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► **To cite this version:**

Anne Goffard, Valérie Lambert, Julia Salleron, Stéphanie Herwegh, Ilka Engelmann, et al.. Virus and cystic fibrosis: Rhinoviruses are associated with exacerbations in adult patients. *Journal of Clinical Virology*, 2014, 60 (2), pp.147-153. 10.1016/j.jcv.2014.02.005 . hal-02266763

HAL Id: hal-02266763

<https://hal.science/hal-02266763>

Submitted on 3 Jan 2024

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Virus and cystic fibrosis: Rhinoviruses are associated with exacerbations in adult patients[☆]



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ARTICLE INFO

Article history:

Received 18 November 2013

Received in revised form 27 January 2014

Accepted 17 February 2014

Keywords:

Cystic fibrosis

Pulmonary exacerbation

Rhinovirus

PCR

ABSTRACT

Background: Few studies have suggested the potential role of respiratory viruses in cystic fibrosis (CF) exacerbation, but their real impact is probably underestimated.

Method: Sixty-four sputum samples collected from 46 adult patients were included in the study: 33 samples were collected during exacerbation of CF, and 31 during the stable phase. After extraction, nucleic acids were tested for the presence of respiratory viruses. When rhinovirus (HRV) was detected, the 5'UTR and VP4/2 regions were sequenced, and phylogenetically analyzed. The characteristics of patients in exacerbation and stable phase were compared.

Results: Viruses were found in 25% of samples. The HRV viruses were the most frequently detected followed by coronaviruses. Only the HRV detection was significantly associated with the occurrence of CF pulmonary exacerbation ($p < 0.027$). Characterization of 5'UTR and VP4/2 regions of the HRV genome specified that HRV-A, -B, -C were detected. All HRV-C were recombinant HRV-Ca.

Conclusions: HRV were the most frequently detected viruses; their detection was significantly associated with the occurrence of an exacerbation. The reality of viral recombination between HRV was demonstrated in CF patients for the first time, raising the role of viruses in lung microbiota. Further studies are now warranted to decipher virus impact in CF.

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1. Introduction

Cystic fibrosis (CF) is the major genetic inherited disease in the European Caucasian population. In addition to bacteria, which are well known to cause recurrent exacerbations of CF-associated pulmonary disease and often determine the vital prognosis of patients

[1], many airborne particles such as viral entities or fungal spores can also colonize the respiratory tract of patients [1,2].

For a long time, the detection of respiratory viruses, especially in exacerbations of chronic lung diseases has been underestimated because of the lack of sensitive methods for virus detection. Since the sensitive molecular methods for detection of viruses are more and more common, several recent studies highlight the clinical importance of respiratory viruses especially during exacerbation of asthma, chronic obstructive pulmonary disease (COPD) or CF [3–9]. In addition, viruses that were not previously sought are now detected, such as human rhinoviruses (HRVs) within the *Picornaviridae* family. Among the HRV genus, a new species of viruses named HRV-C has been described recently, is involved in severe respiratory infections in elderly and in immunocompromised patients [5,10–12]. Furthermore,

[☆] A part of this work was presented as a poster at “Journées Francophones de Virologie”, Paris April 2013.

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HRV-C has been described in patients with CF exacerbation but the real impact in the progression of the lung disease remains unclear [7].

2. Objectives

The primary objective of our study was to investigate the frequency and species of viruses present in sputa collected from CF adult patients with either pulmonary exacerbation or stable disease, and to determine the clinical relevance of viruses. As the most frequently detected viruses were HRVs, we phylogenetically characterized them and identified potential clinical associations, expressly for a significant relationship between HRV detections and occurrence of CF exacerbations.

