Supplementary Appendix

The incidence and clinical impact of respiratory viruses in adults with cystic fibrosis

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METHODS

Respiratory Virus Polymerase Chain Reaction (PCR) Assays

The PCR assays used in this study were all established in routine clinical practice prior to the start of the study. All PCR assays have been validated in accordance with guidance published by the UK Health Protection Agency (now Public Health England).[1] Details of the primers and probes are given in Table S1. Each PCR run included negative and positive control specimens to exclude false negative results due to the presence of inhibitory compounds within clinical specimens. Positive controls were obtained from the National Institute for Biological Standards & Control (Potters Bar, UK). The PCR assays were performed in a series of duplex and triplex reactions in the following combinations:

- Respiratory Syncytial Virus (RSV) and Metapneumovirus
- Adenovirus and Rhinovirus
- Parainfluenza 1, 2 and 3
- Influenza A and B
- Influenza A Haemagglutinin and Neuraminidase lineage

The PCR assays for parainfluenza 1-3 and influenza B were developed in-house in accordance with the standards set out by the UK Health Protection Agency.[1] The PCR assays for adenovirus, influenza A, metapneumovirus, RSV and rhinovirus followed methods published by other research groups as follows:

- Adenovirus — Heim et al[2]
- Influenza A — WHO/CDC 2009 protocol[3]
- Metapneumovirus — Maertzdorf et al[4]
- Respiratory Syncytial Virus — van Elden et al[5]
- Rhinovirus — Sheltinga et al[6]

Rhinovirus Sub-Typing Methods

Sequencing of the P1-P2 region of the rhinovirus 5' untranslated region (UTR) was performed using a modification of the method described by Lee *et al.*[7] Total nucleic acids were extracted from respiratory specimens using the QIAamp® Virus Biorobot® MDx instrument (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Rhinovirus RNA was converted to cDNA and amplified using a two-step PCR assay containing the pan-rhinovirus P1 forward primer (CAAGCACTTCTGTYWCCCC) and the P3 reverse primer (ACGGACACCCAAAGTAG). Primers and PCR reagents were sourced from Life Technologies Ltd (Paisley, UK). The product of the first round PCR assay was amplified using a semi-nested PCR comprising the P1 forward primer and three reverse primers: P2-1 (TTAGCCACATTCAGGGGC), P2-2 (TTAGCCACATTCAGGAGCC) and P2-3 (TTAGCCGCATTCAGGGG). Electrophoresis using a 1.5% agarose gel was performed to confirm successful amplification of cDNA. The semi-nested PCR product was treated with exonuclease/shrimp alkaline phosphatase to remove excess primers and deoxyribonucleotide triphosphates.

Genetic sequencing of the 5'UTR region was performed using the Applied Biosystems 3130xl Genetic Analyzer (Life Technologies Ltd, Paisley, UK). The P1, P2-1, P2-2 and P2-3 primers were employed in the sequencing reaction. Raw sequencing data was edited manually using Sequencher v4.7 (Gene Codes Corporation, Michigan, USA) to correct mis-called bases. Individual DNA fragments were trimmed to 270 bp to match the length of the 5' UTR sequences of the reference rhinovirus strains reported by Lee *et al*.[7] The resulting sequences were compared with the National Center for Biotechnology Information (NCBI) GenBank database using the BLASTn interface. A local BLAST database was also created

using the sequences reported by Lee *et al* to allow a further means of identifying individual rhinovirus strains.[7] Phylogenetic analysis using the neighbour-joining method with 500 bootstraps was performed in ClustalW to assign individual sequences to the appropriate rhinovirus major group.[8] Phylogenetic trees were edited in MEGA v5.1.[9]

Additional Statistical Methods

A number of additional analyses are reported in this supplementary appendix. Firstly, generalised estimating equation (GEE) models as described in the main paper were used to assess the difference in clinical outcomes between episodes of rhinovirus A and B infection. The GEE models used logistic regression structures for binary variables and linear regression structures for continuous variables.

Receiver operating characteristic (ROC) curve analysis was used to determine the diagnostic utility of the upper respiratory tract infection (URTI) score. The area under the ROC curve was calculated as well as the sensitivity, specificity, positive- and negative predictive values of the URTI score at each cut-off.

