analyze the dynamics of the "transient" members.

METHODS: We conducted a single-center, prospective, longitudinal investigation of community-acquired respiratory viruses detected in nasopharyngeal swabs, swabs of explanted and donor lungs, and serial bronchoalveolar lavages post-transplant.

RESULTS: Fifty-two consecutive lung transplant recipients were recruited (bilateral:heart–lung: bilateral lung–liver = 48:2:2) (age [mean ± SD] 48 ± 15 years, range 20 to 63 years; 27 males and 25 females). Follow-up was 344 ± 120 (range 186 to 534) days. Seventeen of 45 explanted lungs were positive for influenza A and/or B (A = 14, B = 2, A+B = 1), despite recipient vaccination and negative nasal swabs, and 4 of 45 had human rhinovirus and 2 of 45 parainfluenza. Donor swabs showed influenza (A = 1, B = 1) and rhinovirus ($n = 3$). Day 1 lavage showed influenza A ($n = 28$), rhinovirus ($n = 9$), and parainfluenza ($n = 1$). Forty-seven of 52 recipients had a positive lavage for virus (38 of 47 on multiple lavages). Influenza persisted for 59 ± 38 (range 4 to 147) days in 27 of 52, and 14 had a single isolate. Rhinovirus persisted for 95 ± 84 (range 22 to 174) days in 13 of 52, and 13 had a single isolate. Analysis of 118 paired transbronchial biopsies and lavage demonstrated no association between viruses and acute cellular rejection (Fisher's exact test, 2 tailed, $p = 1.00$).

CONCLUSIONS: Using a sensitive uniplex polymerase chain reaction we found that the transplanted pulmonary virome often includes community-acquired respiratory viruses, including influenza, which are variably persistent but not associated with acute rejection.

J Heart Lung Transplant 2018;37:1111–1118

Reprint requests: Allan R. Glanville, MBBS, MD, Department of Thoracic Medicine, St. Vincent's Hospital, Sydney, NSW 2010, Australia. Telephone: +614-149-10321. Fax: +612-838-23084.

E-mail address: allan.glanville@svha.org.au

1053-2498/
https://doi.org/10.1016/j.healun.2018.06.002

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Overview

This thesis has focused on the impact of respiratory viral infections in at-risk patient groups. There are two separate components within this project. Firstly, the development and evaluation of a novel methodology to utilise exhaled breath to detect airborne respiratory viruses, and secondly, the characterisation of virus prevalence and the description of transient constituents of the human respiratory virome in patients with respiratory disease.

Aspects from both these areas have been explored throughout each chapter, however for clarity, they will be discussed separately in this section.

6.2 Exhaled breath sampling methodology for respiratory viruses

My research focuses upon the utility of exhaled breath analysis to detect airborne respiratory viruses and builds on our previous work which demonstrated the efficacy of electret filter material as a viral sampling medium.[419,420] My study integrated prior knowledge with clinical applicability, to evaluate the use of spirometry filters[421].

We found that spirometry filters can be used to detect airborne respiratory viruses that have been exhaled during a forced expiratory manoeuvre. We report high rates of virus detection in this study compared with previous exhaled breath studies, likely due

to using a cohort of patient who all had clinically significant underlying chronic respiratory conditions, performing forced expiratory manoeuvres rather than tidal breathing.[422] Furthermore, when compared with nasal washing, sputum and BAL sampling, which are all used currently in a clinical environment, this exhaled breath sampling methodology could detect viruses present in both the upper and lower respiratory tract.

The exhaled breath analysis methodology was evaluated within a cohort of patients with bronchiectasis and gave an ancillary method of sampling which is complementary to the sputum samples typically collected. Within this cohort of patients, viruses were detected extremely frequently within sputum samples. Only some of these were concurrently detected in the exhaled breath samples. During the winter months, multiple respiratory viruses were found within sputum samples, however, only one of these viruses was usually detected in the exhaled breath sample collected at the same time. During the summer months, Influenza A was detected in sputum samples from 5/15 patients, which was concordant in 3/5 exhaled breath samples. We hypothesise this represents higher viral load within the airways, as patients with exhaled breath and respiratory secretion sample viral concurrence usually had a higher viral load of the specific virus present within the respiratory secretion sample as determined by CT value on qPCR.