3. Materials and methods

3.1. Patients and samples

Sixty four sputum samples collected from 46 CF patients in Lille (31 samples, 24 patients), Dunkerque (3 samples, 3 patients), and Grenoble (30 samples, 19 patients) between October 2006 and December 2010 were prospectively screened for respiratory virus carriage (reference number of the institutional ethics committees of Lille Hospital, CPP 06/84 – Written informed consent was obtained from each patient). All patients had a well-documented diagnosis of CF with either the mutations identified in the CFTR gene or an abnormally high sweat chloride test. They were included by physicians according to the same criterion (an annual check-up or an exacerbation situation that required an expectorated sputum sample that allowed us to search also for bacterial and fungal microorganisms). Clinical data including spirometry, therapeutic, radiological and biological data were collected by clinic staff at each time of the visit. At each sampling time, the clinical status of the patients was defined as pulmonary exacerbation or stable period according to the physician defined requirement (i.e. recent changes in clinical parameters and/or modification of the pulmonary function provide criteria of exacerbation according to ERS statement) [13].

3.2. Microbial analysis of sputa

Twenty eight patients had one sputum examined, and 18 patients had two sequential sputa examined, which were considered as independent events since the delay between the samples was at least 6 months [2,14]. Samples were performed by expectoration into a sterile cup after a water rinse to prevent excessive salivary contamination, analyzed according to a standardized protocol as previously described, and screened for respiratory virus carriage [2].

3.3. Molecular analysis of sputa

Total nucleic acids were obtained after mechanical lysis of sputum sample. Briefly, 200 μ L of each sample were subjected to fluidification in a MagNAlyser (Roche Diagnostics) according to the manufacturer's instructions. QIAampMin Elute Virus Spin kit (QIAGEN) was used on crude lysate according to the manufacturer's instructions. Purified nucleic acids were frozen at -80°C until use. Reverse transcription was performed with RevertAid First Strand cDNA Synthesis kit (Fermentas) using 8 μ L of previously obtained RNA. Detection of respiratory viruses was performed with Seeplex RV15 ACE Detection kit (Seegene) in three independent PCR reactions targeting influenza viruses A and B, RSV A and B, human adenovirus, human metapneumovirus (hMPV), human coronavirus

Table 1
Primers used for amplification of HRV VP4/VP2 and 5'UTR regions.

5'-3' sequences	Region	Orientation	Position ^a
CCGGCCCTGAATGYGGCTA	VP4/VP2	Outer sense	458
ACATRTTYSNCCAAANAYDCCCAT	VP4/VP2	Outer antisense	1125
ACCRACACTTTGGGTGCCGTG	VP4/VP2	Inner sense	547
TCWGGHARYTTCAMCACCANCC	VP4/VP2	Inner antisense	1087
HCAAGYACTTCTGTWCCCCSG	5'UTR	Outer sense	178
GAAACACGACACCCAAAGTAGT	5'UTR	Outer antisense	573
TTAGCCRCATTACAGGGCCGG	5'UTR	Inner antisense	477

^a The 5' base position numbered according to the HRV-B serotype 14 genome (GenBank accession number NC.001490).

(HCoV 229E, NL63, OC43 and HKU1), human parainfluenza viruses 1–4, HRV, human enteroviruses, and human bocaviruses in three independent PCR reactions. Amplification products were visualized after semi-automated capillary electrophoresis in a TapeStation Lab 901 (Eurobio) and analysis was performed with Tape DS12 software.

Samples that were positive for HRV were identified, based on sequencing of the VP4/VP2 and 5'UTR regions, according to the previously described nested RT-PCR strategy and the primers listed in Table 1 [15]. The three HRV-specific VP4/VP2, 5'UTR inner sense primers were included in the second amplification step. The amplicons were directly sequenced using ABI Prism 3500XL (Life Technologies) and results were analyzed with the SeqScape 2.7 software (Applied Biosystem).

3.4. Phylogenetic analysis of HRV-A and C genome sequence

Phylogenetic trees of the VP4/VP2 and 5'UTR regions were constructed with unambiguously aligned sequences (516 and 285 sites for VP4/VP2 and 5'UTR, respectively) using neighbor joining of pairwise maximum composite likelihood method implemented in MEGA5 program [16]. The reliability of internal branches was assessed using the bootstrap method with 1000 replicates. Accession numbers of the sequences included in the dataset are listed in Fig. 2.