No imputation of missing data was performed throughout the analysis of this study.

Table S1. Primers and probes for respiratory virus polymerase chain reaction assays.

	FORWARD	REVERSE	PROBE
Influenza A	GAGTCTTCTAACMGAGGTCGAAACGTA	GGGCACGGTGAGCGTRAA	FAM–TCCTGTCACCTCTGAC-MGB NFQ
Influenza B	AATGTTYCAAATATCAGACAAAAACAAA	CTGTGTCCCTCCCAAAGAAGAA	VIC-AATTAAGCAGACCATCCC-MGB
Rhinovirus	(1)GACARGGTGTGAAGAGCC (2)GACATGGTGTGAAGACYC	CAAAGTAGTYGGTCCCATCC	VIC-TCCTCCGGCCCCTGAATGYGGCTAA-TAMRA
Adenovirus	GACATGACTTTCGAGGTCGATCCCATGGA	CCGGCTGAGAAGGGTGTGCGCAGGTA	FAM-CACCGCGGCGTCAT-TAMRA
RSV-A	GTGCAGGGCAAGTGATGTTAC	CACCCAATTTTTGGGCATATTC	FAM-ACAACTTGTTCCATTTCTGC-MGB
RSV-B	TTCAGGGCAAGTAATGCTAAGATG	CCTCCCAACTTCTGTGCATACTC	
MPV ALT	CAACAACATAATGCTAGGACATGTATC	CCGAGAACAACACTAGCAAAGTTG	VIC-TGGTGCGAGAAATGGGTCCTGAATCTGG-TAMRA
MPV N	CATATAAGCATGCTATATTAAAAGAGTCTC	CCTATTTCTGCAGCATATTTGTAATCAG	VIC-TGYAATGATGAGGGTGTCACTGCGGTTG-TAMRA
Parainfluenza 1	ACAGATGAAATTTTCAAGTGCTACTTTAGT	GCCTCTTTTAATGCCATATTATCATTAGA	NED–ATGGTAATAAATCGACTCGCT-MGB
Parainfluenza 2	CTATGAAAACCATTTACCTAAGTGATGGA	CCTCCYGGTATRGCAGTGACTGAA	VIC-TCAATCGCAAAAGCT-MGB
Parainfluenza 3	ACAGTGGATCAGATTGGGTCAAT	ATGGTTGTGAGGTCATTTCTGCT	FAM-CGGTCTCAACAGAGCT-MGB

RSV: respiratory syncytial virus; MPV: metapneumovirus

SUPPLEMENTARY RESULTS

Dual Viral Infections

A total of nine dual viral infections were seen during the study. Details of these dual infections are given in Table S2 below. In five of these cases, the two different viruses were detected in a single specimen (i.e. sputum, nose- or throat-swab). In the remaining four cases, two different viruses were detected in two separate specimen types.

Table S2. Combinations of viruses seen in dual viral infection

Virus 1	Virus 2	Number of Episodes
Rhinovirus	Metapneumovirus	4
Rhinovirus	Adenovirus	1
Rhinovirus	Parainfluenza 3	1
Rhinovirus	Influenza A/H1N1	1
Influenza A/H1N1	Influenza B	1
Influenza A (unidentified)	Metapneumovirus	1

Table S3. Comparison of clinical outcomes between rhinovirus A and B infection using generalized estimating equation models

	Rhinovirus A	Rhinovirus B	Odds Ratio	95% CI	p
Number of visits	29	11	-	-	-
Pulmonary exacerbation n (%)	18 (62.1)	5 (45.5)	1.82	0.37 to 8.97	0.461
Any ABx; n (%)	24 (82.8)	6 (54.5)	4.44	0.27 to 73.4	0.298
IV ABx; n (%)	8 (27.6)	1 (9.1)	3.54	0.51 to 24.5	0.200
	Rhinovirus A	**Rhinovirus B**	**Adjusted Mean Difference**	**95% CI**	**p**
Percent fall in FEV_1 relative to baseline	13.8 (12.6)	15.8 (9.9)	-3.17	-9.8 to +3.5	0.352
URTI score (out of 27)	12.5 (7.1)	6.5 (5.6)	3.82	+0.76 to +6.89	0.014
PEx score (out of 12)	4.5 (2.3)	3.5 (1.8)	0.88	-0.76 to +2.53	0.292
log CRP (mg/l)*	2.77 (1.27)	1.77 (0.75)	0.98	+0.25 to +1.71	0.008
White cell count ($x10^9$)	10.6 (10.6)	9.8 (2.3)	0.88	-0.31 to +2.07	0.147

Data are presented as n (%) for binary variables and mean (SD) for continuous variables. Rhinovirus B was the comparator in each analysis.

