Chapter 7

Respiratory Virus in Cystic Fibrosis — A Review of the Literature

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Abstract

Life expectancy in Cystic Fibrosis (CF) has improved dramatically in the last few decades; this is very much due to the emergence of disease-modifying treatments, optimisation of nutritional status and the inception of specialist CF units. However, progressive obstructive lung disease characterised by chronic inflammation, bacterial colonisation and recurrent infections of the lung, resulting in irreversible pulmonary damage, remains the major cause of mortality in individuals with CF. Historically, bacterial infections are the major pathogens accounting for clinical deterioration in CF. More recently, there has been emerging evidence to support respiratory viruses being accountable for the colonisation of bacteria and progression of lung disease in CF. This chapter sought to provide an overview on the impact of respiratory viruses in CF lung disease, the interaction between viruses and bacteria, the preventative and therapeutic measures that are currently available for the management of viral lung disease in CF.

Keywords: Cystic fibrosis, respiratory virus, bacteria, Pseudomonas aeruginosa

1. Introduction

Cystic Fibrosis (CF) is the most commonly inherited potentially lethal disease amongst the Caucasian ancestry. The prevalence of CF is reported as 0.737 per 10,000 in 27 European Union countries [1]. The United States (US) Cystic Fibrosis Patient Registry reports a similar prevalence of 0.797 CF patients per 10,000 people [2]. It is an autosomal recessive disease and is

caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator gene (CFTR) [3]. The most common mutation is caused by deletion of phenylalanine at position 508 (Delta F508) of the CFTR on chromosome 7, which accounts for approximately 70% of CF cases. The primary function of CFTR in many tissues is to regulate and participate in the transport of chloride ions across epithelial cell membranes. To date, more than 1,900 mutations have been described in this gene.

CF is a multisystem disease as CFTR is expressed in different organs [4]; however, the lungs are the predominant organs that bear the brunt of the disease [5]. Recurrent pulmonary infections may start at very early stages in the lives of patients with CF. It has been hypothesised that low airway surface liquid volume and impaired mucociliary clearance are responsible for the pathogenesis of lung infections. These in turn lead to impaired bacterial clearance from respiratory epithelial cells [6]. Pulmonary infections remain the greatest cause of poor life quality, morbidity and mortality in CF that eventually lead to premature death in this condition [7].

Apart from chronic lung disease with recurrent exacerbations, exocrine pancreatic insufficiency is also a feature that leads to malabsorption and subsequently growth retardation and maturation. Endocrine pancreatic insufficiency is another feature of CF with the manifestation of diabetes. Obstructive azoospermia in male CF patients leads to male infertility.

The median survival from CF has taken great strides over the past 40 years as a consequence of the introduction of specialist centre care, nutritional optimisation, prevention and aggressive management of pulmonary exacerbations [8]. In the United Kingdom (UK) CF population in 2012, the median survival was reported as 43.5 years, compared to 38.8 years for the population in 2008 as per the UK CF Registry [9]. It has been postulated that the continuing improvement in survival of CF patients in successive cohorts means that the previous prediction of patients with CF living beyond a median age of 50 years is not impossible. The recent introduction of Ivacaftor to the management of CF patients with G551D CFTR mutations may further enhance the overall survival [10].

Historically, bacteria have been the predominant cause for respiratory exacerbations. The presences of some organisms including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Burkhoderia cepacia* in the airways have been shown to lead to clinical deterioration [11-13] and may subsequently lead to morbidity and mortality. Pulmonary exacerbations are associated with acquisition of new organisms and increased concentration of airway flora [14]. The new acquisitions of *P. aeruginosa* in CF have been demonstrated to occur in the winter months coinciding with the peak of respiratory viral infections [15, 16]. In the event of a pulmonary exacerbation the absence of pyrexia, raised inflammatory markers and systemic response, pathogens other than bacteria can be the potential cause. Respiratory viruses have been implicated by a number of studies in the last 30 years as potentiators for CF exacerbations [17-29]. *Influenza* is a substantial health threat; it is associated with approximately 36,000 deaths and 220,000 hospitalisations in the USA on an annual basis [30]. The recent emergence of novel *influenza virus (H1N1)* further heightened the awareness of influenza-like illness. CF Pulmonary exacerbation rates have also been shown to be significantly increased during the winter months and are highly associated with the influenza season [31]. Respiratory viruses that are

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associated with the exacerbations of CF include *influenza A and B, respiratory syncytial virus (RSV), parainfluenza virus (PIV) types 1 to 4, rhinovirus, metapneumovirus, coronavirus* and *adenovirus*.

In the last 30 years, there have been a number of published studies depicting the impact of respiratory viruses in CF. A number of studies have also demonstrated the relationship between respiratory viruses and bacteria in the pathogenesis of CF exacerbations [15, 32]. The introduction of molecular diagnostic technologies has further enhanced the awareness of respiratory viral aetiology in CF exacerbations as they have much higher detection rates than traditional methods. However, further understanding is required to appreciate their relationship in order to allow the development of potential novel treatment. If indeed respiratory virus does lead to secondary bacterial infection in CF, viral vaccinations and anti-viral therapies would be important therapeutic options for CF. On the other hand, the currently commercially available vaccines and anti-virals for the prevention and treatment of respiratory viral infections are limited; they are primarily for influenza infection. The potential development of new vaccines and anti-virals is an exciting field which may offer alternate therapeutic opportunities for CF exacerbations.

This chapter will focus on the literature regarding respiratory viruses in CF and their clinical implications, the detection techniques for viruses and their differences in sensitivities, the interaction between viruses and bacteria, and the management of viral infections.

2. Viral respiratory infections in CF

Early studies looking at respiratory viruses in CF relied on repeated serological testing, either alone [20] or in combination with viral cultures for viral detection [21-25]. These methods are relatively insensitive and more recent studies have utilised molecular-based methodologies [18, 26-28, 33-36]. All these studies produced different results in terms of prevalence of respiratory viruses in CF. The differences can be due to different methodologies, different sampling methods; the differences can also be accountable by different populations studied as the prognosis for CF has improved with each successive birth cohort.

