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Respiratory viral infections in patients with chronic, obstructive pulmonary disease

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Summary Objectives. The purpose of the present study was to apply reverse transcription-PCR (RT-PCR) assays to clinical specimens collected from patients with acute respiratory illness and chronic obstructive pulmonary disease (COPD).

Methods. One hundred and ninety-four samples from two different study cohorts were analysed using RT-PCR assays for picornaviruses, coronaviruses 229E and OC43, influenza A and B viruses, respiratory syncytial virus, parainfluenza types 1-3 viruses, and human metapneumovirus and a PCR assay for adenoviruses. The results were added to results obtained previously using cell culture and serologic methods.

Results. RT-PCR assays identified an additional 35 respiratory virus-associated illnesses not identified previously by cell culture or serology (n = 46). Picornaviruses and coronaviruses were the most common viral infections identified only by RT-PCR. Overall, 41.8% of the acute respiratory illnesses evaluated were associated with a respiratory virus infection, with picornaviruses, coronaviruses and influenza viruses being the most common infections recognized. No human metapneumovirus infections were identified by RT-PCR assay.

Conclusions. Respiratory viral infections are commonly associated with acute respiratory illness in COPD patients, and the use of RT-PCR assays significantly increases the ability to diagnose these infections.

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Introduction

The fourth most common cause of death in the United States is chronic lower respiratory disease.\textsuperscript{1,2}
Patients with chronic obstructive pulmonary disease (COPD) also suffer recurrent exacerbations that increase the morbidity of the disease and contribute to its mortality. Acute exacerbations of COPD are associated with acute infections of the respiratory tract caused by bacteria, viruses, or both bacteria and viruses. The frequency in which a respiratory virus infection has been identified in association with acute worsening of respiratory function in patients with COPD has varied in different studies.

We previously performed two studies that evaluated the prevalence of acute respiratory viral infections in patients, including those with COPD. Both studies utilized cell culture and serologic methods to identify acute respiratory virus infections. Study 1 examined the frequency of specific respiratory virus infections in persons with acute respiratory tract condition that lead to hospitalization. In study 2, a longitudinal cohort of COPD patients were tested for RTVIs in an ambulatory setting, although some patients were also hospitalized. Respiratory viral infections were identified in 19% of COPD subjects with acute respiratory illness.

Reverse-transcription polymerase chain reaction (RT-PCR) assays can identify respiratory virus infection missed by cell culture. The number of respiratory viral infections identified in asthmatic patients with acute exacerbations of disease increase significantly when RT-PCR assays are used in addition to other diagnostic methods. A similar observation has been made for acute exacerbations of COPD. In order to extend our understanding of the prevalence of respiratory viral infection in acute respiratory illnesses in patients with COPD, we used RT-PCR assays to evaluate samples from the previous two prospective studies for evidence of respiratory virus infection.

Materials and methods

Study population

Patients with COPD were enrolled in two prospective clinical studies designed to identify the respiratory viruses associated with exacerbations of their lung disease. Study 1 evaluated the prevalence of RTVIs among patients hospitalized with an admitting diagnosis of acute respiratory illness or congestive heart failure between July 1991 and June 1995. Patients were identified by review of the admission log. Persons with exacerbations of COPD enrolled in this study were identified using discharge diagnosis codes (ICD9 codes 491.2, 491.21, 492, 493.2, 496). Study 2 was a longitudinal study conducted between September 1991 and May 1995 to evaluate the incidence of RTVIs in a cohort of 62 patients with COPD. The criteria for diagnosis of COPD were as defined by the American Thoracic Society. An exacerbation of COPD was defined as meeting one or more of the criteria from Anthonisen et al. Increased dyspnea, sputum production or sputum purulence. RTVIs in both studies were identified by virus culture of combined nasal wash/throat swab specimens and by serologic assays as previously described.