The ICU study evaluated this methodology further, utilising similar filter material, but this time contained within a ventilator circuit rather than a spirometer. The ventilator filters are included on both the inspiratory and expiratory loops of the circuit, and act

as barriers to stop the spread of pathogens, allergens and other airborne particulate matter, both in the air reaching the patient on the inspiratory arm and from spreading into the ventilator equipment on the expiratory arm. A previous study has indicated, in a lab-based model, that the filters on the ventilator circuit have an filtration efficiency of 99.999%,[418] implying that the majority of viruses should be collected from the air passing through the circuit. Our study found that it is technically feasible to detect airborne viruses in the ventilator filters collected from patients receiving mechanical ventilation but did not have the same filtration efficiency as seen in the lab-based model. We detected airborne viruses in 44% of the ventilator filters collected from mechanically ventilated patients with suspected, but unconfirmed viral infection. In some cases, the airborne viruses detected reflected the carrier status of the patients, with the same virus found in both the ventilator filter and the respiratory secretions. Therefore, this study was able to demonstrate the efficacy of utilising similar filters in detecting respiratory viruses both from the intubated patient and for sampling airborne respiratory viruses in the community that were detectable in the inspiratory arm of the circuit. As all these patients were intubated in the ICU and therefore critically ill, it is important to be able to accurately and non-invasively monitor viral shedding as a measure of infectivity to guide infection control strategies. Further studies are needed to clarify the clinical utility of this testing.

Taken together, these studies have explored the efficacy of utilising commercially available electret filter material contained within spirometry and ventilator filters. There are clear applications of this methodology within the patient populations described which should allow a reduction of invasive and uncomfortable sampling for

patients who might otherwise require either nasopharyngeal aspirates or swabs, or BAL. There is also the scope for utilising this methodology in children, who are able to breathe or blow through a mouthpiece and who are not very compliant with uncomfortable sampling techniques described above. Furthermore, within clinical trials, where patients regularly complete lung function testing it would facilitate viral sampling without extra testing, allowing further investigation of confounding factors such as viral infections. At this point in time, current sampling methodologies such as nasopharyngeal swabs and aspirates appear to have a higher sensitivity for upper respiratory tract infection, however as the exhaled breath sampling methodology is further refined, it may offer a clear alternative to nasopharyngeal swabs in the future.

6.3 Transient aspects of the human respiratory virome

The other main research aim of my PhD, was to investigate the impact of respiratory viruses in several groups of patients at particular risk of adverse sequelae of respiratory virus infection (patients with bronchiectasis and patients undergoing lung transplantation).

In all the cohorts investigated, we detected high rates (up to 59% in the bronchiectasis cohort) of asymptomatic community acquired respiratory viruses (CARV).

Furthermore, these CARV were seen to persist for up to 24 weeks, suggesting that these viruses are often present within the respiratory tract of “healthy” individuals, are more common in patients with chronic respiratory disease or who are immunocompromised, and given their persistence, are part of the transient populations which compose the human respiratory virome.

In the studies carried out on patients with bronchiectasis, patients in the ICU and the lung transplant cohort, there was a high frequency of respiratory virus detection. This appeared to be even greater during the winter months, likely reflecting the known seasonality of many respiratory viruses.[257] For the bronchiectasis cohort, it is possible that respiratory viruses were detected at a higher frequency than in other studies due to our sampling techniques. We utilised sputum samples to determine viral presence, however this is not used in many studies due to the inability of many patients to expectorate sputum on demand. Furthermore, bronchiectasis is a suppurative condition, whereby the sputum produced is more purulent which possibly encourages replication of microbes within a relatively immune-protected environment.[77]

