The role of viruses in the aetiology of community-acquired pneumonia in adults

Maria Angeles Marcos[1], Marta Camps[1], Tomás Pumarola[1], José Antonio Martinez[2], Esteban Martinez[2], Josep Mensa[2], Elisa Garcia[2], Georgina Peñarroja[2], Póvilas Dambrava[3], Inmaculada Casas[4], Maria Teresa Jiménez de Anta[1] and Antoni Torres[3]*

[1]Department of Microbiology, Hospital Clínic i Provincial de Barcelona, Spain
[2]Department of Infectious Diseases, Hospital Clínic i Provincial de Barcelona, Spain
[3]Department of Pneumology, Institute of Pneumology and Thoracic Surgery, Hospital Clínic i Provincial de Barcelona, Institut d´Investigacions Biomédiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Spain
[4]Department of Virology Centro Nacional de Microbiología, Institute de Salud Carlos III, Madrid, Spain

*Corresponding author: Tel: +34 93 227 5522; Fax: +34 93 227 9372; E-mail: mmarcos@clinic.ub.es

Background: The role of viruses in community-acquired pneumonia may have been previously underestimated. We aimed to study the incidence and clinical characteristics of community-acquired pneumonia (CAP) due to respiratory viruses in adults adding PCR to routine conventional laboratory tests.

Methods: Consecutive adult patients diagnosed of CAP from January 2003 to March 2004 were included. Conventional tests including cultures of blood, sputum, urine antigen detection of *Streptococcus pneumoniae* and *Legionella pneumophila*, and paired serologies were routinely performed. Nasopharyngeal swabs were processed for study of respiratory viruses through antigen detection by indirect immunofluorescence assay, isolation of viruses in cell culture and detection of nucleic acids by two independent multiplex RT-PCR assays. According to the aetiology, patients were categorized in 4 groups: group 1, only virus detected; group 2, only bacteria detected; group 3, viral and bacterial; and group 4, unkown aetiology.

Results: Of 340 patients diagnosed with CAP, 198 had nasopharyngeal swabs available and were included in this study. Aetiology was established in 112 (57%) patients: group 1, *n*=26 (13%); group 2, *n*=66 (33%); group 3, *n*=20 (10%). The most common aetiological agent was *S. neumoniae* (58 patients, 29%), followed by respiratory viruses (46 patients, 23%). Forty-eight respiratory viruses were identified: influenza virus A (*n*=16), respiratory syncytial virus A (*n*=5), adenovirus (*n*=8), parainfluenza viruses (*n*=5), enteroviruses (*n*=1), rhinoviruses (*n*=8) and coronavirus (*n*=5). There were two patients coinfected by two respiratory viruses. Serology detected 6 viruses, immunofluorescence 8, viral culture 12, and PCR 45. For the viruses that could be diagnosed with conventional methods, the sensitivity and specificity of RT-PCR was 85% and 92%, respectively. The only clinical characteristic that significantly distinguished viral from bacterial aetiology was a lower number of leukocytes (*P*=0.004).

Conclusion: PCR revealed that viruses represent a common aetiology of CAP. There is an urgent need to reconsider routine laboratory tests for an adequate diagnosis of respiratory viruses, as clinical characteristics are unable to reliably distinguish viral from bacterial aetiology.

Introduction

Community-acquired pneumonia (CAP) is a common disease, representing the most frequent cause of hospital admission and mortality of infectious origin in developed countries [1]. Despite enormous efforts in well-designed prospective studies, the aetiology of CAP has remained elusive in a high proportion of patients [2,3].

Recently, several studies have stressed that the involvement of viruses in CAP may have been underestimated, and this underestimation has been attributed to a lack of appropriate diagnostic methods. The use of conventional laboratory methods such as viral isolation in cell culture, antigen assays and serology for the diagnosis of viral respiratory infections has shown major limitations, including low sensitivity, low specificity and prolonged time for achieving results [4–7]. Nonetheless, the introduction of rapid molecular techniques such as gene amplification has improved the diagnostic yield of viruses causing CAP [8,9].