Wang et al. [25] described the relationship between respiratory viral infections and deterioration in clinical status in CF almost 30 years ago. In this 2- year prospective study [25], viruses were identified through serology and nasal lavage in 49 patients with CF (mean age 13.7 years) on a quarterly basis and at the onset of exacerbations. Although the CF patients had more respiratory illnesses than sibling controls (3.7 versus 1.7/year), there were no differences in virus identification rates (1.7/year). The rate of proven virus infection was significantly correlated with the decline in lung functions, nutritional status, radiology score, and frequency and duration of hospitalisation.

More recent studies suggest no difference in the frequency of either upper respiratory tract illness (URTI) episodes [22] or proven respiratory viral infections [24] between children with CF and healthy controls, but children with CF have significantly more episodes of lower airway

symptoms than controls [22, 24]. Ramsey et al. [24] prospectively compared the incidence and effect of viral infections on pulmonary function and clinical scores in 15 school-age patients with CF aged between 5 and 21 years and their healthy siblings. Over a 2-year period, samples were taken at regular two monthly intervals and during acute respiratory illnesses (ARI) for pharyngeal culture and serology for respiratory viruses. There was a total of 68 ARI episodes that occurred in the patients with CF and in 19 episodes there was an associated virus identified. A total of 49 infective agents were identified either during ARIs or at routine testing in the patients with CF; 14 were identified on viral isolation (*rhinovirus* on 11 occasions), whilst 35 were isolated on seroconversion (*PIV* on 12, *RSV* on 9 and *M. pneumoniae* on 6 occasions). At the time of an ARI, the virus isolation and seroconversion rates were 8.8% and 19.1%, respectively, in children with CF compared to 15.0% and 15.0%, respectively, for the healthy siblings. In contrast, the rates of virus isolation and seroconversion at routine 2 monthly visits were 5.6% and 16.2%, respectively, for children with CF and 7.7% and 20.2%, respectively, for the healthy siblings. There was no significant difference in the rate of viral infections between the patients with CF and their sibling controls, as measured either by culture or serology. The rate of viral infections was higher in younger children (both CF and controls); however, the rate of decline in pulmonary function and severity score was both lower in the younger children with CF and the ones with more viral infections. The authors concluded that there were no significant adverse effects with viral infections in CF.

Likewise, Hiatt [22] assessed respiratory viral infections over three winters in 22 infants less than 2 years of age with CF (30 patient seasons), and 27 age-matched controls (28 patient seasons). The average number of acute respiratory illness per winter was the same in the control and CF groups (5.0 vs. 5.0). However, only 4 of the 28 control infants had lower respiratory tract symptoms in association with the respiratory tract illness, compared with 13 out of the 30 infants with CF (Odd ratio – 4.6; 95% confidence interval 1.3 and 16.5; p-value <0.05); 7 of the infants with CF cultured *RSV*, of whom 3 required hospitalisation. In contrast, none of the controls required hospitalisation. Pulmonary function measured by rapid chest compression technique was significantly reduced in the infants with CF after the winter months and was associated with two interactions; *RSV* infection with lower respiratory tract infection and male sex with lower respiratory tract infection.

From previous reports, two viral agents appear to have the greatest effect on respiratory status in CF, namely *RSV and influenza*, possibly because the uses of viral culture and serology have underestimated the effects of rhinovirus. In younger children, *RSV* is a major pathogen resulting in an increased rate of hospitalisation. Abman et al. [37] prospectively followed up 48 children with CF diagnosed through newborn screening and documented the effect of *RSV* infection. Eighteen of the infants were admitted into hospital a total of 30 times over a mean follow-up of 28 months (range 5-59]. In 7 of these infants *RSV* was isolated, and their clinical course was severe with 3 requiring mechanical ventilation and 5 necessitating chronic oxygen therapy. Over the next 2 years, these infants had significantly more frequent respiratory symptoms and lower Brasfield chest radiograph [38] scores than *non-RSV*-infected counterparts.

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In older children and adults with CF, *influenza* seems to have the greatest effect. Pribble et al. [23] assessed acute pulmonary exacerbation isolates from 54 patients with CF. Over the year of the study, 80 exacerbations were identified, of which 21 episodes were associated with an identified viral agent (*influenza A* – 5 episodes; *influenza B* – 4 episodes; *RSV* – 3 episodes) with most agents identified on serology. Compared to other respiratory viruses, infection with *influenza* was associated with a more significant drop in pulmonary function (FEV_1 declined by 26% compared with 6%). There were also a higher proportion of patients with a greater than 20% drop in FEV_1 within the *influenza* infected cohort. A retrospective study in older patients with chronic *P. aeruginosa* infection reported an acute deterioration in clinical status in association with *influenza A* virus infection, which was confirmed by serology [39].

Over a 1-year period, Smyth et al. [27] prospectively investigated 108 patients with CF (mean age of 7.9 years) using a combination of viral immunofluorescence, culture and seroconversion to identify respiratory viruses. With the exception of *rhinovirus*, a semi-nested reverse transcriptase PCR technique was used. During the study, 76 subjects had 157 respiratory exacerbations (1.5 episodes/patient/year) and a viral agent was identified in 44 episodes, 25 of which were *rhinovirus* and an equal distribution of other viruses was identified almost always on seroconversion. Rhinovirus identification was associated with significantly more days of intravenous antibiotics, whereas, those children in whom a non-rhinovirus was identified had a significantly greater decrease in FEV_1 over the year of the study. When all viruses were considered as a whole, patients had significantly greater decline in Shwachman score [40] and days of intravenous antibiotics use.