RT-PCR studies

The following RT-PCR or PCR assays (limits of detection) were used to assay the clinical specimens for the following: influenza A and B viruses (1-10 tissue culture 50% infectious dose [TCID50]), respiratory syncytial virus (10-50 TCID50), parainfluenza virus types 1, 2 and 3 (10-50 TCID50), coronavirus OC43 and 229E (1-10 TCID50), picornaviruses (<1 TCID50), adenoviruses (10-50 TCID50), and human metapneumovirus (10-50 TCID50) (Table 2). These assays consist of three major steps: (1) nucleic acid extraction; (2) complementary DNA (cDNA) synthesis and amplification (RT-PCR); and (3) identification of virus-specific amplicons by Southern blot hybridization. Nucleic acids were extracted from nasal wash/throat swab samples using the QIAamp Viral RNA mini kit (Qiagen Inc.; Valencia, CA) according to the manufacturer's instructions. RT-PCR/PCR assays were performed as previously described using primers listed in Table 1. For detection of human metapneumovirus infection, cDNA was synthesized for 1 h at 43 °C. After an initial 5-minute heat denaturation at 94 °C, 40 cycles of heat denaturation at 92 °C for 15 s, annealing at 55 °C for 30 s, and primer extension at
### Table 1: Primers and probes used to identify respiratory viruses in clinical specimens