Most clinical reports focusing on viruses as potential aetiological agents of CAP have either analysed

selected subgroups of patients, such as children or immunosuppressed patients, or have focused on a specific diagnostic method [10–13]. For several years our group has been working on improving the diagnosis of CAP in adults. With this aim in mind, we designed a prospective observational study to specifically determine the incidence of CAP due to respiratory viruses, the differential characteristics compared with CAP of bacterial origin and the yield of several laboratory methods for the aetiological diagnosis of viral CAP in adults.

Materials and methods

Patients and study design

From January 2003 to March 2004, consecutive patients older than 14 years admitted to our hospital with a diagnosis of CAP were prospectively studied. Patients with any potential cause of immunosuppression were also included [14]. For the purpose of the study, only patients with nasopharyngeal swabs for study of respiratory viruses available have been included in the current report. Despite special efforts to get nasopharyngeal swabs for every patient admitted with CAP, some patients, particularly those who arrived during busy days at the emergency ward and those who arrived at night, did not have the swab, as this was not a routine procedure.

CAP was defined as the presence of a new infiltrate visualized on chest radiography together with clinical symptoms suggestive of lower respiratory tract infection and no alternative diagnosis in a patient not admitted to hospital within the previous month and in whom no alternative diagnosis was established during follow-up. Clinical, laboratory and radiological features at presentation as well as other epidemiological data were recorded in a specific questionnaire and entered in a computer database. Part of this study population has been included in previous studies with objectives other than those of the present study [2,15,16]. The Ethics Committee from Hospital Clinic had previously approved the study.

Microbiological evaluation

Regular sampling for microbiological diagnosis included sputum, blood for culture, urine samples for the detection of *Legionella pneumophila* antigen (Binax NOW Legionella Urinary Antigen Test) and *Streptococcus pneumoniae* antigen (Binax NOW *S. pneumoniae* Urinary Antigen Test) [15] and nasopharyngeal swabs. Pleural puncture, tracheobronchial aspiration (BAS) and bronchoalveolar lavage (BAL) clinical samples were obtained according to clinical indication or judgement of the attending physician. For serological testing, acute and convalescent phase samples were analysed in the same assay with a commercially available type-specific complement fixation kit (Diagnostica Senese SpA, Monteriggioni, Italy). Serological studies for *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Coxiella burnetti*, *Legionella pneumophila* and respiratory viruses including influenza virus A and B, parainfluenza virus 1, 2 and 3, respiratory syncytial virus (RSV) and adenovirus were performed. Processing of samples and diagnostic criteria of bacterial aetiology have been described elsewhere [7,15,16].

Nasopharyngeal samples were obtained by inserting a swab into both nostrils parallel to the palate (Mini-Tip Culture Direct, Becton-Dickinson Microbiology Systems, MD 21152 USA) and a second swab from the posterior pharyngeal and tonsillar areas (Viral Culturette, Becton-Dickinson Microbiology Systems, MD, USA). Both nasal and pharyngeal swabs were placed into a same tube with viral transport medium (minimal essential medium with 2% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 20 μg/ml, neomycin 40 μg/ml, $NaHCO_3$ buffer). Within 24 h after admission, the homogenized samples were processed for antigen detection by indirect immunofluorescence assay (IFA), for isolation of viruses in cell culture and for detection of nucleic acids by two independent multiplex RT- PCR.

For IFA, the specimens were spotted onto glass slides and then dried and fixed in acetone. Monoclonal antibodies were used for detection of influenza A and B viruses, parainfluenza viruses 1, 2 and 3, RSV and adenovirus (Chemicon International Temecula, CA). The presence of viral antigen in respiratory cells was indicated by the appearance of characteristic intracellular apple-green fluorescence in ≥1 cell.

