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The role of respiratory viruses in cystic fibrosis

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Abstract

Background: Previous studies have suggested a role played by respiratory viruses in the exacerbation of cystic fibrosis (CF). However, the impact of respiratory viruses could have been underestimated because of the low detection rate by conventional laboratory methods.
Methods: Children with CF had nasal swabs and sputum samples obtained on a routine basis and when they developed respiratory exacerbations. Nucleic Acid Sequence Based Amplification (NASBA) was used to detect respiratory viruses from nasal swabs. The definition of a respiratory exacerbation was when the symptom score totalled to 4 or more, or if the peak expiratory flow fell by more than 50 l/min from the child's usual best value, or if the parent subjectively felt that the child was developing a cold.
Results: 71 patients had 165 reported episodes of respiratory exacerbations. 138 exacerbation samples were obtained of which 63 (46%) were positive for respiratory viruses. In contrast, 23 of 136 asymptomatic nasal swabs (16.9%) were positive for respiratory viruses. There was significantly more viruses being detected during respiratory exacerbations, in particular influenza A, influenza B and rhinovirus ($p<0.05$).

Upper respiratory symptoms significantly correlated with positive respiratory viral detection ($p<0.05$). This study also showed that viral respiratory exacerbations in CF could be independent from bacterial infections.
Conclusions: Respiratory viruses are associated with exacerbations in CF and upper respiratory symptoms are strong predictors for their presence. 'Real-time' NASBA has a rapid turn-around time and has the potential to aid clinical decision making, such as the use of anti-virals and administration of antibiotics.

Keywords: Influenza vaccination; Cystic fibrosis; NASBA

1. Introduction

Life expectancy for Cystic Fibrosis (CF) patients has increased dramatically over the last 40 years [1]. However, progressive pulmonary damage with eventual respiratory failure is still the major cause of morbidity and mortality in CF [2] and bacterial infection is generally thought to be the major cause of clinical deterioration [3]. Recently, evidence from literature shows that respiratory viruses lead to pulmonary exacerbation and disease progression [4–7] and an increase in bacterial adherence to the CF airways [8,9] from in vitro studies. This results in prolonged hospitalizations [10] of CF patients infected by viruses but their true impact on CF may yet have been underestimated by previous studies for a number of reasons: Firstly, respiratory viruses may trigger bacterial infections [11–13] and so the presence of bacteria does not necessarily exclude viral infections. Secondly, many previous studies used tissue culture and immunofluorescence techniques which are relatively

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Table 1
Sequences for primers and molecular beacons