It is difficult to draw conclusions regarding the symptomatology of respiratory viruses in the ICU cohort and in the early (POD 1) samples from the lung transplant cohort, as all patients were intubated when these samples were taken which restricts the ability to observe respiratory virus-related symptoms. However, in the lung transplant patients who had viruses detected at time points later than this and in the bronchiectasis cohort, the majority of patients did not demonstrate symptoms suggestive of respiratory viral infection. We hypothesise that in the lung transplant recipients, early induction immunosuppression and maintenance immunosuppression may have contributed to reducing the immune response to viral presence and therefore masked symptomatology. However, in the bronchiectasis patients, all patients were immuno-competent but still had reasonable levels of asymptomatic viral detection. Explanatory hypotheses include the possibility of decreased viral

symptomatology due to immune evasion and modulation by the virus within the lower airways particularly within altered lung microenvironments due to mucus plugging, with a possible contribution of previous immune system downregulation by the colonising bacteria such as *Pseudomonas aeruginosa*;[423] or due to a mischaracterisation of symptoms due to persistent respiratory signs such as cough and mucus production, as a result of both bronchiectasis and often primary ciliary dyskinesia (PCD) in our cohort. Further controlled studies may help to shed some light on the contributions of the disease, and treatments in mediating viral-related immune responses.

In both the bronchiectasis and lung transplant cohorts, we also investigated clinical outcomes after respiratory virus detection. In both studies, we found no significant correlation between detection of a respiratory virus and acute events. For patients with bronchiectasis, there was no statistically significant relationship between respiratory exacerbations within the two months following virus detection. Furthermore, within the lung transplant cohort, while there were some cases of acute cellular rejection (ACR), when analysed using Fishers exact test there was no relationship between concurrent ACR and detection of respiratory viruses within the lower respiratory tract on BAL. There is limited literature exploring the relationship of viral infections with ACR and the available studies show conflicting conclusions, therefore more studies are needed in this area to reach a consensus. Some previous studies in the area have suggested a possible link,[97,383] while our work agrees with that of Bridevaux et al and Soccal et al, who also did not demonstrate a relationship between viral detection and acute allograft rejection.[96,424]

In the bronchiectasis and lung transplantation studies presented, the follow up period has not yet allowed analysis of long-term outcomes but early Influenza A was not associated with an increased risk of subsequent ACR after lung transplantation. The immune response to the virus is important in determining symptomatology and lung injury may result from an aberrant inflammatory response.[247] It has not yet been determined whether detection of virus may lead to low-level inflammation or a sub-acute lung injury which has long-term implications for patients that were not observed within the current follow-up periods.