Specimens were inoculated into two human epithelial cells (Hep-2 and A-549) and into Madin–Darby canine kidney cells (MDCK) for primary viral isolation of influenza viruses, parainfluenza viruses, RSV and adenoviruses and incubated at 35ºC during 3 weeks. Conventional cultures were examined twice weekly for the development of a cytopathic effect. In cultures with positive results, virus was identified by staining with IFA. When Hep-2 cells had a cytopathic effect and the IFA for respiratory viruses was negative, IFA for herpes simplex virus was performed.

For multiplex RT-PCR, viral genomic RNA and DNA was extracted from a total volume of 200 μl of sample, by the guanidinium thiocyanate extraction method [17]. The lysis buffer included 500 molecules of the cloned amplified product used as internal control in each reaction tube and then excluded false-negative results due to non-specific inhibitors or extraction failure. Two independent multiplex reverse

transcription nested RT-PCR assays able to detect from 1 to 10 copies of viral genomes were performed as described previously [18,19]. We used specific primers for influenza viruses types A, B and C, RSV type A, RSV type B, adenovirus in one RT-PCR assay, and specific primers for parainfluenza viruses 1, 2, 3 and 4, coronaviruses 229E and OC43, and for generic detection of rhinoviruses and enteroviruses in another RT-PCR assay. In each assay, negative (viral transport medium containing no nucleic acid) and positive controls (cDNAs obtained from our viral lysates or from reference strains) were treated with the same procedure. All positive results were confirmed in two sequential assays.

Viral aetiology was considered definitive if at least one of the following criteria was fulfilled: a four-fold increase in the IgG titer for respiratory viruses; virus isolation in cell cultures; detection of respiratory viruses by RT-PCR in two different and independent assays; detection of antigens by IFA plus virus isolation or detection by RT-PCR.

Definition of groups of CAP according to aetiology

For the purpose of the study, patients were categorized in 4 groups: group 1, only virus detected; group 2, pathogenic bacteria detected; group 3 or mixed, virus and bacteria detected; and group 4, unkown aetiology.

Statistical analysis

Quantitative characteristics were described for each of the four groups (virus, bacteria, mixed and without aetiology) with median and interquartile ranges (IQRs). Comparisons between virus, bacteria and virus plus bacteria groups were made using the Kruskall–Wallis test and the Mann–Whitney test was used in each of the three pairwise comparisons. Qualitative characteristics were reported as frequencies and percentages for all four groups and compared between the three groups [1,–3] using the Fisher´s exact or χ^2 test; and the same test was used for pairwise comparisons.

All the tests were two-tailed and the confidence level was set at 95%. Bonferroni´s correction of the significance level was used for three pairwise comparisons. The sensitivity and specificity of RT-PCR compared with the conventional methods were calculated using standard methods [20]. The comparison of the proportion of viruses detected by RT-PCR with the proportion of viruses detected by the other assays was calculated using the McNemar's test. For the purpose of the analysis, coronaviruses, rhinoviruses and enteroviruses (group 1 and 3) were included in the comparison of clinical characteristics (Table 1 and Table 2), but they were excluded from the comparison of diagnostic techniques due to the fact that these viruses are exclusively detected by RT-PCR.

Results

Patients and samples

Of the 340 patients with CAP admitted to our hospital during the study period, 198 patients with nasopharyngeal swabs for studying the respiratory viruses were included in this study.

Urine samples for detection of *L. pneumophila* and *S. pneumoniae* antigens and blood for culture were obtained from all the 198 patients. Sputum was obtained from 116 (59%) patients with 70 (60%) samples with good quality criteria (21). Pleural fluid was obtained from 8 (4%), BAS from 6 (3%) and BAL from 3 (2%) patients. Paired serology was obtained in 66 (33%) patients.

Aetiology of CAP

The aetiology of CAP was established in 112 (57%) patients (Table 3). According to the aetiological diagnosis, 26 (13%) cases of CAP were due to viruses (group 1), 66 (33%) to bacteria (group 2), 20 (10%) to viruses and bacteria concomitantly (group 3), and 86 (43%) were of unknown aetiology (group 4).