Virus	Primer identification nuceotide no.	Sequence 5′→ 3′	Function
HPIV1	P1: 55	CGATGGCTGAAAAAGGGA	RT reverse primer
	P1: 139	CACCAGCAGGAAGGACACA	RT-PCR reverse primer
	P1: 857	GGCAAGGAGCATAACTGATAA	RT-PCR forward primer
	P1: 549	***AATTCTAATACGACTCACTATAGGGAGAAGG***GAACCCCTACTGAGCAACAAC	NASBA P1 primer T7 RNA polymerase tail
	P1: 801	***GATGCAAGGTCGCATATGAG***CCTGTTGTCGTTGATGTCATA	NASBA P2 primer ECL detection tail
	PR1: 669	CTTCCCTATATCTGCACATCC	HPIV1 capture probe
	HPIV1MB	***CCATGCGC***TTCCCTATATCTGCACATCC***CGCATGGT***	HPIV1 molecular beacon
HPIV2	P2: 309	CACAGCAAGGCATTATTCA	RT reverse primer
	P2: 738	CAATGGGGATAATACAACAAT	RT-PCR reverse primer
	P2: 1371	ATGCAGACCACCAAGAGG	RT-PCR forward primer
	P2: 848	***AATTCTAATACGACTCACTATAGGGAGAAGG***CCAGGAGGTTGTGTCTTGTAT	NASBA P1 primer T7 RNA polymerase tail
	P2: 1058	***GATGCAAGGTCGCATATGAG***GAGACCACCATATACAGGAAA	NASBA P2 primer ECL detection tail
	PR2: 979	CCCTGTTGTATTTGGAAGAGA	HPIV2 capture probe
	HPIV2 MB	***CCAAGC***CCCTGTTGTATTTGGAAGAGA***GCTTGG***	HPIV2 molecular beacon
HPIV3	P3: 770	ATAACTGTAAACTCAGACTTGGT	RT reverse primer
	P3: 896	ACTCCCAAAGTTGATGAAAGA	RT-PCR reverse primer
	P3: 1548	GACAGATGACACAATGCTCC	RT-PCR forward ptimer
	P3: 1052	***AATTCTAATACGACTCACTATAGGGAGAAGG***GGACCAGGGATATACTAYAAA	NASBA P1 primer T7 RNA polymerase tail
	P3: 1201	***GATGCAAGGTCGCATATGAG***TTGACCATCCTYCTRTCTGAA	NASBA P2 primer ECL detection tail
	PR3: 1129	CACCCAGTTGTRTTGCAGATT	HPIV3 capture probe
	HPIV3MB	***CCATGCGC***ACCCAGTTGTRTTGCAGATT***CGCATGGT***	HPIV3 molecular beacon
HPIV4A and HPIV4B	P4: 359	GCTTATGGGATCAGACACACA	RT reverse primer
	P4: 531	GAAAGAGGCTTGGGTTACACA	RT-PCR reverse primer
	P4: 1147	GCTCTTATCACAGTCTCCAAA	RT-PCR forward primer
	P4: 910	***AATTCTAATACGACTCACTATAGGGAGAAGG***CCTGGAGTCCCATCAAAAGTA	NASBA P1 primer T7 RNA polymerase tail
	P4: 1088	***GATGCAAGGTCGCATATGAG***CATCTATACGAACACCTGCTC	NASBA P2 primer ECL detection tail
	PR4: 1045	GGTTCCAGAYAAWATGGGTCT	HPIV4 capture probe
	HPIV4 MB	***CCAAGC***GGTTCCAGAYAAWATGGGTCT***GCTTGG***	HPIV4 molecular beacon
229E	229E : 1949	TAAGGCGTCTTCAATAGT	RT reverse primer
	229E : 1872	CATAAGTGGAGCAATCAA	RT-PCR reverse primer
	229E : 1281	TTGTATGTTTCTTGGAGT	RT-PCR forward primer
	229E : 1658	***AATTCTAATACGACTCACTATAGGGAGAAGG***AGTGCCATTGGACGCATAGAA	NASBA P1 primer T7 RNA polymerase tail
	229E : 1391	***GATGCAAGGTCGCATATGAG***TTATGATGTATCTGGTGTGGG	NASBA P2 primer ECL detection tail
	PR229E	ACCATCTACTCTATCACTCCT	229E capture probe
	229E MB	***CCATGC***ACCATCTACTCTATCACTCCT***GCATGGT***	229E molecular beacon
RSV A and B	RSV RT	GATCAAAAGTGCTCTACTAT	RT reverse primer
	RSV RT+	GATCAAAAGTGCTCTACTAT	RT-PCR reverse primer
	RSV RT-	AGTTTTGCCATAGCATGACA	RT-PCR forward primer
	RSV T7 A+B	***AATTCTAATACGACTCACTATAGGG***GAYAGAGGATGGTAYTGTGA	NASBA P1 primer T7 RNA polymerase tail
	RSV ECL (A)	***GATGCAAGGTCGCATATGAG***CAATGGCTCCTAGAGATGTGA	NASBA P2 primer ECL detection tail
	RSV ECL (B)	***GATGCAAGGTCGCATATGAG***CTATAGCTCCAAGAGAAGTAA	NASBA P2 primer ECL detection tail
	RSV A+B BIO	CDGAGCTGCTTAYRTCTGTTT	RSV A and B capture probe
	RSV MB A+B	***CCATGC***CDGAGCTGCTTAYRTCTGTTT***GCATGG***	RSVA and B molecular beacon
Influenza A	Flu A RT	AGCAGGGTAGATAATCACTC	RT reverse primer
	Flu A RT+	AGCAGGGTAGATAATCACTC	RT-PCR reverse primer
	Flu A RT−	TTGTGCHGCTGTTTGRAATT	RT-PCR forward primer
	Flu A T7	***AATTCTAATACGACTCACTATAGGG***AGCAGGGTAGATAATCACTC	NASBA P1 primer T7 RNA polymerase tail
	Flu A ECL	***GATGCAAGGTCGCATATGAG***ATYTCRKTDGCATTCTGGCG	NASBA P2 primer ECL detection tail
	Flu A Bio	TAAGAYCGTTTGGTGCCTTG	Influenza A capture probe
	Flu A MB	***CCAAGC***TAAGAYCGTTTGGTGCCTTG***GCTTGG***	Influenza A molecular beacon

(continued on next page)

Table 1 (*continued*)