| Virus                          | Virus gene target | Oligonucleotide name | Oligonucleotide sequence | Reference |
|-------------------------------|-------------------|----------------------|--------------------------|-----------|
| Influenza A                   | Matrix            | FAM1                 | 5'-CAG AGA CTT GAA GAT GTC TTT GC-3' | 17        |
|                               |                   | FAM2                 | 5'-GCT CTG TCC ATG TTA TTT GRA T-3' | 17        |
|                               |                   | AH2 (probe)          | 5'-TCC TGT CAC CTC TGA CTA AGG GGA TTT TG-3' | 17        |
|                               |                   | Matrix               | INFA-1a                  | 21        |
|                               |                   |                      | 5'-GGA CTG CAG CGT AGA CGC TT-3' |           |
|                               |                   | Matrix               | INFA-2a                  | 21        |
|                               |                   |                      | 5'-CAT YCT GTT GTA TAT GAG GCC CAT |           |
|                               |                   | Matrix               | INFA probea              | 21        |
|                               |                   |                      | 5'-FAM-CTC AGT TAT TCT GCT GGT GCA CTT GCC A-TAMRA |           |
| Influenza B                   | Matrix            | B1                   | 5'-GAA AAA TTA CAC TGT TGG TTC GGT G | 16        |
|                               |                   | B2B                  | 5'-AGC GTT CCT AGT TTT ACT TGC ATT GA | 16        |
|                               |                   | HYB365               | 5'-TTC TAG CTG AGA GAA AAA TGA GAA GAT GT | 16        |
|                               |                   |                      | INFB1a                   | 21        |
|                               |                   |                      | 5'-AAA TAC GGT GGA TTA AAT AAA AGC AA |           |
|                               |                   |                      | INFB2a                   | 21        |
|                               |                   |                      | 5'-CCA GCA ATA GCT CCG AAG AAA |           |
|                               |                   |                      | INFB probea              | 21        |
|                               |                   |                      | 5'-FAM-CAC CCA TAT TGG GCA ATT TCC TAT GGC TAMRA |           |
| Parainfluenza virus type 1    | Matrix            | PF526                | 5'-ATT TCT GGA GAT GTC CCG TAG GAG AAC | 19        |
|                               |                   | PR678                | 5'-CAC ATC CTT GAG TGA TTA AGT TTG ATG A | 19        |
|                               |                   | P640 (probe)         | 5'-TAC CTT CAT TAT CAA TTT GTA AGT CAA | 19        |
| Parainfluenza virus type 2    | Matrix            | P2 upstream          | 5'-CAT GTA CTA TAC TGA TGG TGG | 18        |
|                               |                   | P2 downstream        | 5'-GTT AGT AAC TTA AAT AGG GTA AC | 18        |
|                               |                   | P2 probe             | 5'-AAT GGA ACA TGC AAC ATC ACC | 18        |
| Parainfluenza virus type 3    | Matrix            | P3HN1                | 5'-CTC GAG GTT GTG AGG ATA TAG | 40        |
|                               |                   | P3HN2                | 5'-CTT TGG GAG TGG AAC ACA GTT | 40        |
|                               |                   | P3 (probe)           | 5'-CAT CTG TAT TTA GGA GTG CTA G | 40        |
| Virus               | Virus gene target | Oligonucleotide name | Oligonucleotide sequence                                                                 | Reference |
|--------------------|-------------------|----------------------|----------------------------------------------------------------------------------------|-----------|
| HCoV OC43          | Nucleoprotein     | CVP3                 | 5'-IIA AAT TGC TII TCT TGT TCT GGC                                                   | 13,41     |
|                    |                   | CVP4                 | 5'-CCA AAA TTC TGA TTA GGG CCT CTC                                                    | 13,41     |
|                    |                   | CVPP                 | 5'-AAG CAI ATG GCC AAA IAA GTC AGI CAG AAA ATT TT                                       | 13,41     |
|                    |                   |                      | Nucleoprotein O1.1a                                                                   | 42        |
|                    |                   |                      | 5'-CAT CAG GAG GGA ATG TTG TAC C                                                     |           |
|                    |                   |                      | O3.1a                                                                                 | 42        |
|                    |                   |                      | 5'-TAC TGG TCT TTA GCA TGC GGT C                                                      |           |
|                    |                   |                      | Probea                                                                                |           |
|                    |                   |                      | 5'-FAM-CAG CAG TCG AGC TCG GTT GCC ATC TAMRA                                         | This study|
| HCoV 229E          | Nucleoprotein     | E7                   | 5'-TCT GCC AAG AGT CTT GCT CG                                                          | 42        |
|                    |                   | E9                   | 5'-AGC ATA GCA GCT GGT GTC GC                                                        | 42        |
|                    |                   | E8 (probe)           | 5'-AGC ATA GCA GCT GGT GTC GC                                                        | 42        |
|                    |                   | E7a                  | 5'-TCT GCC AAG AGT CTT GCT CG                                                         | 42        |
|                    |                   | E9a                  | 5'-AGC ATA GCA GCT GGT GTC GC                                                        | 42        |
|                    |                   |                      | Probea                                                                                |           |
|                    |                   |                      | 5'-VIC-TGG CCA CAA CAC CTG CAC GTC C-TAMRA                                           | This study|
| Picornavirus       | 5' Non-coding     | R1                   | 5'-AGC GAC ACC CAA AGT A                                                              | 13        |
|                    | region            | R2                   | 5'-AGC ACT TCT GTT TCC C                                                              | 13        |
|                    |                   | R4                   | 5'-TCC TCC GGC CCC TGA ATG                                                           | 13        |
| RSV A and B        | Fusion            | Forward primer       | 5'-ATT GGC ATT AAG CCT ACA AAG CA-3'                                                 | 20        |
|                    |                   | Rev primer           | 5'-CTT GAC TTT GCT AAG AGC CAT CT-3'                                                 | 20        |
|                    |                   | Probe A              | 5'-TAG YCC AAA TGG AGC CWG AAA ATT ATA GTA-3'                                         | 20        |
|                    |                   | Probe B              | 5'-CRG TTA AGA AGG AGC TAA TCC ATT TTA GTA-3'                                        | 20        |
| Adenovirus         | Hexon             | Hex1deg              | 5'-GCC SCA RTG GKC WTA CAT GCA CAT C-3'                                               | 43        |
|                    |                   | Hex2deg              | 5'-CAG CAC SCC ICG RAT GTC AAA-3'                                                    | 43        |
|                    |                   | AD2 (probe)          | 5'-GCC ACC GAG AGC TAC TCC AGC CTG-3'                                                | 44        |

(continued on next page)
72 °C for 30 s were followed by a final primer extension at 72 °C using the PTC 200 thermal cycler (MJ Research, Inc., Cambridge, MA). Virus-specific PCR products were detected by Southern hybridization using digoxigenin-labeled probes (see Table 1). Human metapneumovirus-specific amplimers were identified following hybridization with a virus-specific digoxigenin-labeled oligoprobe at 50 °C.