The most common aetiological agent was *S. pneumoniae* (58 patients, 29%), followed by respiratory viruses (46 patients, 23%). Forty-eight respiratory viruses were identified: influenza virus type A (n=16), RSV type A (n=5), adenovirus (n=8), parainfluenza viruses (n=6), enteroviruses (n=1), rhinoviruses (n=8), and coronaviruses (n=4). Multiple viral aetiology was found only in two cases, in the group 1 (influenza type A plus coronavirus 229E and RSV plus coronavirus OC43). We identified herpes simplex virus type 1 in 2 cases of CAP both of which also had *S. pneumoniae* and other viruses.

Comparison of laboratory techniques for diagnosis of viral CAP

Table 4 summarizes the yield of different laboratory techniques for the diagnosis of influenza type A virus, RSV, adenovirus and parainfluenza virus. Hence, 35 respiratory viruses were detected by at least one of the several diagnostic methods used. Immunofluorescence detected 8 respiratory viruses (23%) and the positive results were also confirmed with at least one of the other techniques used (Table 4). Viral culture allowed the isolation of 12 respiratory viruses (34%; Table 4).

Adequate paired serology samples were collected only in 7 out of the 35 (20%) patients with a diagnosis of viral CAP. Serology identified six respiratory viruses (Table 4). RT-PCR allowed the detection of 32 out of 35 viruses (91%; Table 4). Three viruses were detected by methods

Table 1. Baseline characteristics of the patients according to the aetiology of CAP

	Viral CAP (*n*=26)	Bacterial CAP (*n*=66)	Mixed CAP (*n*=20)	Unknown aetiology (*n*=86)	Total (*n*=198)	*P*-value
Median age, years (IQR)	64.5 (45–78)	65 (46–79)	74 (46–78)	73 (52–81)	70 (49–79)	0.972
Sex						
Female	13 (50)	28 (42)	10 (50)	32 (37)	83 (42)	0.750
Male	13 (50)	38 (58)	10 (50)	54 (63)	115 (58)	
Any comorbidity	21 (81)	51 (77)	19 (95)	68 (79)	159 (80)	0.206
Diabetes mellitus	5 (21)	12 (18)	4 (22)	17 (20)	38 (20)	0.835
Renal	2 (8)	1 (2)	0 (0)	17 (20)	20 (10)	0.191
Hepatic cirrhosis	2 (8)	5 (8)	1 (6)	2 (2)	10 (5)	1.000
CNS	2 (8)	3 (5)	3 (17)	12 (14)	20 (10)	0.173
Pulmonary	12 (50)	36 (55)	13 (72)	44 (52)	105 (54)	0.346
Heart	3 (13)	7 (11)	2 (12)	20 (24)	32 (17)	0.913
HIV	3 (13)	11(17)	4 (22)	6 (7)	24 (13)	0.666
Neoplastic disease	3 (13)	2 (3)	0 (0)	5 (6)	10 (5)	0.132
Alcohol use >80 g/day	0 (0)	5 (8)	2 (11)	2 (2)	9 (5)	0.295
Current smoker	6 (24)	25 (38)	6 (30)	13 (15)	50 (26)	0.427
Antibiotics prior to admission	3 (13)	15 (23)	2 (10)	13 (15)	33 (17)	0.395
Previous pneumonia	6 (24)	14 (21)	5 (25)	21 (25)	46 (23)	0.857
PSI class						
I+II+III	15(65)	36 (55)	9 (47)	42 (49)	102 (53)	
IV+V	8 (35)	30 (45)	10 (53)	43 (51)	91 (47)	0.493
Season						
Spring–summer	10 (38)	37 (56)	8 (40)	54 (63)	109 (55)	
Autumn–winter	16 (62)	29 (44)	12 (60)	32 (37)	89 (45)	0.221
Influenza vaccine	3 (12)	14 (22)	5 (25)	29 (34)	51 (26)	0.459

Data are number (percentage) unless otherwise specified. *P*-values refer to the comparison among viral community-aquired pneumonia (CAP), bacterial CAP and mixed CAP. CNS, central nervous system; IQR, interquartile range; PSI, pneumonia severity index score.

other than RT-PCR: two influenza type A virus (both by cell culture) and one adenovirus (by serology). In addition, RT-PCR was the only technique allowing for the detection of enteroviruses (*n*=1), coronaviruses (*n*=4) and rhinoviruses (*n*=8).