Collinson et al. [26] followed 48 children with CF over a 15-month period using viral cultures for viral detection, with the exception of *picornaviruses* where polymerase chain reaction (PCR) was used; 38 children completed the study and there were 147 symptomatic upper respiratory tract infections (URTIs), 2.7 episodes/child/year, with samples available for 119 episodes. *Picornaviruses* were identified in 51 (43%) of these episodes, of which 21 (18%) were *rhinoviruses*. In those children old enough to perform spirometry, there was significant reduction in both FVC and FEV_1 in association with URTIs, with little difference in severity of reduction whether a picornavirus was identified or not. Maximal mean drop in FEV_1 was 16.5%, at 1-4 days after onset of symptoms, but a deficit of 10.3% persisted at 21-24 days. Those with more URTIs appeared to have greater change in total Shwachman score [40] and Chrispin-Norman score [41] over the study. Six children isolated a *P.aeruginosa* for the first time during the study, 5 at the time of a URTI and only 1 was asymptomatic at the time of first isolation. However, the data from this study have to be handled with care as the term 'URTI' does not necessarily imply a positive viral isolation.

Punch et al. [42] used a multiplex reverse transcriptase PCR (RT-PCR) assay combined with an enzyme-linked amplicon hybridization assay (ELAHA) for the identification of seven common respiratory viruses in the sputum of 38 CF patients; 53 sputum samples were collected over 2 seasons and 12 (23%) samples from 12 patients were positive for a respiratory virus (4 for *influenza B*, 3 for *parainfluenza type 1*, 3 for *influenza A* and 2 for *RSV*). There were no statistical associations between virus status and demographics, clinical variables or isolation rates for *P. aeruginosa, S. aureus or A. fumigatus*.

Olesen and colleagues [28] obtained sputum and laryngeal aspirates from children with CF over a 12-month period in outpatient clinics. They achieved a viral detection rate of 16%, with *rhinovirus* being the most prevalent virus. FEV_1 was significantly reduced during viral infection (-12.5%, p=0.048), with the exception of *rhinovirus* infection. The authors were not able to demonstrate a positive correlation between respiratory viruses and bacterial infections in their studied population as the type or frequency of bacterial infection during or after viral infections were not altered. They also concluded that clinical viral symptoms had a very poor predictive value (0.39) for a positive viral test.

Our group in 2004 [36] utilised 'real-time' multiplex Nucleic Acid Sequenced Based Amplification to examine the role of respiratory viruses in CF children. Over an 18-month period, a viral detection rate of 46% was achieved during reported episodes of respiratory illness. The results compared favourably with previous studies and it may be that earlier studies relied heavily on repeated serological testing, either alone [20] or in combination with viral isolation [21-25]. The viral detection rate was 18.3% from routine nasal samples. However, this was comparable to the seroconversion rate of 12.3% as reported by Wang et al. [25]. Ramsey and colleagues [24] also achieved a similar seroconversion rate of 16.2% from asymptomatic samples. These results suggest that a laboratory method with a higher sensitivity for viral detection does not increase the detection rate in asymptomatic samples, implying that false positives are not necessarily more common than less sensitive diagnostic methods. *Influenza A and B* viruses were the major viruses in causing respiratory exacerbations in CF and both viruses are more commonly detected during pulmonary exacerbations; 22 of 88 [23%) viruses found in this study were *influenza viruses (A & B)*. The result is consistent with majority of the previous studies which showed that *influenza* virus represented between 12% and 27% of all viruses detected. However, the findings are in contrast with other studies where rhinovirus is the major virus in CF exacerbations [26, 28, 43]. The *influenza* vaccine uptake rate during the study period was up to 70% [36]. It is possible that the detection rate for *influenza* virus could have been higher had the vaccine coverage not been this high.

Asner et al. [44] performed an observational cross-sectional study of CF children from a large paediatric referral centre investigating the association between respiratory viruses and pulmonary exacerbations by taking mid-turbinate swabs, sputum and throat swab samples that were tested by a direct immunofluorescent antibody assay and a multiplex PCR panel. Forty-three patients were recruited into the study. Pulmonary function tests, quality of life and severity scores were recorded. Sputum cell counts, bacterial density and cytokines were measured. Twenty-six (60.5%) subjects were tested positive for at least one respiratory virus by any diagnostic method applied to any sample type. Of the 26 virus positive subjects, 17 (65.4%) were positive for one virus and the remaining 9 (34.6%) were positive for two or more viruses. *Coxsackie/echovirus* was the most commonly identified pathogen (29.4%) amongst the 17 subjects that were positive for one virus. Virus-positive patients were younger (p=0.047) and more likely to be male (p=0.029). They were also more likely to present with fever (p=0.019), have higher CF clinical severity (p=0.041) and lower quality of life scores (p=0.022). However, virus-positive and negative patients had similar IL-8, neutrophil percentage, elastase levels and 26 additional cytokines levels between both groups. The authors reported a higher rate of

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viral detection with mid-turbinate swabs than with sputum samples. The study was primarily conducted in the outpatient setting and subjects may have milder form of exacerbations which may in turn explain the lack of inflammatory response in virus-positive subjects. There was also a significant median age difference between the virus-positive and negative groups (6.9 vs. 13 years, p=0.047), with respiratory viral infection being more common in younger children presumably related to the delay maturation of the immune system.

A CF centre in Milan (Italy) led by Esposito and colleagues [43] showed that human *rhinovirus* was the most frequently isolated virus from CF patients <25 years of age during respiratory exacerbations and when subjects were clinically stable over a 1-year period. Molecular techniques were utilised to isolate viruses from nasopharyngeal samples. The authors demonstrated that human *rhinovirus* was more common among patients with pulmonary exacerbations than among clinically stable patients. The human *rhinovirus* viral load was however similar in subjects with or without acute respiratory exacerbations (p=0.46). There were no correlations between the associated clinical condition and viral load as well as between bacterial colonisation, colonising bacterial, and viral infections between the 2 groups. This finding is similar to de Almeida et al. [17] who did not show a difference in viral infection between the exacerbation and clinically stable groups. Therefore, this raises a possibility that isolation of *rhinovirus* from nasopharyngeal swabs does not always indicate that it is a cause of exacerbation as it may be explained by a coincidental upper airways infection, a carrier state, or prolonged shedding of a pathogen that caused a previous infection [45].