* CRP values were log-transformed to correct for non-normal distribution. Mean CRP levels were 32.9 (42.2) for rhinovirus A and 7.7 (6.7) for rhinovirus B.

ABx: antibiotics; IV: intravenous; FEV_1: forced expiratory volume in 1 sec; URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; CRP: C-reactive protein

Table S4. Odds of individual symptoms being present at virus-positive compared with virus-negative visits

PEx Score[10]	OR	95 % CI	p value[#]
Change in sputum	2.04	1.5 – 2.8	<0.001
Haemoptysis	1.26	0.8 – 2.0	0.307
↑ Cough	2.08	1.5 – 2.9	<0.001
↑ Dyspnoea	1.48	1.1 – 2.0	0.010
Malaise	1.20	0.9 – 1.6	0.233
Pyrexia >38^{o}C	1.73	0.95 – 3.2	0.072
Anorexia	1.72	1.1 – 2.6	0.013
Sinus pain	1.38	0.98 – 1.9	0.063
Sinus discharge	2.05	1.4 – 3.0	<0.001
New signs	0.59	0.3 – 1.06	0.079
Fall in FEV_1 >10%	0.91	0.6 – 1.4	0.650
New CXR findings	1.3	0.4 – 4.6	0.682

URTI Score[11]	OR	95% CI	p value[#]
Runny nose	1.47	1.1 – 2.1	0.023
Sneezing	1.59	1.1 – 2.3	0.013
Blocked nose	1.39	0.99 – 2.0	0.058
Itchy eyes	1.19	0.8 – 1.7	0.331
Sore throat	2.52	1.7 – 3.8	<0.001
Hoarse voice	2.15	1.6 – 3.0	<0.001
Fever/shivers	1.46	1.02 – 2.1	0.037
Headache	1.17	0.9 – 1.6	0.309
Myalgia	1.19	0.8 – 1.7	0.347

PEx: pulmonary exacerbation; URTI: upper respiratory tract infection; FEV_1: forced expiratory volume in 1 sec; CXR: chest x-ray; OR: odds ratio; CI: confidence interval

Level of significance set at 0.01 *a priori* to take account of multiple comparisons