Virus	Primer identification nuceotide no.	Sequence 5′→ 3′	Function
Influenza B	Flu B RT pol	ACACAATGGCAGAATTTAGTG	RT reverse primer
	Flu B RT+	ATCCTGAAYTACARCCAGCA	RT-PCR reverse primer
	Flu B RT−	AGGTCCYCCCATTTCAACTT	RT-PCR forward primer
	Flu B pol T7	***AATTCTAATACGACTCACTATAGGGAGAAGG***CTATTCAACATCTGCGTCCATC	NASBA P1 primer T7 RNA polymerase tail
	Flu B pol ECL	***GATGCAAGGTCGCATATGAG***ATYACTTCATAYTGTGGTCTCA	NASBA P2 primer ECL detection tail
	Flu B pol Bio	CCTTGTCCTTCTAATGCTGTAT	Influenza B capture probe
	Flu B pol MB	***CCAAGC***CCTTGTCCTTCTAATGCTGTATA***GCTTGG***	Influenza B molecular beacon
HRV	HRVJ P1	AATTCTAATACGACTCACTATAGGGAGACCAMYWTTYTGYSTWGAWAC	NASBA P1 primer T7 RNA polymerase tail
	HRVJ P2	GATGCAAGGTCGCATATGAGCTCCGGCCCCTGAATGYGGCT	NASBA P2 primer ECL detection tail
	HRVJ Pro	GAYGGGACCRACTACTTTGG	HRV capture probe
	HRVJMB-FAM	***CCAAGC***GAYGGGACCRACTACTTTGG***GCTTGG***	HRV molecular beacon FAM
	HRV X PR1	CGCGCAAGTCCGTGGCGGAA	HRV cross primer 1
	HRV X PR2	TGGGYAACTCTGCAGCGGAA	HRV cross primer 2
	HRV X PR3	CGGGCAACTCTGCAGCGGAA	HRV cross primer 3

insensitive methods for viral detection, but these were the techniques used in most published studies in CF [6,7,11,14]. Thirdly, few studies have used sensitive methods such as molecular based technique for viral detection [12,15–17]. Finally, many previous studies were poorly designed with a small study sample and used invalidated criteria to define pulmonary exacerbations, therefore providing biased results.

Identifying respiratory viruses in CF is important in clinical decision making and is potentially important as new anti-virals are becoming readily available [18].

1.1. Aims

1. To investigate the incidence of respiratory viruses in a CF paediatric population during pulmonary exacerbations and routine (asymptomatic) settings.
2. To investigate if upper respiratory tract symptoms are associated with viral infections.
3. To investigate if bacterial infection is associated with viral infection.

2. Materials and methods

2.1. Patients

This was a prospective study conducted for 17 months between December 2002 and May 2004 involving patients with CF below the age of 18 years from four CF centres, namely University Hospital of Wales in Cardiff, Royal Gwent Hospital in Newport, and Singleton Hospital in Swansea and Neville Hall Hospital in Abergavenney.

2.2. Sample collection

Nasal swabs were obtained from the patients whenever they developed symptoms suggestive of respiratory exacerbations and whenever they attended for routine outpatient assessment on a 2 monthly basis. Each nasal swab was obtained by inserting a sterile cotton swab into one of the nostrils to a depth of 2 to 3 cm. The swab was then subjected into 0.5 ml of guanidium thiocyanate lysis buffer. It was transported with ice packs to the laboratory and then stored at −80 °C until undergoing nucleic acid extraction on a later date. A paired sputum/cough swab sample was also obtained at the same time for bacteriology and was analysed by the National Public Health Service (NPHS) of Wales.

2.3. Virus identification

Respiratory virus nucleic acid materials were extracted from the nasal swabs using silica slurry as described by Boom et al. [19]. Extracted materials were amplified at 'real-time' using Nucleic Acid Sequence Based Amplification (NASBA) in conjunction with molecular beacons [20–23]. The panel of respiratory viruses studied included influenza A, influenza B, respiratory syncytial virus (RSV), parainfluenza viruses (PIV) types 1 to 4, rhinovirus and coronavirus. The sequences of primers and molecular beacons used in 'real-time' NASBA assays are shown in Table 1. Analysis of results was undertaken using the NucliSens® Easy Q Analyser (BioMérieux Ltd) isothermically at 41 °C and results were available within 120 min. The NASBA assays have been found to be highly sensitive with the Tissue Culture Infective Dose being between 1 to 1 in 10^{-5} virus input ($TCID_{50}$) and 100 to 0.1 copies of synthetic RNA. The assays are also specific with no background signal detected with a cross-reacting panel including a range of respiratory viruses and a lysis buffer negative control. The cut-off threshold for a positive result was defined as 20% above the negative control wild-type signal [22].