3.5. Statistical analysis

Quantitative variables were described by median, mean and standard deviation; qualitative variables were described by frequency and percentage. The comparison of sub-population regarding HRV carriage were performed using the Mann–Whitney *U* test for quantitative variables. Qualitative variables were compared using Chi-square test or Fisher's exact test when appropriate. A *p* value of <0.05 was considered significant. All statistical analyses were performed under SAS software (SAS Institute, Cary, NC, USA; version 9.2).

Interactions between rhinovirus infection and the different variables were investigated with one interaction term with respective subterms in the model (univariate model) and then repeated with adjustments to dyspnea, S-K score, ABPA, segmental or sub-segmental bronchiectasis, fungus detection, intravenous antibiotics or azithromycin treatment or systemic steroids regimens (multivariate model).

4. Results

4.1. Study population characteristics

Forty six patients (23 males and 23 females) were included in this study, with a mean age of 25 years (mean \pm SD: 25 ± 11.4). Their characteristics at enrolment and at each sampling time are summarized in Table 2. A majority of patients (82%) were homozygous or heterozygous for the del-F508 mutation in the CFTR gene

Table 2
Whole population data and comparison of subjects with and without detectable HRV RNA in sputum samples.

Patient characteristic	Whole population	Comparison of sub-population regarding HRV carriage		
		HRV-positive (n=7)	HRV-negative (n=38)	p value
At enrolment	n=46			
Age (mean; median, SD in years)	25; 29 (11.4)	23; 24.8 (5.7)	26; 29.9 (12.1)	0.181
Sex (Male)	23 (50%)	3 (37.5%)	20 (52.6%)	0.699
Sweat chloride test (mean; median, SD in mmol/L)	112; 117.6 (58.6)	128; 126 (6.2)	103.5; 116.2 (63.4)	0.366
CFTR mutations (Δ F508 homozygous)	36 (82%)	6 (85.7%)	30 (81.1%)	1
At each sampling time	n=64	HRV-positive (n=9)	HRV-negative (n=55)	p value
BMI ^b (mean; median, SD)	19.6; 19.7 (2.3)	19.7; 19.8 (2.6)	19.4; 19.7 (2.2)	0.983
S-K score ^a (mean; median, SD)	65.0; 64.2 (16.0)	66.0; 62.7 (19.8)	65.0; 64.5 (15.5)	0.963
FEV1 ^c (mean; median, SD in % of predicted value)	43.0; 50.7 (25.9)	53.0; 54.6 (28.5)	41.0; 50.1 (25.7)	0.535
FVC ^d (mean; median, SD in % of predicted value)	66.0; 69.9 (24.7)	74.5; 74.1 (25.9)	63.5; 69.2 (24.7)	0.586
Dyspnea evaluated using Sadoul scale	2.0; 1.7 (1.2)	1.0; 1.8 (1.9)	2.0; 1.6 (1.0)	1.0
Pulmonary exacerbation (%)	33 (51.6)	8 (88.9)	25 (45.4)	0.027
Wheezing (%)	19 (41.3)	3 (42.5)	16 (41.0)	0.318
Ronchi (%)	15 (30.0)	1 (14.3)	14 (32.6)	0.244
Crackles (%)	21 (42.0)	3 (42.7)	18 (41.7)	0.316
Co-morbidities				
CF-related diabetes (%)	25 (41.0)	2 (25.0)	23 (43.4)	0.452
ABPA ^e (%)	17 (26.6)	2 (22.2)	15 (27.3)	1.000
Microbial colonization at enrolment				
<i>Staphylococcus aureus</i> (%)	16 (30.2)	1 (16.7)	15 (31.9)	0.654
<i>Pseudomonas aeruginosa</i> mucoid strain (%)	29 (54.7)	3 (50.0)	26 (55.3)	1.000
<i>Pseudomonas aeruginosa</i> non-mucoid strain (%)	16 (30.2)	1 (16.7)	15 (31.9)	0.655
<i>Aspergillus fumigatus</i> (%)	29 (45.3)	4 (44.4)	25 (45.4)	1.000
<i>Penicillium</i> sp. (%)	10 (15.6)	0 (0.0)	10 (18.2)	0.333
<i>Candida albicans</i> (%)	44 (68.7)	5 (55.6)	39 (70.9)	0.443
Other yeast (%)	46 (71.9)	5 (55.6)	41 (74.5)	0.255
Treatments				
Nebulized rhDNase (%)	40 (65.6)	4 (50.0)	36 (67.9)	0.186
Inhaled steroids (continuous) (%)	43 (70.5)	5 (62.5)	38 (71.7)	0.267
Azithromycin (sub-therapeutic dosage) (%)	42 (70.0)	7 (87.5)	35 (67.3)	0.190
Oral antifungal ^f (%)	11 (18.0)	2 (25.0)	9 (17.0)	0.297
Systemic steroids (continuous or as bolus) (%)	28 (45.9)	3 (37.5)	25 (47.17)	0.264
Intravenous antibiotics (1–14 regimens) (%)	33 (75.0)	5 (71.4)	28 (75.7)	1