Real-time RT-PCR assays also were performed for influenza A and B viruses and coronavirus OC43 and 229E viruses. The influenza virus-specific assays were performed as previously described.21 For the coronavirus-specific assays, cDNA was generated in a single reaction in the thermal cycler at 43 °C for 1 h. Specimens were assayed in duplicate in a 50 µl reaction containing 25 µl of 2 × Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 5 µl of cDNA, 900 nM forward and reverse primers, and 100 nM fluorogenic probes. Amplification and detection were performed using the GeneAmp 5700 sequence detection system (Applied Biosystems) at the following conditions: 10 min at 95 °C for denaturation followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Extraction and reagent positive and negative controls were used in each experiment to assure the validity of the experimental results. Steps to prevent carryover contamination were used routinely such as separation of sample preparation, separate PCR set-up and post-PCR analysis areas, the use of plugged pipette tips, traffic control from ‘clean’ (pre-PCR) to ‘dirty’ (post-PCR) areas, and the use of extraction and reagent positive and negative controls. For an assay to be considered valid, negative controls yielded negative results and positive controls yielded positive results.

**Results**

There were 96 study subjects with 194 separate illnesses for which samples were available for analysis (Table 2). Participants in study 1 were younger (61.3 vs. 66.7 years, \( P < 0.005 \)), more likely to be non-white (71% vs. 21%, \( P < 0.05 \)), and less likely to have received influenza vaccination (49% vs. 89%, \( P < 0.001 \)) than those in Study 2. The majority of subjects in both studies had a smoking history, but a larger percentage of persons in Study 1 were still smoking at study enrollment (46% vs. 15%, \( P < 0.01 \)). Paired sera were available for analysis for fewer illnesses in Study 1 than in Study 2 (38% vs. 91%, \( P < 0.001 \)).

Eighty-eight respiratory viral infections were identified in 81 illnesses in the two studies; there were seven illnesses in which dual respiratory infections were identified (Table 3). Fifty (57%) of the 88 infections were identified by culture or serologic methods while the RT-PCR assays identified an additional 38 infections. Overall, picornaviruses (rhinoviruses and enteroviruses) were the most common respiratory virus infections identified and the most frequent virus group identified only by RT-PCR. Twenty-eight of the isolated picornaviruses were further classified to the genus level: 24 were rhinoviruses and 4 were enteroviruses.

Influenza viruses and coronaviruses were the next most commonly identified viruses. Fourteen of the 16 influenza virus infections were caused by influenza A viruses. Influenza virus infection was more common in Study 1 where none of the influenza virus-infected subjects were documented to have received influenza vaccine in the prior year. Twelve of the 16 coronavirus infections identified were caused by OC43-like strains. Only one of the four infections with 229E-like strains occurred in hospitalized subjects, and all four of these infections were identified by RT-PCR.

Parainfluenza virus infections occurred in less than 5% of the illnesses. Two, two and five parainfluenza virus infections were seen with types 1, 2 and 3 strains, respectively. RT-PCR identified two additional type 3 infections and one additional type 1 infection. RSV infections were

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**Table 1 (continued)**

| Virus                        | Virus gene target | Oligonucleotide name | Oligonucleotide sequence | Reference  |
|------------------------------|-------------------|----------------------|--------------------------|------------|
| Human metapneumovirus        | Nucleoprotein     | N3F                  | 5’GTC TCT TCA AGG        | This study |
|                              |                   | N479R                | 5’-ATT ATI GGT           | This study |
|                              |                   | N101F                | 5’-CAG TGA CAC CYT CAT CAT TG-3’ | This study |

* For real-time PCR assay.
seen in 3.6% of the subjects. No additional RSV infections were identified by RT-PCR. RT-PCR was the only assay used to identify human metapneumovirus infections, and no infections with this virus were identified.