Figure 6.1
Applications of exhaled breath viral sampling methodologies

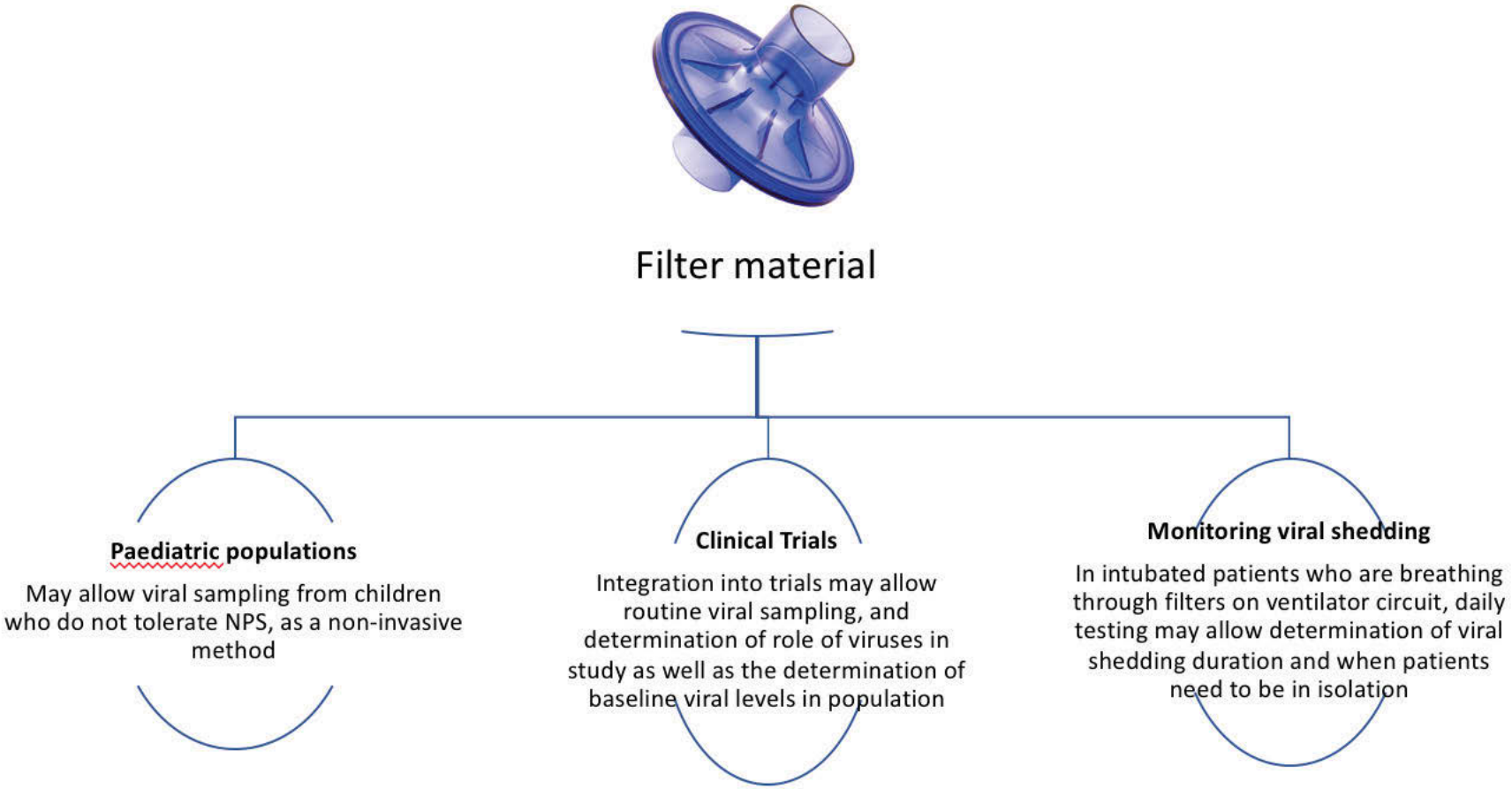

Fig 6.2 The electret filter contained within spirometry mouthpieces explored in this thesis may have many downstream applications following validation and refinement.