^a S-K score: Shwachman-Kulczycki score.

^b BMI: body mass index.

^c FEV1: forced expiratory volume in 1 second.

^d FVC: forced vital capacity.

^e ABPA: allergic bronchopulmonary aspergillosis as referred in [17].

^f Oral antifungal treatments were: itraconazole in 8 cases, voriconazole in 1 case, and fluconazole in 2 cases.

associated with an abnormally high sweat chloride test (median \pm SD: 112 \pm 58.6 mmol/L). Our population was composed of patients with moderate CF disease, during a state of clinical stability (31 episodes) and during acute exacerbation (33 occurrences) according to ERS statement [13]. Most subjects had exocrine pancreatic dysfunction (88.4%), while diabetes mellitus or impaired glucose tolerance was observed in 37.2%, in agreement with published data [17]. All, but one (a 22 year-old woman), patients had microbial colonization in their sputa as determined by culturing. Colonization with *Pseudomonas aeruginosa* was mainly detected (53.1%); *Aspergillus fumigatus* and *Candida albicans* were observed in 45.3% and 68.7% of patients respectively, as previously published [1,2,17]. Regarding therapy management, most of the patients received rhDNase (65.6%) and/or 70.5% inhaled steroids; 70.0% received low dose of azithromycin. Whereas oral antibiotic regimens remained the most often prescribed therapeutics (86.9%), intravenous regimens of antibiotics were prescribed in 75.0% of episodes. Antifungal drugs were prescribed for 11 (18.0%) of cases in agreement with allergic bronchopulmonary aspergillosis (ABPA) diagnosis [18]. In 28 cases (45.9%), patients were under systemic steroids. None other systemic immunosuppressive therapy was used.

4.2. Virus detection

Overall, viruses were identified from 16 out of 64 sputum samples (25%). Rhinoviruses (9/64; 14%) and HCoV (5/64; 8%) were the

main identified agents. Eight HRVs were isolated from patients with acute exacerbation (8/33 occurrences; 24%, Fig. 1A) and 1 from a patient during a stable state (1/31 episodes; 3.2%, Fig. 1B). Two (2/33; 6%) OC43/HKU1 or 229E/NL63 HCoVs have been detected from sputa collected during pulmonary exacerbation (Fig. 1A). Three (3/31; 10%) HCoVs, 1 (1/31; 3%) influenza A virus and 1 (1/31; 3%) parainfluenza 2 virus (PIV-2) were detected in sputa collected from patient in stable clinical condition (Fig. 1B). Detection of respiratory viruses occurred mainly in the winter season (Fig. 1C). Indeed, detection of influenza A virus and PIV-2 occurred in October 2008 and February 2009, respectively. Coronaviruses were exclusively detected during the two winter seasons. HRV-A viruses were observed in the autumn 2007 and in springs 2008 and 2009, while detection of other HRVs occurred during the winter 2007 and the autumn 2008 (Fig. 1C), in agreement with the phylogenetic characterization (see Section 4.4). No co-infection with respiratory viruses was detected.