In 2009, a novel swine pandemic *influenza A virus (H1N1)* was identified. Nash et al. [46] showed that the symptoms of CF patients infected with H1N1 tend to be mild. There was no significant reduction in FEV_1 % predicted, FVC % predicted and body mass index regardless of whether the patients were positive or negative for *H1N1*. Colombo et al. [47] performed a multi-centre survey showing that diagnostic testing did not identify clinical characteristics specifically associated with *H1N1* infections. Similarly, they did not show a significant decline in lung function associated with this infection. To date, the significance of *H1N1* infection in CF remains undefined.

In contrast, the data regarding respiratory viral infection in adults is sparse. An observational study conducted by Hoek et al. [48] over a 1-year period amongst adult CF patients yielded a viral isolation rate of 33% [8/24] utilising molecular techniques and conventional methods. Etherington and colleagues [18] from an adult CF centre published a retrospective case control study looking at the prevalence of respiratory viruses during exacerbations. Viral throat swabs were taken from all patients presenting with an acute pulmonary exacerbation requiring intravenous antibiotic treatment over a 12-month period. Viral isolation was performed by PCR. There were 432 pulmonary exacerbations in 180 adults. In total, there was a total positive viral isolation in 42 exacerbations indicating a prevalence of 9.7%. *Rhinovirus* was the commonest isolated virus and was found on 29 occasions (69%). *Influenza A/H1N1* was isolated in seven patients (16.7%). They demonstrated a measurable impact of viral infections in CF as exacerbations associated with a positive viral PCR had a greater fall in lung function at presentation with higher levels of inflammatory markers. These patients also received more

days of intravenous antibiotics, showed less response to treatment and had a shorter time to next pulmonary exacerbation compared to matched controls.

Flight et al. [35] followed up 100 adult CF patients prospectively for 12 months. Sputum, nose swabs and throat swabs were collected every 2 months and at the onset of pulmonary exacerbation for virus detection. PCR assays for *adenovirus, influenza A&B, human metapneumovirus, parainfluenza 1-3, respiratory syncytial virus* and *human rhinovirus* were performed on each sample. Symptom scores, spirometry and inflammatory markers were measured at each visit. Overall, virology results were available for 626 of 649 completed study visits. Of these, 191 (30.5%) were positive for a respiratory virus including 9 episodes of dual viral infection. Human *rhinovirus* accounted for 72.5% of viruses. Overall incidence of viral respiratory infection (VRI) was 1.66 (95% CI 1.39 to 1.92) cases/patient-year. VRI was associated with increased risk of pulmonary exacerbation (OR=2.19; 95% CI 1.56 to 3.08; p<0.001) and prescription of antibiotics (OR=2.26; 95% CI 1.63 to 3.13; p<0.001). Virus-positive visits were associated with higher respiratory symptom scores and greater C-reactive protein levels. Virus-positive exacerbations had a lower acute fall in FEV_1 than virus-negative exacerbations (12.7% vs. 15.6%; p=0.040). The incidence of exacerbations, but not VRI, was associated with greater lung function decline over 12 months (-1.79% per pulmonary exacerbation/year; 95% CI -3.4 to -0.23; p=0.025).

Experimental data on the effects of viral infections in CF are limited. Toll-like receptors (TLRs) have recently been identified as key mediators of the innate response and they recognise pathogens through detection of conserved microbial structures that are absent from the host. Kurt-Jones et al. [49] found that *RSV* persisted longer in the lungs of infected TLR4-deficient mice compared to normal mice. Haynes et al. [50] also demonstrated that TLR4-deficient mice when challenged with *RSV* exhibited impaired natural killer cell trafficking and impaired virus clearance compared to normal ones. Limited human studies have demonstrated the important role of TLRs in host response against many major groups of mammalian pathogens [51]. The relationship between TLR and respiratory virus including *RSV* in humans will require further studies before it can be established.

Some studies have suggested a higher viral replication when there is an impairment of the innate host defence in CF. *Influenza* titres were significantly increased in a mouse model which were chronically infected with *P. aeruginosa* compared to control model [52]. This in turn led to an increase in susceptibility to fatal streptococcus pneumonia infection. Increased virus replication was also found after *PIV* infection of CF human airway epithelial cells, compared to controls [53]. One of the possible causes of increased virus replication and of virus persistence might be a reduced production of respiratory nitric oxide (NO), which is a vital part of innate antiviral defence mechanism [54]. Increased production of NO protects against viral infections. In CF patients, expression of the NO producing enzyme NO synthase type 2 (NOS2) is considerably reduced.

Xu et al. [55] showed that CF cells that were infected with influenza A had less IFN-related antiviral gene induction at 24 h but more significant inflammatory cytokine gene induction at 1 h after infection. Therefore, the lesser antiviral and greater early inflammatory response may explain the severe respiratory illness of CF patients with viral infections. Sutanto and co-

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workers [56] showed that CF airway epithelial cells had a marked increase in IL-8 production, a reduction in apoptosis and an increased viral replication compared with airway epithelial cells from healthy children following exposure to human rhinovirus. This is despite the fact that CF and healthy airway epithelial cells have similar basal and stimulated expression of IL-8 in response to pro-inflammatory stimuli. The increment of IL-8, together with a reduction of apoptotic responses by CF cells to human rhinovirus, could contribute to augmented airway inflammation in the setting of recurrent viral infections early in life.

Azithromycin has previously been shown to offer anti-rhinoviral activity in bronchial epithelial cells and, during rhinovirus infection by increasing the production of interferon-stimulated genes [57]. However, the role of anti-viral properties of Azithromycin in CF is not clearly defined. Schögler et al. [58] showed that primary bronchial epithelial cells from CF children that were pre-treated with Azithromycin had a seven-fold reduction in rhinovirus replication without inducing cell death. Azithromycin also increased RV-induced pattern recognition receptor, IFN and IFN-stimulated gene mRNA levels when measured by real-time quantitative PCR. Therefore, it is likely that Azithromycin pre-treatment reduces RV replication in CF bronchial epithelial cells, possibly through the amplification of the antiviral response mediated by the IFN pathway.