Figure 6.2
The Human Respiratory Virome Interactions after Lung Transplantation

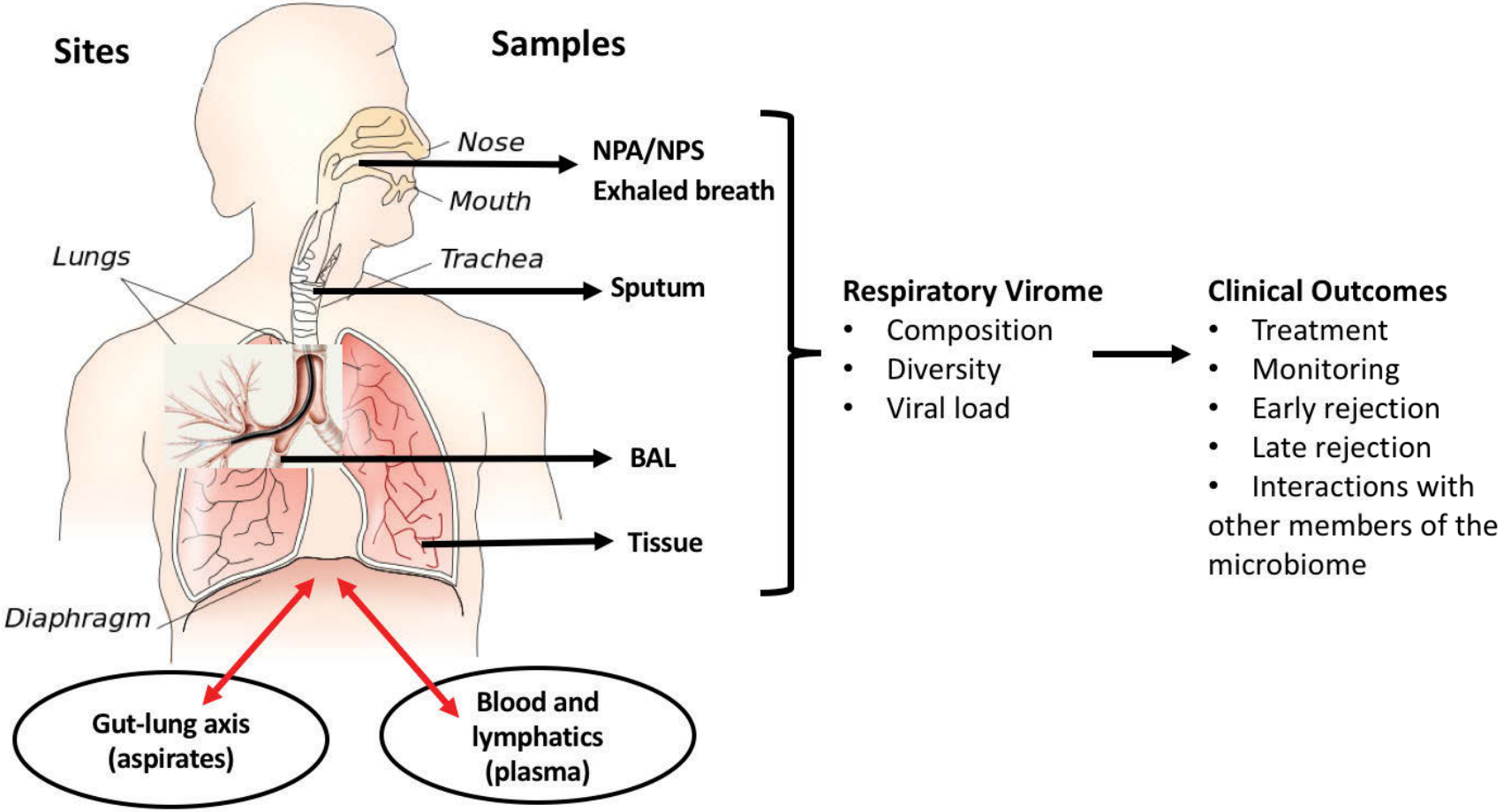

Fig 6.2 Samples collected from a range of sites within the respiratory tract may give information regarding the composition and diversity of the respiratory virome. In lung transplantation, the presence of viral species may be important for clinical outcomes.

6.4 Limitations of the current research

There are distinct limitations to the research contained within this thesis. While this work provides preliminary evidence to support the use of exhaled breath captured via electret filter as a viral sampling medium, and for the role of community acquired respiratory viruses as common, albeit likely transient, members of the human respiratory virome, these studies simply provide an introduction to the importance of this area.

Firstly, in regards to evaluating the filter methodology, the exhaled breath samples did not always correlate exactly with the respiratory secretion samples or available clinical data. In the original study describing the methodology, only one type of sample was taken from most patients, either a nasal washing, sputum sample or a BAL sample. This meant that we were could not discern the site of origin of the virus within the respiratory tract, or whether there was contamination from the surrounding environment. All precautions were taken to ensure that samples were not contaminated during transport or processing, and negative controls were included at all steps, including using clean spirometry filters which had not been used, to ensure that the stock mouthpieces were sterile before use. However, we cannot entirely exclude the possibility of airborne environmental contamination.

Similarly, in the ventilator filters on the inspiratory loop, we assumed that we were detecting airborne environmental viruses either from other patients, visitors or

healthcare staff as these often did not match with viruses detected on other clinical samples.

In the lung transplant cohort, we were unable to process ventilator filters from patients who were intubated following their lung transplantation surgery. This was due to the use of a different type of ventilator filter which did not contain an electret filter. These filters were unable to be removed from the plastic casing in which they were housed, and therefore unable to be processed as per protocol. It would have been interesting to have compared exhaled breath which had passed through these ventilator circuits for each transplanted patient, directly with the post-operative day (POD) 1 BAL. It would be important, in the future, to determine more clearly, the impact of environmental contamination by this means.

As mentioned in the previous section, there is a short follow up time in the current studies which does not allow the determination of robust associations between viral detection and chronic outcomes. There were no statistically significant correlations between viral presence and the risk of acute events in both the bronchiectasis cohort and the lung transplant cohort, in regards to exacerbations and development of acute allograft dysfunction respectively. However, longer follow up is required in both studies to be able to draw conclusions about whether patients in whom a virus was detected have adverse long-term outcomes. There is a body of literature describing an association between viral infection and the subsequent development of BOS after lung transplantation,[97,102,409,425] however there are conflicting opinions based on these studies regarding the contribution of CARV to the development and progression of BOS. Our prospective, longitudinal study design with ongoing sample collection and

clinical outcome data collection does provide an exciting opportunity to explore this aspect further.