4.3. Association between respiratory viruses and CF pulmonary exacerbation: importance of HRVs

No significant differences were found in baseline values between the subgroups composed of patients with and without virus detection. Viral respiratory infections in our population of CF patients were not significantly associated with either respiratory symptoms, or deterioration of body mass index, of Shwachman-Kulczycki score, and of spirometry parameters, or

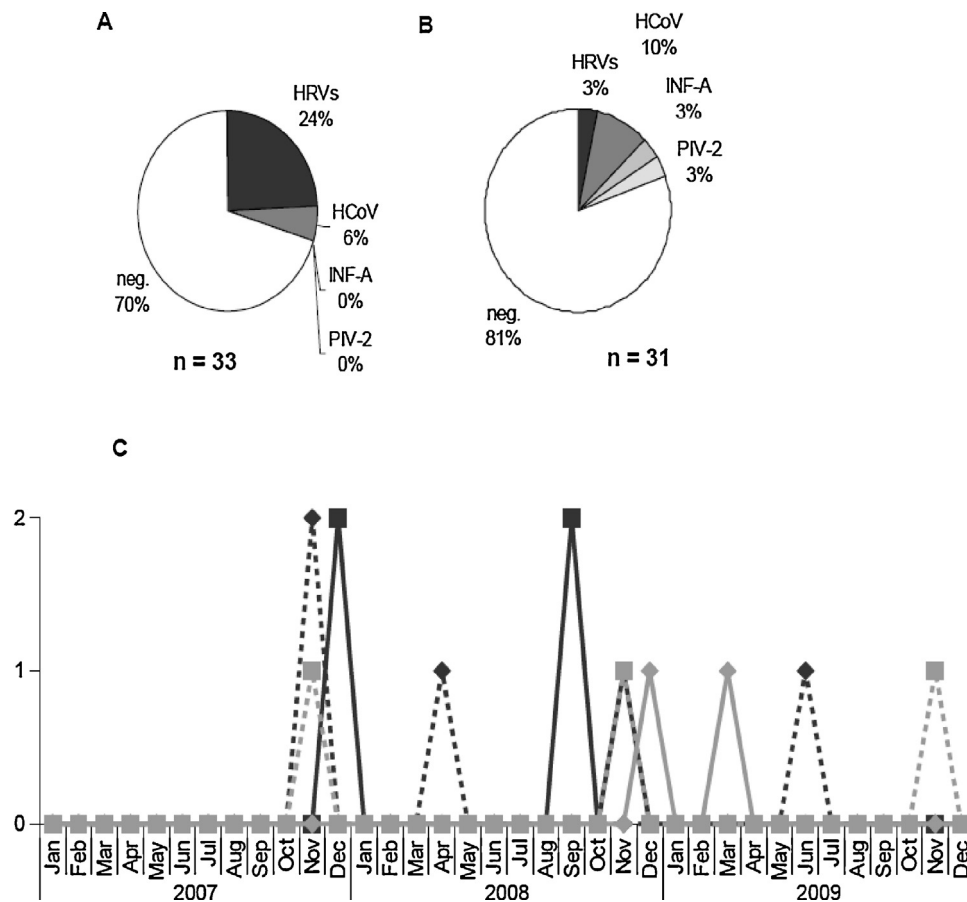


Fig. 1. Frequency and seasonal distribution of respiratory viruses detected from sputa of CF patients. In each subgroup composed of sputa collected during pulmonary exacerbation (A) or of sputa collected during stable period (B), positive and negative samples are presented as percentages from the total in each subgroup. Seasonal distribution (C) of HRV-A, dotted black line with diamond; of HRV-C, black line with square; HCoV-229E/NL63, gray line with diamond; and of HCoV-OC-43/HKU1, dotted gray line with square.