2.4. Bacterial isolation

Bacterial isolation in this study was performed by the NPHS of Wales and the methods were based on the 'Antibiotic treatment for CF' published in the CF Trust Report in 2002 (www.cftrust.org.uk) and the standard operating procedures (BSOP 57) from the 'National Standard Procedure on the Investigation of Bronchoalveolar Lavage, Sputum and Associated Specimens' issued by the Standards Unit, Evaluations

and Standards Laboratory Specialist and Reference Microbiology Division (www.hpa.org.uk).

2.5. Definition of an exacerbation

In this study, each patient (and parents) had been provided with a symptom diary card which comprised of upper and lower respiratory symptoms including runny nose, blocked nose, sore throat, hoarse voice, fever/shivering, cough (daytime and night-time), wheeze (daytime and night-time), shortness of breath and school absenteeism [24]. Patients and parents had been asked to score their symptoms using the diary card regularly. The symptoms were scored as 0 for no symptoms, 1 for mild symptoms, 2 for moderate symptoms and 3 for severe symptoms for each criterion. Each patient above the age of 5 years was also provided with a mini-Wright Peak Flow Meter and was asked to record their best of three readings every morning and evening. The definition of a respiratory exacerbation was when the symptom score totalled to 4 or more, or if peak expiratory flow fell by more than 50 l/min from the child's usual best value, or if the parent subjectively felt that the child was developing a cold [25]. Under these circumstances, the parents or patients were encouraged to contact the investigators to have a nasal swab taken.

Patients and parents were reminded regularly throughout the study by telephone and by letters to contact the investigators in the event of a respiratory exacerbation and to record their peak expiratory flows and symptom scores.

2.6. Ethics

The study protocol was approved by the Committee of Ethics of each individual hospital and research funding was granted by the Welsh Office of Research and Development (WORD). Informed consent was obtained from at least one parent at the beginning of the study and children and parents information sheets were also provided for clarity.

2.7. Statistics

The statistical analysis of this study was performed using the software package GraphPad InStat Version 3.0 for Windows (GraphPad software, San Diego, CA) and the author, FC. Chi-square test and Fisher's exact test were used to compare the incidence of respiratory viral infections between routine and exacerbation samples. Fisher's exact test was also used to compare the reasons used to define respiratory exacerbations between the virus positive and virus negative groups. Chi-square test was again used to study the correlation between respiratory viruses and bacteria. P-value of <0.05 was taken to be statistically significant.

3. Results

3.1. Patient demographics

There were 151 patients with CF under the care of these four centres who were all invited to participate in the study. Of these 151 patients, 71 (and parents) kindly agreed to enrol. There was a male predominance, in total, 53 boys (74.6%) and 18 girls (25.4%) participated with a median age of 9 years. 46% of the study population were homozygous for delta F508 and 49% were heterozygotes for this genetic mutation. 69% had an FEV_1 predicted of greater than 80%. 21% and 10% of the study population were colonised with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively (Table 2). The demographics of those who did not participate in this study ($n=71$) were similar to those who joined the study.

Table 2
Demographics of patients who enrolled in the study and those who declined to join

	Patients enrolled in study ($n=71$)	Patients who declined to join the study ($n=80$)
Median age in years (range)	9 (0–18)	7 (0–18)
M:F ratio	2.9:1	1.2:1
% of F508 homozygous**	46	50
% of F508 heterozygotes***	49	41
% of $FEV_1>80\%$	69[#]	70[##]
% of *Pseudomonas* colonization	21	18
% of Staph colonisation	10	15

**denotes percentage of delta F508 homozygous in Caucasian population only.
***denotes percentage of delta F508 heterozygote in Caucasian population only.
#51 patients out of 71 in the SNOT population were greater than 5 years of age who were able to perform lung function.
40 out of 80 patients who declined to join the study were greater than 5 years of age who were able to perform lung function test.

3.2. Prevalence of respiratory viruses

There were 165 reported cases of respiratory exacerbations from 71 volunteers over the 17 month study period. The maximum number of reports was 8 from one child. 138 samples were obtained from 165 reported exacerbation cases. The discrepancies in numbers arose when 4 volunteers refused nasal swabs to be taken; 4 were not at home to have samples collected and 2 were asleep at the point of sample collection. 17 samples were missed. The median number of samples taken from each patient was 2 with a range between 0 and 8. Respiratory viruses were detected by 'real-time' NASBA in 63 of 138 cases (46%); in six cases (4.3%) two viruses were detected, and in three cases (2.2%) three were detected (Table 3). Influenza A, influenza B, PIV 4 and rhinovirus were the major viruses detected.