3. Detection of respiratory viruses

The diagnostic accuracy and sensitivity of respiratory viral detection is determined by several factors:

1. Appropriate respiratory specimen for testing – Nasal swabs, nasopharyngeal aspirates, nasal swabs and mid-turbinate sampling are reasonable sampling methods in young children who may not be able to expectorate. However, in older patients, sputum is easy to obtain, painless and quick. Bronchoalveolar lavage (BAL) is a useful intervention to obtain specimen in the distal airways but it is more invasive. However, it can provide useful information regarding the activities of respiratory viruses and bacteria in the distal airways.
2. Appropriate specimen transport method – There are recognised procedures for transporting clinical specimens for diagnostic virology testing. These procedures should be adhered to closely to enhance the chances of isolating the viral organism. For instance, nasal swabs should be transported in viral transport medium and all specimens should be refrigerated if there is a delay of more than 2 hours in reaching the laboratory.
3. Detection methods – Molecular techniques have superseded many conventional methods such as viral culture and serology as they are far more sensitive and specific; in addition, they have a more rapid turn-around, allowing diagnostic technology to have an immediate impact on clinical management. The advantages of such technology will allow the appropriate utilisation of anti-virals, many of which are virus-specific; it may play a role in complying with hospital infection control policy; finally, it can provide useful infor-

mation to public health authorities such that public health policies can be adjusted accordingly, e.g. the outbreak of SARS and influenza H5N1 virus.

4. Utilisation of real-time multiplex amplification technique allows multiple viruses being quantified even if the copy number of the viral target is low.

More recently, Virochip has been shown to be a pan-virus microarray platform that is capable of detection of known as well as novel viruses in a single assay simultaneously [59]. Probes chosen for Virochip can identify nodes in the viral taxonomy at the family, genus and species levels. As the Virochip probes are updated regularly, the extent of probes that can be covered are ever increasing, up to 36,000. It has a diagnostic sensitivity comparable to PCR for detecting respiratory genomes at levels as low as 100 genome copies. At the present time, Virochip is very much a research tool, and several issues must be addressed before it can be used as a routine test for virus detection in the clinical setting, including cost, diagnostic accuracy, repeatability, and sensitivity/specificity for virus detection. In addition, the clinical implication of novel viruses in the human respiratory tract is not yet defined. Therefore, the accurate interpretation of Virochip in the clinical setting remains a formidable task. For example, where specimens are polymicrobial or viral material are present at low levels, clinical and epidemiological information might be required to draw clinically meaningful conclusions.

4. Interaction between respiratory viruses and bacteria

In a 25-year retrospective review from the Danish CF clinic, the first isolation of *P. aeruginosa* was most likely between October and March [16] coinciding with the peak of the *RSV* season. However, there are a number of other possible viral agents that would broadly fit the winter season, most notably *influenza, rhinovirus* and *metapneumovirus;* therefore, these findings must be interpreted with caution.

An increase in immunoglobulin A (IgA) antibodies to the O-antigen of *P. aeruginosa* is noted in 62% of viral infections [60]. This suggests a possible 'microbial synergism' between bacterial infections and infections with respiratory viruses in CF.

The first bacterial isolation of a given organism in CF has also been shown to often follow a viral infection. In the 17-month prospective study reported by Collinson et al. [26], 5 of the 6 first isolations of *P. aeruginosa* were made during the symptomatic phase of an upper respiratory tract infection or three weeks thereafter. In contrast, only one of the 6 initial infections with *P. aeruginosa* was identified during the asymptomatic period. Similarly, *H. influenzae* was recovered for the first time from 3 children within 3 weeks of an upper respiratory tract infection and the one new *S. aureus* infection was identified immediately following a viral infection.

Armstrong and colleagues have reported that 50% of CF respiratory exacerbations requiring hospitalisation are associated with isolation of a respiratory virus [21]. In their prospective study of repeated BAL in infants over a 5-year period, a respiratory virus was identified in 52% of infants hospitalised for a respiratory exacerbation, most commonly *RSV*; 11 of the 31

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hospitalised infants (35%) acquired *P. aeruginosa* in the subsequent 12-60 month follow-up, compared to 3 of 49 (6%) non-hospitalised infants (Relative risk 5.8). This indicates that RSV infection was identified immediately following a viral infection.

Respiratory viruses can disrupt the airway epithelium and precipitate bacterial adherence. *Influenza A* infection has been shown to cause epithelial shedding to basement membrane with submucosal oedema and neutrophil infiltrate [61], while both influenza and adenovirus have a cytopathic effect on cultured nasal epithelium leading to destruction of the cell monolayer [62]. This epithelial damage results in an increase in the permeability of the mucosal layer [63, 64] and possibly facilitating bacterial adherence. Bacteria can also utilise viral glycoproteins and other virus-induced receptors on host cell membrane as bacterial receptors in order to adhere to virus-infected cells [65, 66].

Kim et al. [67] found that invariant natural killer T cells induce a type of macrophage activation driving the secretion of interleukin-13 leading to the production of globlet cell metaplasia and airway hyperactivity following infection with Sendai virus. The term 'invariant' stems from the fact that all invariant natural killer T cells in humans and mice use a unique T cell receptor that is essential for interaction with CD1d. CD1d molecules present lipid antigens to T lymphocytes rather than peptide antigens as in the case of major histocompatibility complex (MHC) class I and II molecules. Historically, MHC class II dependent CD4 and T lymphocytes, through their response to stimulation by environmental allergens, are keys to the pathogenesis of human asthma. The findings by the authors lead to the notion of the use of anti-interleukin-13 therapy as a potential therapy in patients.