Fifteen of the respiratory illnesses in study 2 were upper respiratory illnesses only, and all were managed in the outpatient setting. Eighty percent (12/15) of these illnesses were associated with a respiratory viral infection compared to 38% (39/102) of the acute exacerbations of COPD in this study. There were six picornavirus, four coronavirus, two parainfluenza virus, one influenza virus, and one RSV infections (two dual) associated with these common cold illnesses.

### Discussion

The application of RT-PCR assays to clinical specimens collected from patients with COPD and acute respiratory illness significantly increased the number of illnesses in which an associated respiratory viral infection was identified. When culture and serologic studies were used for virus identification, 23.4% of the sampled illnesses were associated with a respiratory virus infection. The addition of RT-PCR assays increased the overall percentage of

### Table 2  
**Study population demographics**

|                  | Study 1 | Study 2 | Both   |
|------------------|---------|---------|--------|
| Number of patients | 63      | 33      | 96     |
| Number of illnesses | 77      | 117     | 194    |
| Mean (SD) age in years | 61.3    | 66.7    | 63.1   |
| Gender—# (% male) | 35 (56%) | 12 (36%) | 47 (49%) |
| Race/ethnicity     |         |         |        |
| Asian              | 1       | 0       | 1      |
| Black              | 39      | 7       | 46     |
| Hispanic           | 5       | 0       | 5      |
| White              | 18      | 26      | 44     |
| Number (%) with a history of smoking | 55 (87%) | 26 (79%) | 81 (84%) |
| Number (%) current smokers | 29 (46%) | 5 (15%) | 34 (35%) |
| Number (%) with a history of influenza vaccination in last year | 38 (49%) | 104 (89%) | 142 (73%) |
| Number (%) hospitalized | 77 (100%) | 29 (25%) | 106 (55%) |
| Number (%) with serologic studies | 29 (38%) | 106 (91%) | 135 (70%) |

* Based on number of illnesses.

### Table 3  
**Number of respiratory viral infections identified in Studies 1 and 2**

|                  | Study 1 Hospitalized (n = 77) | All (n = 117) | Study 2 Hospitalized (n = 29) | Outpatient (n = 88) | Culture or serology | Both Studies | RT-PCR only (n = 194)a |
|------------------|-------------------------------|---------------|-------------------------------|---------------------|---------------------|-------------|------------------------|
| Picornavirus     | 9                             | 30            | 3                             | 27                  | 17                  | 22          | 39 (20.1%)             |
| Influenza A and B virus | 12                           | 4             | 1                             | 3                   | 13                  | 3           | 16 (8.2%)              |
| RSV              | 2                             | 5             | 2                             | 3                   | 7                   | 0           | 7 (3.6%)               |
| Coronavirus 229E and OC43 | 6                           | 10            | 2                             | 8                   | 6                   | 10          | 16 (8.2%)              |
| Parainfluenza virus types 1-3 | 3                             | 6             | 0                             | 6                   | 6                   | 3           | 9 (4.6%)               |
| Adenovirus       | 1                             | 0             | 0                             | 0                   | 1                   | 0           | 1 (0.5%)               |
| Human metapneumovirus | 0                             | 0             | 0                             | 0                   | ND                  | 0           | 0                      |
| Any respiratory viral illness | 30                           | 51            | 8                             | 43                  | 46                  | 35          | 81 (41.8%)             |

* Seven dual infections involving parainfluenza viruses (N = 4), picornaviruses (n = 4), coronaviruses (n = 4), RSV (n = 1), influenza type B virus (n = 1), and adenovirus (n = 1).
respiratory virus-associated illness to 41.8%. This is similar to the ~40% of patients Seemungal et al.\textsuperscript{3} noted to have a respiratory virus infection in association with an acute exacerbation of COPD and somewhat lower than the 56% prevalence rate Rohde et al.\textsuperscript{22} noted in COPD patients hospitalized with an acute exacerbation and the 64% reported by Seemungal et al.\textsuperscript{9} in a later study. The percentage of dual respiratory infections (7/81, 8.6%) is also similar to rates reported in previous studies.\textsuperscript{23}