272 routine samples were collected from the patients. The median number of routine sample collected from each patient was 4 with a range between 2 and 6. Alternative routine sample was analysed in this study due to the financial constraint. There were 23 (16.9%) samples positive for respiratory viruses from 136 routine collections (Table 3). PIV 4 and rhinovirus were the major viruses detected.

There were significantly more respiratory viruses detected from exacerbation samples than from routine samples (p-value $=0.001$). In particular, there were significantly more

Table 3
Respiratory viral detection in different sample types

Virus	Number detected (%)		*p*-value*	Odds ratio
	Routine, *n*=136	Exacerbation, *n*=138		
FLU A	2 (1.5%)	11 (8.0%)	**0.011**	0.1723
RSV	3 (2.2%)	4 (2.9%)	1.000	0.7556
PIV 1	1 (0.7%)	2 (1.4%)	1.000	0.5037
PIV 2	2 (1.5%)	1 (0.7%)	0.621	2.015
PIV 3	0 (0.0%)	1 (0.7%)	1.000	0.3358
PIV 4	5 (3.7%)	11 (8.0%)	0.130	0.4407
FLU B	0 (0.0%)	10 (7.2%)	**0.002**	0.045
Rhinovirus	10 (7.4%)	22 (15.9%)	**0.027**	0.4185
Coronavirus	2 (1.5%)	1 (0.7%)	0.621	2.015
Any	24 (17.6%)	50 (36.2%)	**0.001**	
Total	25	63		
Collected	136	138		
Detection rate	18.3%	46%		

("Any" in the table refers to the number of samples that contained one or more virus of any type.) **p*-values are for Chi-squared tests in the case of FLU A, PIV4, RHINO and ANY and Fisher's exact test for all other variables, since expected counts were low.

influenza A ($p=0.011$), influenza B ($p=0.002$) and rhinoviruses ($p=0.0027$) detectable from exacerbation samples (Table 3) Influenza A was detected in eleven exacerbation samples and only on two occasions from routine samples ($p=0.011$). Similarly, influenza B was detectable from eleven exacerbation samples compared to none from routine samples ($p=0.002$). Rhinoviruses were also more commonly found in exacerbation samples ($p=0.027$). PIV 4 was isolated from nasal specimens on 16 occasions, with 11 from exacerbation and 5 from routine samples. The difference between the two sample types was not significant ($p=0.13$).

3.3. The association of upper respiratory tract symptoms and viral infections

Of the 138 episodes of respiratory exacerbations, the summated symptom score was used on 92 occasions (67%) to define respiratory exacerbations; peak flow fall by greater than 50 l/min from usual best was used on 25 occasions (18%) and subjective evaluation was used on 21 occasions (15%) to define the other episodes of respiratory exacerbations.

When the exacerbation nasal swab samples were divided into virus positive group (where at least one virus was identified from a nasal swab, [$n=50$, 36%])and virus negative group ([$n=88$, 64%], where no virus was identified from a nasal swab), there were significantly more patients in the virus positive group using the summated symptom score to define exacerbations; 39 of 50 episodes (78%) of exacerbations in the virus positive group in comparison to 53 of 88 episodes (60%) in the virus negative group ($p=0.04$). The mean symptom score during respiratory exacerbations in the virus positive group was 7 compared to that of 6 for the virus negative group, though the difference was not significant. There was no difference in the proportion of patients using peak expiratory flow reduction or using subjective evaluation to define an exacerbation between the two groups.

There was a significantly higher proportion of patients in the virus positive group complaining of upper respiratory tract symptoms such as a runny nose (77% versus 38%, p-value=0.0003) and sore throat (62% versus 38%, p-value=0.03) during exacerbations. The frequencies in lower respiratory tract complaints were similar between the two groups.

A subgroup analysis was performed comparing the samples that were positive for either influenza A, influenza B or both and the group that was positive for other viral infections. Of the 50 virus positive exacerbation samples (accounting for 63 respiratory viruses detected), 18 (36%) were positive for influenza A, B or both. 4 of the 18 samples were excluded from analysis because they also had other respiratory viruses identified in addition to influenza virus. One of the 14 remaining samples contained both influenza A and B viruses. 32 samples (64%) were positive for other respiratory viruses only. The reasons used to report respiratory exacerbations were similar between the influenza and other viral groups. Summated symptom score was the major reason used to define respiratory exacerbations in both groups (79% in the influenza group and 81% in the other viral group). However, there were significantly more upper respiratory symptom complaints including runny nose (p-value=0.02), blocked nose (p-value=0.01), sore throat (p-value=0.01), hoarse voice (p-value=0.0002) and fever/shivery episodes (p-value=0.0002) in the influenza group compare to the other viral group. There was no difference in the lower respiratory symptom complaints between the groups.