Viral infections might predispose to secondary bacterial infections by impairing mucociliary function and triggering host inflammatory receptors [68, 69]. This phenomenon has been demonstrated both in vivo and in vitro [70, 71]. Avadhanula et al. [72] showed that different respiratory viruses use different mechanisms to enhance the adherence of bacteria to respiratory epithelial cells. In particular, *RSV* and *PIV type 3* up-regulate intercellular adhesion molecule-1 (ICAM-1), carcinoembryonic adhesion molecule 1 (CEACAM1) and platelet activating factor receptor (PAFr) but not mucin on the surfaces of A549, BEAS-2B and NHBE but not SAE cell lines. Much of the increased bacterial adhesion following *RSV* infection could be blocked by antibodies directed against these receptors. A549 and BEAS-2B are transformed cell lines derived from type II alveolar and normal bronchial cells, respectively. NHBE and SAE cells are primary epithelial cells obtained from bronchi and distal bronchial tree and are likely to include a heterogeneous population of cells.

Mechanisms independent of the expression of conventional receptors for bacteria, such as binding to viral proteins, could be responsible for enhanced adhesion [73]. Immunofluorescence microscopy demonstrates that bacteria binding to *RSV*-infected A549 cells adhere not only to these cells expressing viral antigens but also to uninfected epithelial cells. These data suggest that the ability to augment bacterial adhesion may result from a factor served by infected cells that exert a paracrine effect on adjacent epithelium. Cytokines or other inflammatory molecules are potential good candidates for such a mediator.

Rhinovirus has been shown to potentiate bacterial infections by inhibiting the secretion of TNF alpha and interleukin-8 by macrophages in vitro following co-infection with gram negative bacterial products, lipopolysaccharide (LPS), and gram positive bacterial products, lipoteichoic acid (LTA) [74]. This rhinovirus-dependent impairment of the macrophage immune response was not mediated by autocrine production of the anti-inflammatory cytokines interleukin-10 and PGE2, or by down-regulation of the cell surface receptor for LTA and LPS. In addition, the authors also show that rhinovirus inhibit the phagocytosis of bacterial products by macrophages. These findings support the notion that *rhinovirus* exposure resulted in a reduced ability to innate and adaptive immune responses against bacterial products, hence promoting the occurrence of bacterial and viral co-infections.

The lower respiratory tract is protected by local mucociliary mechanisms that involve the integration of the ciliated epithelium, periciliary fluid and mucus. Mucus acts as a physical and chemical barrier onto which particles and organisms adhere. Cilia lining the respiratory tract propel the overlying mucus to the oropharynx where it is either swallowed or expectorated. *Influenza* viral infection has been shown to precipitate the loss of cilial beat, and shedding of the columnar epithelial cells generally within 48 hours of infection [75]. Pittet et al. [76] showed that a prior *influenza* infection of tracheal cells in vivo does not increase the initial number of *pneumococci* found during the first hour of infection, but it does significantly reduce mucociliary velocity, and thereby reduces *pneumococcal* clearance during the first 2 hours after *pneumococcal* infection at both 3 and 6 days after an *influenza* infection. The defects in *pneumococcal* clearance were greatest at 6 days after *influenza* infection. Changes to the tracheal epithelium induced by *influenz*a virus may increase susceptibility to a secondary *S. pneumoniae* infection by increasing *pneumococcal* adherence to the tracheal epithelium and/or decreasing the clearance of *S. pneumoniae* via the mucociliary escalator of the trachea, and thus increasing the risk of secondary bacterial infection.

De Vrankrijker et al. [77] showed that mice that were co-infected with *RSV* and *P. aeruginosa* had a 2,000 times higher colony-forming units (CFU) count of *P. aeruginosa* in the lung homogenates compared to mice that were infected with *P. aeruginosa* alone. Co-infected mice also had more severe lung function changes. These results suggest that *RSV* can facilitate the initiation of acute *P. aeruginosa* infection.

Another study also showed that *H. influenzae* and *S. pneumoniae* bind to both free *RSV* virions and epithelial cells transfected with cell-membrane-bound G protein, but not to secreted G protein. Pre-incubation with specific anti-G antibody significantly reduces bacterial adhesion to G protein-transfected cells [78].

Stark et al. [79] showed that mice that were exposed to *RSV* had significantly decreased *S. pneumonia*, *S. aureus* or *P. aeruginosa* clearance 1 to 7 days after *RSV* exposure. Mice that were exposed to both *RSV* and bacteria had a higher production of neutrophil-induced peroxide but less production of myeloperoxidase compared to mice that were exposed to *S. pneumoniae* alone. This suggests that functional changes in the recruited neutrophils may contribute to the decreased bacterial clearance.

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More recently, Chattoraj et al. [15] demonstrated that acute infection of primary CF airway epithelial cells with rhinovirus liberates planktonic bacteria from biofilm. Superinfection with *rhinovirus* stimulates robust chemokine responses from CF airway epithelial cells that were pre-treated with mucoid *P. aeruginosa*. The authors also showed that these chemokine responses lead to a liberation of bacteria from mucoid *P. aeruginosa* biofilm and transmigration of planktonic bacteria from the apical to the basolateral surface of mucociliary-differentiated CF airway epithelial cells. Planktonic bacteria, which are more pro-inflammatory than their biofilm counterparts, stimulate increased chemokine responses in CF airway epithelial cells which, in turn, may contribute to the pathogenesis of CF exacerbations and subsequent prolonged intravenous antibiotic use and hospitalisation.

Contrary to the above reports, Chin et al. [32] performed a prospective study over a 2-year period on 35 adult CF patients. *P. aeruginosa* sputum density was analysed during stable, exacerbation and post-exacerbation assessments. PCR was used to detect respiratory viruses during exacerbations. The sputum density of *P. aeruginosa* in patients with or without a viral infection was compared using quantitative culture or by PCR. Twenty-two patients experienced 30 exacerbations during the study period; 50% were associated with a viral infection. There was no change in sputum density of *P. aeruginosa* from the stable to exacerbation state. Virus-associated exacerbations did not result in significant increases in *P. aeruginosa* sputum density compared to non-viral exacerbations.

Contrary to the above findings, Asner et al. [44] found the mean total bacterial density in sputum samples in virus-positive patients being two logs lower than that found in virus-negative patients ($p=0.299$). However, this could be explained by the fact that the median age of the virus-positive group was significantly lower than the virus-negative group. Virus-positive and virus-negative patients had similar IL-8, neutrophil percentage and neutrophil elastase levels.