comorbidity factors, or microbial colonization, or an increased use of antibiotics, of antifungal or steroids (Table 2). Alterations confirmed by radiology or scanner as proximal bronchiectasis or as segmental/sub-segmental bronchiectasis (identified in 56.4% and 51.3% of the population, respectively) were not significantly associated with virus detection, but missing data were about 39%.

Regarding HRV detection, neither general features of CF patients (such as CFTR mutations or body mass index), nor spirometric values, nor clinical criteria of respiratory function (such as dyspnea, wheezing, ronchi, or crackles), nor radiological and microbial parameters were significantly associated with HRV detection (Table 2). Regarding clinical criteria of respiratory function, missing data were about 23% for dyspnea, and 21.8% for wheezing, ronchi, and crackles, which might explained the absence of statistical significance. Interestingly, detection of rhinoviruses was significantly associated with pulmonary exacerbation ($p = 0.027$, Table 2).

4.4. Genetic diversity of HRVs

Sequencing the VP4/VP2 region (nucleotide positions 547–1087) was successfully performed in the 9 HRV positive samples. The obtained sequences were aligned with 13 published complete genome sequences and 12 available partial VP4/VP2 sequences (Fig. 2A). Four HRV sequences were identified as belonging to HRV-A species, 4 to HRV-C species and 1 to HRV-B species. The 8 samples that were assigned to HRV-C or HRV-A species were characterized further by sequencing the 5'UTR region (nucleotide position 178–477; Fig. 2B). Using a bootstrap value of

70% or greater to define clades, topologies of the trees constructed from VP4/VP2 and 5'UTR regions were in agreement with previous published results [19–21]. Surprisingly, the 4 sequences obtained classified as HRV-C by comparative analysis of VP4/VP2 sequences were related more closely to HRV-A strains when their 5'UTR regions were analyzed. All query sequences grouped within the HRV-A clade, with bootstrap value of 90 and 84% (Figure 2), and belong to HRV-Ca variants, according to recent published data [19–22].

5. Discussion

In the present study, we reported detection of respiratory viruses from adult patients with CF during either routine visit or acute pulmonary exacerbation. We detected respiratory viruses in 25% of 64 sputum samples. This proportion is close to previously published results obtained using conventional diagnosis methods but notably smaller than other recently published data, which might be related to our sampling method [8,10,23–25]. Upper respiratory tract sample such as nasal washes or oropharyngeal swabs are the most frequent sample types used to detect respiratory viruses. Sputa were considered as lower respiratory tract samples and has been recently described as suitable to identify viruses in CF patients [24,26]. Here, we performed a mechanical pre-treatment of each sputum sample before nucleic acid extraction; this pre-treatment is newly recommended for respiratory virus detection in sputa from patients with asthma or CF [26,27]. Moreover,