3.4. Relationship between bacteria and respiratory viruses

There were 274 paired sputum samples/cough swabs analysed for bacterial isolation (138 exacerbation and 136 routine), consisting of 84 (31%) cough swabs and 190 (69%) sputum samples. 69 of 274 specimens (25%) were positive for bacteria accounting for a total of 86 isolates. 8 of 84 (10%) cough swab specimens were positive and 61 of 190 sputum samples (32%) were positive. *P. aeruginosa* and *S. aureus* were the major bacteria isolated accounting for 54% and 23% of all bacteria identified, respectively.

Of the 74 nasal swabs (exacerbation and routine samples) that were positive for at least one respiratory virus, 19 (26%) of the paired sputum/cough swab samples were positive for bacteria. Similarly, there were 200 nasal swab samples with no viruses identified, 50 (25%) of them were positive for bacteria. Hence there was no statistical difference between the viral and non-viral groups in terms of bacteria isolation ($p=0.909$). There was also no difference in *Pseudomonas* isolation between the viral and non-viral groups.

When nasal swab samples were considered in terms of routine/asymptomatic ($n=136$) and exacerbation ($n=138$) collections, once again no difference was found between the two groups in terms of bacterial infection status ($p=0.728$). Bacteria were found in 33 (24.3%) routine samples and 36 (26.1%) exacerbation samples.

4. Discussion

This is a novel study using 'real-time' NASBA to examine the role of respiratory viruses in CF. It has achieved the highest detection rate of 46% amongst all existing literature concerning respiratory viruses in the CF population during reported episodes of respiratory illness. Our results compare favourably with previous studies and this may be that earlier studies relied heavily on repeated serological testing, either alone [11] or in combination with viral isolation [4,6,7,12,26]. These traditional methods are relatively insensitive and may have underestimated the prevalence of viruses in CF.

We have also achieved a viral detection rate of 18.3% from routine nasal samples and this is comparable to the seroconversion rate of 12.3% as reported by Wang et al. [7]. This value is also similar to the seroconversion rate of 16.2% from asymptomatic samples achieved by Ramsey et al. [6]. Amongst stable asthmatic children, Johnston et al. [25] found a viral detection rate of 12% by PCR. Therefore, a laboratory method with a higher sensitivity for viral detection used in this study has not increased the detection rate in asymptomatic samples, implying that the high detection rate in exacerbation samples is unlikely to be due to false positive results.

PIV 4 has been reported as a cause of severe respiratory infections in previously healthy children and can lead to an increased risk of hospital admission [27,28] To our knowledge, this is the first study showing that PIV4 can be detectable in CF. The lack of data regarding PIV4 in CF may be that it was not actively sought in previous studies and may also be due to the lack of knowledge of this virus. This study shows that PIV4 can be isolated in CF and could lead to pulmonary exacerbation but further studies will be required to fully define the exact role of PIV 4 in CF exacerbations.

This study demonstrates that influenza A and B viruses are 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 are influenza viruses (A and B). This figure is consistent with majority of the previous studies which showed that influenza virus represented between 12 to 27% of all viruses detected. In relation to influenza vaccination, the uptake rate was up to 70% during the 2003/4 season [24] (but we did not have the data for our study population during 2002/2003) and the significance is that the influenza detection rate in our study could easily have been higher had the vaccination uptake not been this high.

Previous studies have shown that RSV were also important viruses in CF exacerbations [4,10] and represented 9 to 58% of all viruses in CF with the highest incidence in young children. In our study, only 7 of 88 (8%) reported viruses were RSV and this low detection rate may be due to nasal swabs used for sample collection having a 20 to 30% lower detection rate than nasopharyngeal aspirate for RSV [29]. RSV is a relatively labile virus and the amount of virus in a small-volume nasal swab specimen may be a lot less than that in a sample obtained by aspiration. In addition, RSV more commonly infects the lower airways and sampling from the upper airways by nasal swabs could have underestimated its true prevalence [30].

The 2002/03 season [31] between October 2002 and May 2003, was a high season for RSV but our study started in December 2003 and could have potentially missed 2 months' worth of valuable specimens. Lastly, 90% of the RSV infections reported were amongst children between the ages of 0 to 4 years [31] so the median age of 9 years in the study population may account for the low RSV detection rate.

However, following a review of the case histories of the volunteers, there were 53 occasions where respiratory exacerbations and diminished peak flows that were not reported. Therefore, the total number of cases of respiratory exacerbation was 218 over 17 months, giving an exacerbation rate per patient per year of 2.05 and this was comparable to that reported by previous studies [5–7,12,13,16,26,32,33].