SUPPLEMENTARY FIGURES

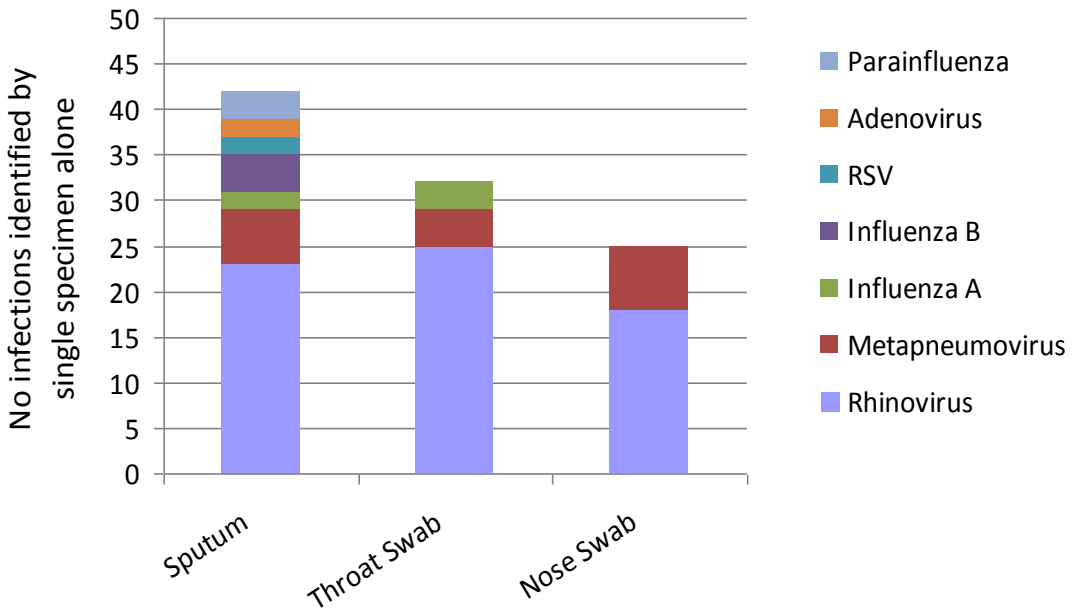

Figure S1. Breakdown of viruses identified by each sample type at study visits where only one specimen was positive

RSV: respiratory syncytial virus

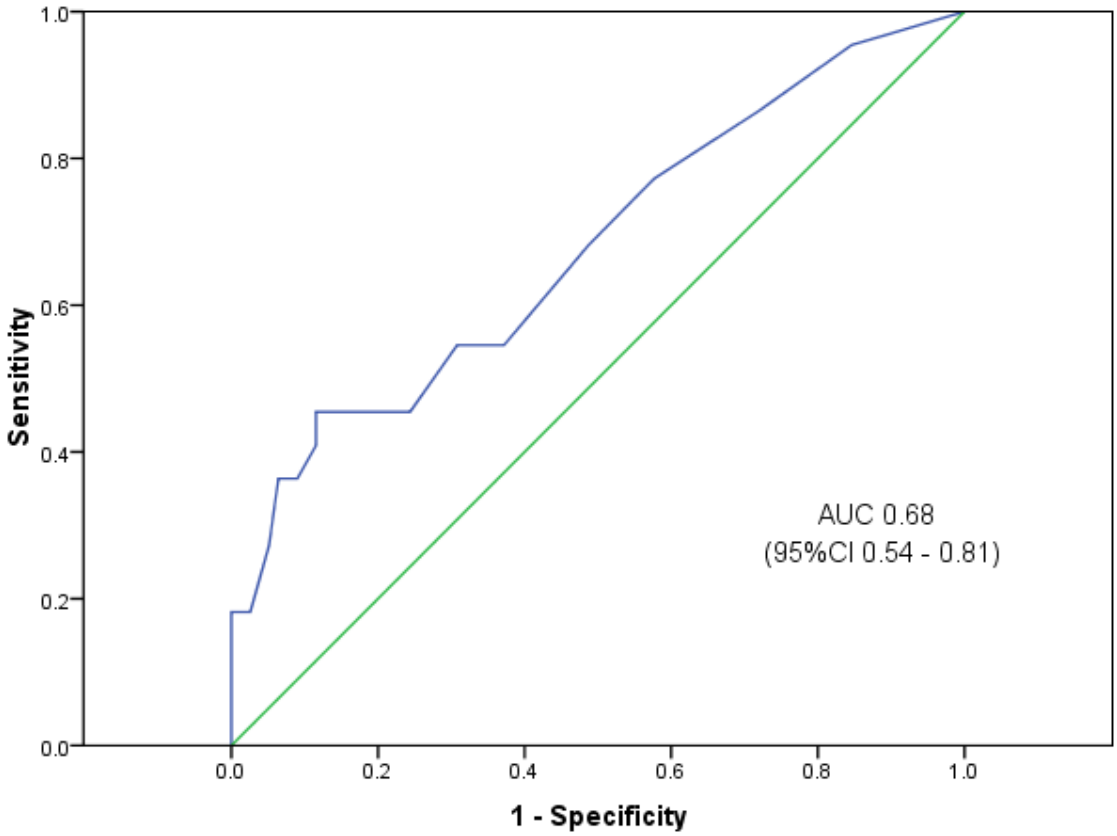

Figure S2. Receiver operating characteristic curve for diagnosis of respiratory virus infection using the Johnston URTI score[11]

AUC: area under the curve; URTI: upper respiratory tract infection

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