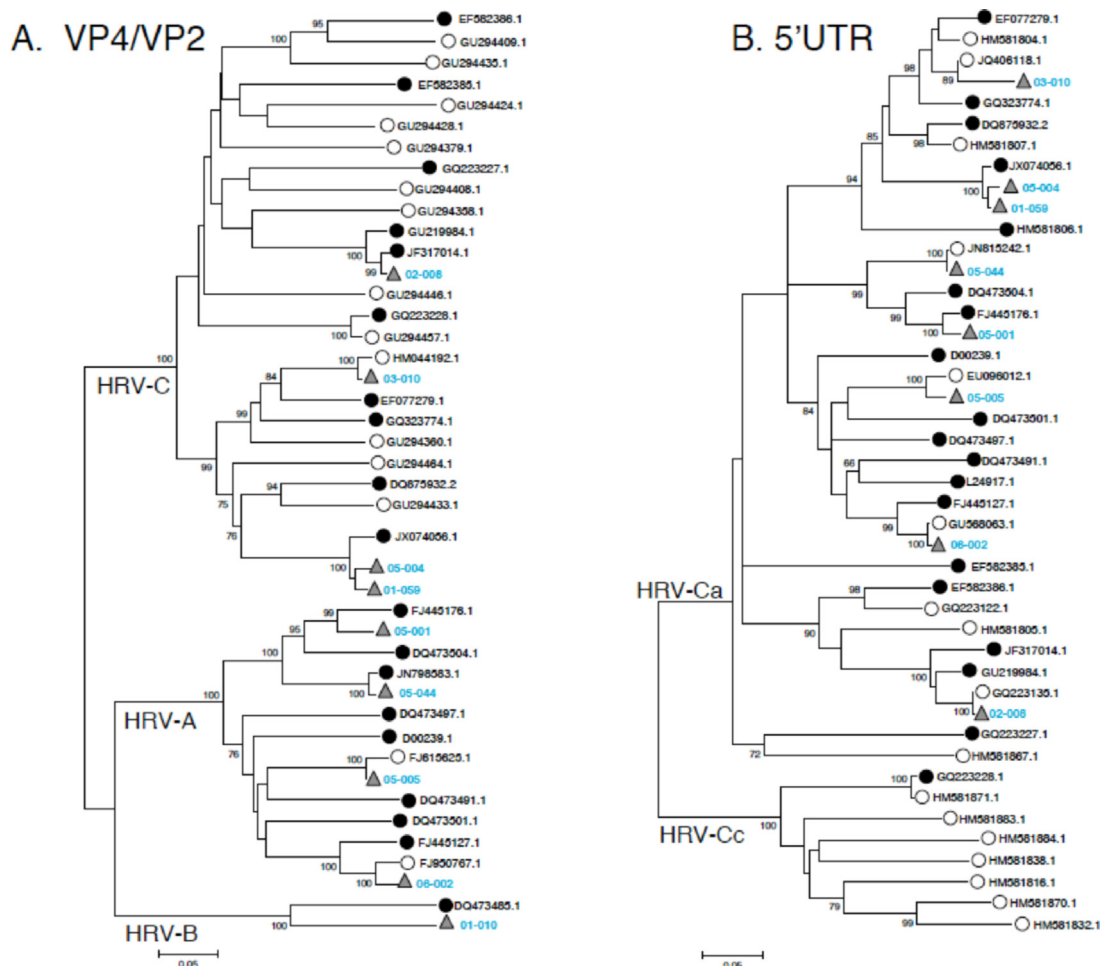


Fig. 2. Phylogenetic trees of VP4/VP2 gene (A) and 5'UTR region (B) of HRVs. Trees were constructed from alignments of available sequences of complete (white circles), partial genomes (black circles) and sequences obtained from HRV-positive samples (gray triangles). HRV sequences were deposited in GenBank under the accession numbers KC492746–62.

sputum analysis has been used for rhinovirus detection in CF patients, who present exacerbation and sputum production [26].

Even if there is only a little data on the incidence of viral pathogens causing exacerbation in adult CF patients, coronavirus, PIV-2, pandemic A/H1N1 were detected in our population as previously reported [6,8,25,28–30]. Our screening for respiratory viruses showed the presence of coronavirus in samples collected both from patients with exacerbation and from patients during stable disease. However, the presence of coronavirus in samples is not associated with the occurrence of an exacerbation, according to previous study [30,31]. We detected influenza A virus in only one case, and therefore did not confirm the link between influenza infection and the occurrence of an exacerbation in CF previously described [29]. Due to the potential severity of influenza infection in CF, it is recommended to vaccinate patients against influenza viruses, but the patient positive to A/H1N1 detection did not follow this recommendation and was not vaccinated. The low influenza A virus detection frequency observed here might be linked to the good matching of the influenza vaccine to the circulating influenza viruses during our study period, even if the vaccination status of our CF patient cohort was not documented. Consistent with previous studies, we detected coronaviruses preferentially during the cooler months, HRV-A mainly in spring and autumn, and HRV-C variants preferentially during the autumn and winter months [15,32,33].