It has been more than 20 years since Wang et al. [7] described the relationship between respiratory viral infections and the deterioration in clinical status in CF. Viruses were identified through repeated serology and nasal lavage for viral isolation in 49 patients with CF (mean age 13.7 years) over 2 years. 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). All their viruses were identified by seroconversions; none were detected by viral cultures. From a total of 1028 (689 acute episodes versus 339 asymptomatic episodes) serum samples, 105 infections (10.2%) were identified. 42 of the 105 infections were identified from asymptomatic periods. Therefore their viral detection rates were 9.1% during acute episodes and 12.3% during asymptomatic episodes, compared to our detection rates of 46% and 18.4%, respectively. Our study also show that rhinoviruses played a major role in exacerbations of CF but in contrast, Wang et al. [7] failed to detect any rhinoviruses. The authors concluded that the high serotypes of rhinoviruses making detection by serology and cultures impossible.

Ramsey et al. [6] prospectively compared the incidence and effect of viral infections on pulmonary function and clinical scores in 15 schoolchildren with CF aged 5–22 years and their unaffected siblings. Over a 2-year period, oropharyngeal cultures and serological tests were taken at regular two monthly intervals and during acute exacerbations for respiratory viruses. A total of 68 acute respiratory illness (ARI) episodes occurred in the patients with CF and in 19 episodes there was an associated virus identified. This gave a viral detection rate of 27.9% during CF exacerbations and a rate of 27.8% when asymptomatic. They were unable to demonstrate any significant adverse effect of viral infections on lung functions in patients with CF. However, they found that patients with CF had more frequent viral infections and infections were associated with a significantly slower rate of clinical decline. The authors also suggested that viral infections may possibly protect against Pseudomonas acquisition.

Rhinovirus has been found to be a major virus in CF exacerbation in this study. To date only 3 studies have prospectively used molecular based methods to identify viral agents [5,12,15,16]. Over a 1-year period, Smyth et al. [15] prospectively investigated 108 patients with CF (mean age of 7.9 years) using a combination of viral culture and immunofluoresence, seroconversion (fourfold

increase in titres) and a PCR based method for rhinoviruses. During the study, 76 subjects had 157 reported respiratory exacerbations (1.5 episodes/patient/year compared to 2.05 in our study), and a viral agent was identified in 44 episodes (28%) (with rhinovirus in 25 [15.9%]), and an equal distribution of other viruses identified almost always on seroconversion. They also found that those children in whom a non-rhinovirus was identified had a significantly greater decline in FEV_1, whereas patients with rhinovirus infection had fewer declines.

Collinson et al. [5] followed 48 children with CF over a 15 month period using a combination of viral culture and PCR for picornaviruses alone. Thirty eight children completed the study and there were 147 symptomatic upper respiratory infections (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. This study confirmed the importance of this virus in CF exacerbation similar to our findings (15.9%) from exacerbation episodes.

There are significantly more patients in the virus positive group using the summated symptom score to define respiratory exacerbations compared to the virus negative group, possibly because the virus positive group had a higher perception and awareness of increased respiratory symptoms. This suggests that patients with more upper respiratory symptoms were more likely to have a virus isolated from nasal swabs.

The difference in symptomology is even more striking when the patients were divided into subgroups of influenza positives and other viral positives. The influenza subgroup had significantly more upper respiratory tract symptoms during respiratory exacerbations compared to the other virus group, but there was no difference in terms of lower respiratory complaints. This is an important observation as this confirmed that influenza is more likely to infect the upper airways. In addition, we have recently shown that influenza vaccination does offer protection against its subsequent acquisition in patients with CF [24] contradictory to a recent Cochrane review [34]. The recent development of intranasal vaccination may be an effective and attractive option as this will offer local protection in the upper airways, which can help arresting infection at an early phase before symptom complications arise.

The data available for the optimal method for respiratory viral detection is limited. The best sites to collect material for viral detection may differ for different viruses, such as using bronchoalveolar lavage (BAL) to obtain lower airways specimens for RSV [30] and nasal swabs for upper airways sampling for rhinovirus and influenza virus [29]. The collection of a nasal swab is simple, convenient, relatively painless, does not require any additional devices such as an electrical suction device for obtaining nasopharyngeal aspirates (NPA) or any specialised skill. Thus far there is no data comparing the sensitivities and specificities of different sampling techniques incorporating molecular detection methods for viral detection.

In future studies, it may be worth obtaining specimens from different respiratory sites using different sampling methods simultaneously as this may increase the diagnostic yield [35,36] and further establish the impact of respiratory virus in CF.