In general, surprisingly little is known about the implications of asymptomatic viral carriage. As PCR has become a more commonly used viral detection methodology, the number of respiratory viruses that are detected in people where there is no suspicion of a possible viral infection has increased substantially, with rates of up to 51% in children.[222] The studies included in this thesis have also demonstrated high rates of asymptomatic viral detection in adults with chronic respiratory diseases and in immunocompromised transplant patients. As the majority of studies of asymptomatic viral detection have focused on children, these studies add to the small body of knowledge in this area and expand our understanding of the occurrence of asymptomatic viral carriage in adults. However, insofar as these descriptive studies provide evidence for asymptomatic carriage as a common phenomenon, they do not explore the mechanisms or biological significance of asymptomatic carriage.

Furthermore, these studies all have limited numbers of participants due to the nature of completing a clinical study in a specialised group of patients. However, while replicating these results in a larger cohort, including healthy controls would be cumbersome, it would permit a greater ability to generalise the findings. As mentioned, it is important to determine the role of respiratory viruses within the groups discussed within this thesis. However, as we do not yet know the true baseline of asymptomatic viral detection in the general public, especially in including healthy controls, the interpretation of results in our studies is somewhat limited. To date, there is almost no evidence regarding which respiratory viruses are transient or

resident members of the pulmonary virome except for TTV, let alone an understanding of which are obligate pathogens within the lungs. Therefore, extended studies with large numbers of patients particularly without underlying respiratory disease would be informative and extend the utility of findings from this PhD.

As part of the prospective, longitudinal, lung transplantation study, extra BAL samples were collected at every time point and stored at -80 degrees Celsius. The intent was to use a next generation sequencing approach to investigate the full DNA and RNA virome contained within the lungs of these patients to give further information to complement the PCR data regarding CARV detection. There were unforeseen limitations inherent in this approach, as virome sequencing is in its infancy, and there are no publically available protocols regarding processing of samples to isolate viral nucleic acids while simultaneously depleting for both human and bacterial reads within the sample. This form of processing is necessary as human and bacterial reads are present at a much greater abundance than viral sequences, and thus any viral genetic material would be lost within these sequences. Furthermore, as viruses do not have a conserved sequence such as the 16S rRNA in bacteria, viral metagenome sequencing is considerably more difficult. At this point in time, we have been unable to process the BAL samples sufficiently to isolate high quality nucleic acid samples adequate for sequencing. The inability to sequence the corresponding BAL samples for the transplant studies during this PhD is a significant limitation to interpreting the importance of the CARV within the resident lung virome post-transplantation. However, we anticipate this will be completed as the field develops and a robust methodology is made available.

The findings from the publications presented within this thesis have added to our overall understanding of the field. However, the study is largely observational with short term clinical correlations. Additional *in vitro* or animal studies to determine mechanisms and causality between the viruses detected and clinical outcomes would provide further insights. Nevertheless, it is now clear that respiratory viruses are ubiquitous within the human respiratory tract. The challenge lies ahead, as we still lack a deep understanding regarding the implications of their presence.

6.5 Future Directions

As outlined in the previous section, there are significant future directions that may be explored to extend and apply the observations that have been reported in this thesis.

Firstly, for the exhaled breath studies, it is important to extend this study in a much larger cohort with direct intrasubject comparison currently used sampling modalities. Comparing exhaled breath, NPS, NPA, sputum and BAL would allow the efficacy of the exhaled breath sampling tool to be determined more clearly in a clinical setting and to determine the comparative sensitivity and specificity for detecting clinically relevant viral infections. As mentioned within the limitations section, incorporating the exhaled breath sampling tool as part of a large clinical trial study may allow its evaluation in a carefully recruited cohort who are undergoing regular follow up, to give a clearer understanding of possible utility.

Further evaluation of the exhaled breath sampling tool within the ICU setting is also important to clarify the role of airborne viruses in the environment and their impact on detection within these filters. Delineation of the origin of detected viruses, and comparison of patient outcomes based on viruses detected within the expiratory loop filters may allow further exploration of the value of the exhaled breath sampling tool. While the current studies have helped establish the utility of the electret filter as a medium for collection and sampling of airborne viruses, further modification of the current methodology may be necessary to increase efficacy in a clinical setting.