HRVs were the most commonly detected viruses, as previously reported in CF children [9,25,26]. Whereas the physiopathology of

virus-induced exacerbation in CF remains unclear, we established a significant association between HRV detection and respiratory exacerbation, confirming the results of recent studies [7,32,33]. The detection of HRVs is more frequent in nasal swabs, especially in patients in a stable phase of CF [9]. However, our results show that the detection of HRV in sputum is associated with the occurrence of an exacerbation suggesting that the analysis of sputum could be more relevant than nasal swabs when a viral cause of pulmonary exacerbation is suspected. Some recent studies have shown that HRV-C are involved in severe respiratory infections especially during exacerbations of asthma [34,35]. Numerous findings underscore the polymicrobial nature of respiratory tract as well as the importance of lung microbiota as a distinctive feature of tissue compartmentalization, but viral microbiota (or virome) has been poorly studied in respiratory tract [22,36]. Respiratory virome appears composed of three major virus families: *Paramyxoviridae*, *Orthomyxoviridae* or *Picornaviridae* for which a novel type of Rhinovirus C was identified [36].

Here, we performed sequence-based typing of 9 HRVs resulting in 1 HRV-B, 4 HRV-A, and 4 HRV-C. As previously described, HRV-A and HRV-C were the most frequently detected viruses compared to the frequency of HRV-B detection [37]. Recent studies have shown recombination between the 5'UTR region of HRV-C genome and the coding region of HRV-A genome leading the authors to propose a new classification of HRVs in two subspecies, HRV-Ca and -Cc [15,19,20,38]. Occurrence of such recombination

was then confirmed, HRV-Ca and HRV-Cc being detected in various upper respiratory diseases [37]. Here, we identified all the HRV-C isolates as belonging to the sub-species HRV-Ca by sequencing both the VP4/VP2 and 5'UTR regions. To our knowledge, the present study describes for the first time the presence of HRV-Ca subtype in patients with CF. Although the clinical significance of such recombination is still matter of debate, our results confirm the well-known recombination ability of enteroviruses (notably HRVs); a fact of particular relevance when phage community represents a key point in the lung microbiota analysis as well as in the improvement of resistosome concept [26,35,38,39]. In fact, human microbiota represents a huge reservoir of mobilizable genes, as proposed by Rolain and co-workers [39]; some of those genes encoding antimicrobial resistance that the authors named 'resistome' [39]. This concept is based on few metagenomic studies that have analyzed viral and bacterial communities in CF sputum samples, revealing the presence of an important bacteriophage community containing genes encoding antimicrobial resistance, and demonstrating these bacteriophages are consistent with the characteristics of multidrug-resistant microbial communities that are commonly observed in CF patients [26,35,38,39].

To conclude, we have reported a relatively high frequency of respiratory viruses in a cohort of CF adult patients from France, and demonstrated for the first time that rhinovirus detection including newly identified HRV-Ca variants are the most frequent and significantly associated with respiratory exacerbations. Our results can be considered as contribution to the characterization the role of viruses in CF pulmonary exacerbation, underscoring the importance of the lung virome that remains poorly understood in the context of CF.

Funding

The authors thank PHRC 1902 – Vaincre la mucoviscidose – Pfizer Laboratories for their financial support.

Competing interests

None.

Ethical approval

Reference number of the institutional ethics committees of Lille Hospital, CPP 06/84. Written informed consent was obtained from each patient.

Acknowledgments

The authors thank all the clinicians from the three hospitals for their cooperation in collecting sputum samples and clinical data. The authors would like to thank Carolyn Engel-Gautier for English editing.

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