The bacterial isolation rate in this study was low at 25% (69 positive samples from 274), compared to a study by Olesen et al. [17] who achieved a bacterial detection rate of 76%. This may be that 30% of the samples obtained for bacterial isolation in this study were cough swabs whereas Olesen et al. used a combination of laryngeal aspirate (20%) and sputum sample (80%) for isolation, which tended to have a higher isolation rate. In the Brompton study [37], cough swabs had a specificity of 100% compared to sputum samples for bacteria isolation but a sensitivity of only 34%. Another study conducted by Maiya et al. [38] showed a similar sensitivity of 35% with cough swabs compared to sputum samples and cough plates.

Bronchoalveolar lavage or laryngeal aspirate may be superior [39,40], but these procedures carry higher risks, costs and require more expertise and these considerations must be taken into account before utilising these procedures for routine screening and research purposes.

In this study, there is no change in incidence of bacterial infection during viral infections. Symptomatic viral infections did not increase the likelihood of bacterial infection. Therefore, the criteria used in this study to define a respiratory exacerbation are poor predictors of bacterial infection. The introduction of additional antibiotics in these situations may be difficult to decide. In addition, Ramsey et al. [6] have shown that CF patients colonised with *S. aureus* did not have an increased risk of viral infection and those colonised with *P. aeruginosa* were protected from viral infections. Hence, the high proportion of *S. aureus* and *P. aeruginosa* found in this study may part explain the lack of association between viruses and bacteria.

NASBA used in this study for viral identification offers several advantages. Firstly, it is developed specifically for RNA target amplification and is particularly suited for the detection of RNA viruses because there is no need for a separate reverse transcription step, unlike PCR. Secondly, the selection criteria for NASBA primers are less stringent, allowing easier primer design in selected less-conserved regions of the gene [41]. Thirdly, it is a continuous isothermic process that does not require a thermocycler [42,43]. Hence a constant temperature throughout the amplification reaction allows NASBA to amplify the targeted RNA or DNA exponentially at each step of the reaction. The NASBA reaction is also more efficient than PCR methods that are restricted to binary increases per cycle. Fourthly, background DNA does not interfere with the NASBA reaction, as single-stranded RNA sequences are specifically targeted, unlike PCR where background DNA may cause false positive results [44]. The closed-tube format of NASBA assay greatly reduces the risk of contamination and thus of false positive results. Finally, the detection of PCR products requires gel electrophoresis, which necessitates the transfer of resolved nucleic acid to an agarose gel. This is very labour intensive and time-consuming and is not as well suited for diagnostic use compares to 'real-time' NASBA.

We did not study the differences in hospitalisation rates between the viral and non-viral group as patients could have been admitted to hospital for non-respiratory reasons as this was not deemed as a robust end-point. Similarly, we did not study

the use of intravenous antibiotics between the viral and non-viral groups as one of the end-points of the study because it was felt that antibiotics were sometimes administered for reasons other than for pulmonary exacerbations, such as before holidays or school examinations in order to improve lung functions and respiratory reserve.

We did not have the assay identifying human metapneumovirus (hMPV), a virus that has recently been reported and it is closely related taxonomically to RSV [45]. This virus possibly accounts for about 10% of unexplained respiratory infections in children during the winter season. Garcia et al [46] has recently shown that this virus behaves similarly to RSV in CF in that it leads to an increased risk of hospitalisation and exacerbation.

5. Conclusions

Our results provide evidence to support respiratory viruses are commonly found during respiratory exacerbations of CF, particularly influenza A, influenza B and rhinovirus. PIV 4 has been detected for the first time in the CF population but its exact role will need to be further defined. However, there is little evidence to suggest that viral infections predispose to bacterial infections, contrary to previous reports.

As the role of respiratory viruses in CF becomes a more prominent entity , 'real-time' NASBA in viral detection may play a pivotal role in the future management of CF because of its rapid turn-around of results. It may provide guidance to clinicians regarding anti-viral administration, thus avoiding the inappropriate use of antibiotics and further support the importance of patient segregation. At the moment, NASBA largely remains as a research tool and for now, clinicians will have to use a matter of judgement and clinical accruement to diagnose viral infection as many currently available diagnostic tools still take days or weeks to provide definitive results. Hopefully, one day NASBA may become widely available to allow respiratory viral testing to become part of the routine investigation during pulmonary exacerbations of CF. In addition, NASBA may also become a very important screening tool for exacerbations in other respiratory illnesses such as chronic obstructive pulmonary disease, asthma and bronchiectasis.

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