As a high frequency of asymptomatic virus detection has been observed in all the studies presented within this thesis, it is important, in the future, to determine the clinical importance of asymptomatic viruses. This may vary between healthy controls and different patient groups, but as detection tools become more sensitive, the discovery that respiratory viruses appear to be ubiquitous leads to a plethora of questions concerning relevance for patient outcomes and treatment decisions. Furthermore, development of a sensitive but general biomarker may also be very helpful. In the ICU study, the IFN27 biomarker is discussed, but appears to be specific for differentiating severe influenza infection from more mild cases in at-risk hosts. A biomarker which aids in distinguishing symptomatic and asymptomatic viral infections for a range of respiratory viruses may be more useful in future diagnostics than molecular techniques to detect viral nucleic acids as we discover how common viruses may be present.

Baseline levels of respiratory viral detection within a general, otherwise healthy population also need to be determined. As mentioned above, it is difficult to discern whether the frequency of virus detection in these studies is unusually high and represent a heavy viral burden during that season, a reflection of the patient's immune state, underlying disease characteristics or if it simply reflects a stable level of resident viral species present at low viral load within the lungs of most individuals. Further studies establishing this baseline detection level, and further comparisons with larger groups of individuals with a range of underlying conditions will allow us to draw stronger conclusions regarding the possibility of resident viral species.

In the lung transplant study, all LTX BAL samples have been stored at -80 degrees Celsius indefinitely, until well-defined protocols are established that will allow sequencing of all DNA and RNA viral material contained within the BAL samples. In future, the aim is to characterise the full respiratory virome from the early post-transplant period (first 84 days) for all patients recruited into the lung transplant study. This will allow the dynamics of virus establishment within the allograft including perturbations and variations that occur during this critical period to be elucidated. Next generation sequencing technologies are becoming more accessible and technologies are improving to allow sequencing to a much deeper level, providing information about microbes present at a low concentration. Due to the low biomass of the viral reads within the lung samples, it is important that complete depletion of human, bacterial and fungal reads occurs and non-specific enrichment for all RNA and DNA viruses can occur before deep sequencing to ensure that the complete respiratory virome can be accurately described. The ongoing nature of this study, with continuing recruitment of patients and sample collection, will allow virome

characterisation in a large cohort of transplant patients at a great number of time points. Furthermore, our current findings regarding asymptomatic viral impact on ACR will be further evaluated, and analysis of the impact of respiratory viruses on the development of chronic rejection events will also be able to be elucidated.

Replication of this work in other solid organ transplant recipients or otherwise immunosuppressed patients may permit insights into the impact of the lung allograft specifically versus generalised immunosuppression, on virome dynamics. Furthermore, other populations, including paediatric lung transplant recipients, have also been explored as possible future avenues to determine the differences in the virome over time. Lung transplant patients are a suitable target group from which to obtain longitudinal lower respiratory tract samples, as they already undergo regular scheduled bronchoscopies as part of a clinical surveillance protocol to monitor the allograft.

In vitro and animal models of lung transplantation are being developed which may help to guide knowledge of some of the mechanisms underlying the observations reported herein. Mechanistic studies of the impact of the influenza vaccination on further attenuation of the inflammatory immune response would be a worthwhile topic for further exploration. Vaccination may contribute to increased asymptomatic viral presence in lung transplant patients, in addition to the immunosuppression previously discussed.

The revolution of ideas, understanding and management that came with acquiring knowledge of the human gut microbiome inspired our research into viruses as important members of the lung microbiome. We currently know very little about whether viruses have the ability to exist symbiotically within the human body but the work presented herein tends to support this concept. As has been uncovered for the bacterial component of the gut microbiome, understanding viral interactions may be important for our ability to influence human health outcomes, and prevent or treat disease states due to a dysbiosis of microbial species. Understanding the human respiratory virome, and the subtle forces which alter interactions between viral species and the human host in conjunction with other constituents of the microbiome will guide the way ahead. Much work is still required in this area, but as the world community focusses on the virome, as it has the Human Microbiome Project, to successfully complete sequencing of the human-viral metagenome, an exciting new area of research will materialise which will lead inevitably to therapeutic options currently beyond our understanding.

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