Similarly, Kieninger et al. [80] performed a comprehensive investigation of the inflammatory response of CF airway epithelial cells on virus infection. Strong cytokine production was found in all cells studied, with the magnitude and type of inflammation differing depending on cell type and virus used. There was no exaggerated inflammatory response in CF, either during cytokine production or at the transcriptional level. Instead, there was a trend towards lower cytokine production in CF airway epithelial cells after virus infection, which was associated with increased cell death. The lower inflammatory response in CF can also be explained by additional pathophysiological mechanisms, such as interactions between anti-viral and pro-inflammatory pathways, which are likely to be involved [81]. It could also be speculated that because of chronic activation of pro-inflammatory pathways, CF airway epithelial cells are not able to respond sufficiently to further stimuli, such as virus infections. This might, in turn, lead to a lack of recruitment of effector immune cells resulting in longer duration and more severe respiratory symptoms.

TLRs are key mediators of type I interferon (IFN) during viral infections by recognizing various viral components. TLR7 and TLR9 have become apparent as universally important in inducing type I IFN during infection with most viruses, particularly by plasmacytoid dendritic cells [82]. New intracellular viral pattern recognition receptors leading to type I IFN production have

been identified. CFTR mutations have been shown to affect the epithelial induction of type I IFN expression by airway cells in response to *P. aeruginosa* infection [83]. This is achieved by abolishing this signalling pathway, an important component of the innate immune system that protects mucosal surfaces. Based on available evidence, chronic colonisation of *P. aeruginosa* in CF airways can be hypothesised to increase the predisposition of viral infections; however, more in-depth studies are required to elucidate this hypothesis.

Taken together, these findings suggest conflicting data regarding the inflammatory response of the CF airway epithelium on virus infection and to some extent the symbiotic relationship between viruses and bacteria. Nonetheless, respiratory viruses may lead to epithelial disruption, increase neutrophil influx, inhibition of macrophage phagocytosis, destruction of mucociliary escalator, down-regulation of cilia beat, liberation of pro-inflammatory planktonic *P. aeruginosa* from biofilm and increased neutrophil-induced peroxide release, indirectly facilitating bacterial infection of the airway.

5. Prevention and treatment for respiratory viruses

The diversity of viral serotypes in causing infection has made vaccine preparation very difficult. Frequent mutations of viral proteins of RNA viruses (e.g. genetic drift and shift of *influenza*) have further hampered the prevention of the illness.

In the UK, it has been reported that 2,150 deaths during the 2011/12 season was attributable to influenza [84], though some of the deaths may be attributed to *RSV*. *Influenza* vaccines are the only commercially available vaccines against common respiratory viruses. They have been used since the mid-1940s and they now have an established role in the prevention of *influenza A and B* infections. Inactivated *influenza* vaccine is effective even in young children including those younger than 2 years [85]. The waning of vaccine-induced immunity over time requires annual re-immunisation even if the vaccine antigens are unchanged.

Recent vaccines contain antigens of two *influenza A* subtypes, strains of the currently circulating *H3N2 and H1N1 (Swine flu)* subtypes, and one *influenza B* virus. The current recommendation for *influenza* vaccination in the UK is to offer it to those over the age of 65, those with chronic heart, respiratory (including CF) or renal diseases and those who are diabetic or immunosuppressed.

Our group [34] recently showed that *influenza* vaccination provides protection against *influenza* acquisition in patients with CF, with 1 of 41 patients vaccinated having a positive nasal swab for influenza compared to 4 of the 22 non-vaccinated patients (p=0.046). Although *influenza* vaccination does not appear to have any impact on respiratory exacerbation rates, it does have a role in preventing live infections. In our study, respiratory exacerbation rates in the preceding 10 months before the study between the vaccinated and non-vaccinated groups were similar, indicating that these were unlikely to be the reasons influencing the decision on immunisation. The decision may be secondary to a combination of patient/parent education, social background, awareness of vaccination and accessibility of vaccination.

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Due to the lack of randomised controlled studies looking at the efficacy of *influenza* vaccine in CF, the Cochrane review recommends clinicians to make their own judgements on the benefits and risks of this therapy in this cohort of patients [86]. In addition to vaccine, neuraminidase inhibitors have been shown to have a role in preventing *influenza A and B* infections [87].

Rhinovirus has more than 100 serotypes; therefore, it will be unlikely that a unifying vaccine can be developed. VP4, one of the non-enveloped capsids, is highly conserved among all of the rhinoviruses; anti-VP4 antibodies have recently been generated and been shown to have the potential for future vaccine development [88].

The development of an *RSV v*accine has been hampered by the experience with formalin-inactivated whole *RSV* vaccine in the 1960s, as it caused 80% of *RSV* vaccinees to become hospitalised compared with 5% of controls, as well as two fatalities [89]. Current major research work has focused on a prophylaxis using a humanised mouse monoclonal antibody, Palizivumab. In patients with CF, monthly Palizivumab injection significantly reduced the hospitalisation rate for acute respiratory illness during the *RSV* season compared to those who were not immunised ($p<0.05$). The former group also had fewer hospital days for acute respiratory illness [90]. However, the Cochrane database systematic reviews were not able to draw any firm conclusions on the safety and tolerability of RSV prophylaxis with Palivizumab in infants with cystic fibrosis up to 2 years of age due to a lack of randomised controlled studies [91]. Further studies are required to evaluate the safety and effectiveness of this treatment in CF patients.

There is currently no licensed *PIV* vaccine. The formalin-inactivated vaccine generated in the 1960s was not able to prevent *PIV* infection and was soon abandoned. Recently, recombinant bovine *PIV type 3* and human *PIV type 3* attenuated vaccines are being evaluated in animal models as vectors for the delivery of other viral antigens such as *RSV*-G and *RSV*-F proteins. This bivalent vaccine combination provides high level of resistance to challenges with *PIV type 3* and *RSV* in animal models [92].