Picornaviruses and coronaviruses were the most likely viral pathogens to be detected using RT-PCR assays. Picornavirus infections, especially those caused by rhinoviruses, are the most prevalent of respiratory virus infections identified in COPD patients with acute respiratory illnesses in this (44%) and other recent studies (36–58%).\textsuperscript{3,22} Many rhinoviruses grow poorly in cell culture and are only detected when RT-PCR assays are used.\textsuperscript{3,12,13,24,25} Similarly, coronaviruses are either difficult to grow (229E) or do not grow at all (OC43) using current cell culture methods, so it is not surprising that the number of infections identified increases with the application of RT-PCR techniques. Coronaviruses are associated with 11–20% of viral illnesses in COPD patients when RT-PCR assays are used.\textsuperscript{3,25}

Influenza virus remains an important cause of virus infection in COPD patients, especially in the hospitalized patient. Fifteen of the 77 (19%) illnesses in Study 1 were associated with an influenza virus infection compared to only four of 117 (3.4%) in Study 2. Less than half of the patients in Study 1 had received influenza vaccine in the previous year while almost 90% of the patients in Study 2 had accepted influenza vaccine in the same time frame. The use of influenza vaccine is safe, effective and recommended for this population.\textsuperscript{26–28} Nevertheless, lack of use of influenza vaccine persists as a modifiable risk factor in patients hospitalized with COPD.\textsuperscript{29}

Respiratory syncytial virus is increasingly being recognized as an important viral pathogen in older adults.\textsuperscript{30–32} Just under 4% of the illnesses evaluated in the two studies were associated with RSV infection, a number similar to that observed in some studies but lower than that reported in others.\textsuperscript{3,22,33} Differences in methods used to identify RSV infection and differences in the sensitivities in the RT-PCR assays likely contribute to the differences observed between studies.\textsuperscript{34}

Human metapneumovirus is a newly recognized paramyxovirus that causes lower respiratory disease in children.\textsuperscript{35,36} The importance of this pathogen in adults is less well defined. Falsey et al.\textsuperscript{37} found up to 10% of subjects admitted with acute cardiopulmonary conditions had serologic evidence of acute human metapneumovirus infection. Prevalence rates were somewhat lower in an ambulatory geriatric population, and there was considerable variability between the two study years. Cases of human metapneumovirus infection also have been observed in elderly patients hospitalized with chronic lower respiratory diseases or immunosuppression.\textsuperscript{38,39} In contrast, we failed to identify any illnesses associated with human metapneumovirus infection. Our RT-PCR assay for human metapneumovirus was designed to detect all known groups of human metapneumovirus and has detected these viruses in another study involving hospitalized children (unpublished data), differences in assay performance could explain the results observed. Alternatively, virus circulation during the seasons studied may have been low enough to prevent detection.\textsuperscript{37}

A potential weakness of this study is that we did not evaluate the impact of respiratory viruses in our two populations during times of clinical stability. Seemungal et al.\textsuperscript{9} reported detection of respiratory viruses other than RSV in 16% of COPD patients during clinical stability and detection of RSV in 23.5%. We have recently completed an evaluation of another cohort of chronic bronchitic patients during periods of clinical stability (unpublished data). The prevalence of RT-PCR positivity (excluding coronavirus 229E and human metapneumovirus) ranged from 0 to 2% for individual agents, with an overall prevalence of 6.4% in 143 encounters. The overall frequency of identification of a respiratory viral infection during illness was similar to that seen in this study. Thus, our experience with the application of RT-PCR assays to cohorts with COPD differs from that described by Seemungal et al.\textsuperscript{9}

In summary, the application of RT-PCR assays to clinical specimens collected from patients with COPD during clinical respiratory illness significantly increased the number of respiratory viruses identified. Rhinoviruses, coronaviruses and influenza viruses were the most common respiratory virus infections in this patient population, and no human metapneumovirus infections were found. Respiratory virus infections are an important cause of respiratory illness in COPD patients.

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