The conventional methods of vaccination are via the intramuscular and subcutaneous routes. Mucosal immunisation has recently been introduced as it represents an attractive manner of delivering vaccines. It is fast, simple, non-invasive and can be carried out by unskilled individuals. The use of mucosal vaccination seems logical in that most of respiratory viral infections initially start at the mucosal sites and therefore induce local immunity. In the autumn/winter of 2014/15 the annual nasal spray flu vaccine (Fluenz Tetra) became available for children aged 2, 3 and 4 year as part of the UK NHS childhood vaccination programme. The nasal spray flu vaccine is also for children aged 2-18 years who are "at risk" from flu, such as children with long-term health conditions.

Amantadine has been the conventional anti-viral against *influenza*. However, it is strain-specific as it is only effective against *influenza A* and has common side effects such as insomnia, poor concentration and irritability. It is now largely being replaced by neuraminidase inhibitors such as Zanamivir and Oseltamivir, which are licensed for the treatment of *influenza A and*

B, including *avian flu H5N1* and *swine flu H1N1*. However, Amantadine still has a role in dealing with Oseltamivir-resistant H1N1 virus. In children and adults, early initiation of neuraminidase inhibitors within 48 hours of the onset of symptoms can reduce the duration of flulike symptoms by 0.5 to 2.5 days [93]. Early use of these medications can also reduce development of complications such as pneumonia [94]. The 2009 pandemic *H1N1* virus remains susceptible to neuraminidase inhibitors, and Oseltamivir has been used extensively for treatment related to this viral infection. Resistance to Oseltamivir has been reported with *H1N1* viral infection but this is mainly restricted to immunocompromised individuals [95]. Zanamivir has a poor oral bioavailability, and intranasal application has been shown to be effective in treating experimental *influenz*a infection with the reduction in symptoms caused, virus shedding and development of otitis media [96]. Intravenous use of Peramivir or Zanamivir could be life-saving in critically ill patients with *influenza* infection [97, 98]. However, currently the Cochrane database of systematic reviews does not recommend the routine use of neuraminidase inhibitors in influenza infection in CF because of the absence of high level evidence for the effectiveness of these interventions [99].

Ribavarin, a synthetic guanosine nucleoside that has a broad spectrum of anti-viral activity, has been used for treatment of infections related to *RSV*, *metapneumovirus, and parainfluenza and influenza viruses* [100]. Potential benefits of ribavarin therapy include the inhibition of RSV-specific IgE production in nasal secretions, which has been associated with the development of hypoxaemia and wheezing [101] and it has improved pulmonary functions [102]. Controlled studies also show that the use of ribavarin is effective in reducing the clinical severity score, duration of mechanical ventilation, supplemental oxygen use and days of hospitalisation [103]. Aerosolised ribavarin has been used for the treatment of *RSV*-related bronchiolitis and pneumonia. Intravenous formulation could be used for treatment of severe pneumonia, caused by infection *RSV*, *metapneumovirus*, *or parainfluenza virus*, on the basis of experience in immunocompromised patients [104]. Bonney et al. have shown that *metapneumovirus* can be successfully treated with a combination of intravenous ribavarin and immunoglobulin [105].

Although *rhinovirus* is the major cause of colds, its vast amount of serotypes has made development of anti-virals against it problematic. A 90% of *rhinovirus* serotypes gain entry into epithelial cells using ICAM-1 cellular receptors, and blockade of these receptors in experimental studies has shown reduced infection severity [106], but further study is required before this treatment option becomes widely available. Macrolide antibiotics, Bafilomycin A1 and Erythromycin have been shown to inhibit ICAM-1 epithelial expression and hypotheses about their potential as anti-inflammatory agents have yet to be definitive, as clinical proof is either negative or inconclusive [107].

Recently, an anti-rhinoviral agent known as Plecoranil, which acts by inhibiting the uncoating of Picornaviruses [108], the RV 3C protease inhibitor, Ruprintrivir[109] and soluble ICAM-1, Tremacamra[106] have shown promising results in early-stage clinical trials, but each of these medications was derailed by a combination of cost, pharmacokinetics, toxicity, drug interactions, and limited efficacy [110].

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A previous study suggests that the increased morbidity in CF patients after virus infection is not due to an exaggerated inflammatory response of the airway epithelium but rather linked to increased cell death. Thus, they provide a rationale for implementing therapies aimed at controlling viruses and their replication rather than primarily targeting inflammation. In this respect, a promising candidate is the macrolide-antibiotic azithromycin, which is increasingly used in CF patients as a beneficial immunomodulatory agent [111] and has recently been shown to possess anti-viral properties [57].

6. Conclusion

As we become increasingly knowledgeable about the impact of respiratory virus infections in the context of CF exacerbations, screening for respiratory viruses should be part of the routine investigations for any CF patients that present with exacerbation symptoms. Using the appropriate sampling method in conjunction with sensitive and specific diagnostic technology will enable us to make appropriate clinical decisions surrounding the use of anti-virals and antibiotics.

Gaining further understanding in the pathogenesis of virus-induced respiratory exacerbations in CF may allow the development of new therapeutic techniques. If viral infection does predispose to bacterial infection, then influencing the interaction between viruses and bacteria could be a next pathway to diminish respiratory morbidity in patients with CF. The development of novel therapies will be exciting and this may improve their quality of life and prolong the lifespan of patients with CF.

However, there are still a number of research dilemmas that remain unanswered:

1. What are the standardised definitions of CF pulmonary exacerbation and pulmonary exacerbation severity score?
2. What is the optimal way for viral sampling?
3. What is the role of Virochip in routine viral identification?
4. How do respiratory viruses influence bacterial activities in chronically infected airways?
5. What influences the rate of respiratory viral clearance in CF respiratory tract?
6. What are the roles of anti-virals in CF?
7. What are the anti-viral properties of Azithromycin in CF?

Further understanding in the pathogenesis of viral infection in CF would be beneficial as this may provide insight to the above unresolved mysteries. At the moment, *influenza* vaccination and the use of neuraminidase inhibitors remain the only evidence based practice, albeit weak for the management of viral infections in CF.

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