The yield of the RT-PCR assay was higher than that of conventional methods (*P*=0.007, McNemar test) with a sensitivity and specificity of this assay being 85% and 92%, respectively.

Characteristics of patients

The characteristics of the patients are summarized in Tables 1 and 2. Of the 198 patients, 115 (58%) were men and 83 (42%) women, with a median age of 70 years (IQR: 49–79). All the patients were admitted to hospital and 17 (9%) required transfer to the intensive care unit. At least one predefined comorbidity or immunosuppression was identified in 159 (80%) patients, with pulmonary comorbidity being the most prevalent (53%). Thirty-three patients (17%) had received antimicrobial therapy prior to hospital admission. We did not find any differences regarding age, gender, toxic habits, comorbidity, previous antimicrobial treatment or PSI score. Only 51 patients (26%) had received influenza vaccine prior to the episode of CAP, and none of them presented with CAP due to influenza virus.

Comparing CAP of viral aetiology versus CAP of bacterial aetiology, the number of leukocytes (×1,000/μl; median, IQR) was significantly lower in viral CAP (9.0, 6.1–11.1) compared with that of bacterial origin (14.3, 10.4–20.0) (*P*=0.004). Although most cases of viral CAP occurred in autumn or winter with a peak incidence in March, there were no significant differences between groups (Figure 1).

Five patients (2.5 %) died: one with CAP due to adenovirus, another one with CAP due to *Escherichia coli* and three with CAP of unknown aetiology. All these 5 patients had presented with any comorbidity.

Discussion

Respiratory viruses have been recently recognized as a potential common cause of pneumonia in adults ranging from 2% to 23% [1,7,22]. In our study, *S. pneumoniae* was the predominant aetiological agent, in both bacterial and in mixed CAP, but viruses

Table 2. Clinical presentation and evolution of the patients according to the aetiology of CAP

	Viral CAP (*n*=26)	Bacterial CAP (*n*=66)	Mixed CAP (*n*=20)	Unknown aetiology (*n*=86)	Total (*n*=198)	*P*-value
Clinical presentation						
Days with symptoms*	4 (3–7)	5 (3–7)	4 (3–7)	4 (2–7)	4 (2–7)	0.986
Leukocytes ×1,000/μl*	9.0 (6.1–11.1)	14.3 (10.5–20.0)	12.4 (9.1–16.7)	13.5 (9.4–17.2)	12.9 (9–17.9)	0.004
C reactive protein*, mg/dl	10.2 (5.9–14.5)	16.8 (6.8–25.7)	20 (14–43.5)	11 (4.2–21.5)	13.8 (6.0–24.7)	0.082
PO_2, mm Hg*	63 (54–69)	63 (56.0–67.0)	62 (58.0–76.0)	65 (56.0–80.0)	64 (56.0–73.0)	0.939
Temperature at admission, °C*	38 (37.5–38.5)	38 (37.3–38.6)	38 (37.8–39.3)	38 (37.2–38.7)	38 (37.3-38.6)	0.363
Creatinine ≥1.5 mg/dl	2 (9)	15 (23)	5 (25)	24 (28)	46 (24)	0.309
Upper respiratory viral illness	7 (29)	16 (25)	6 (32)	13 (15)	42 (22)	0.753
Digestive symptoms	1 (4)	13 (20)	0 (0)	8 (9)	22 (11)	0.020
Mental confusion	1 (4)	5 (8)	4 (20)	6 (7)	16 (8)	0.189
Cough	23 (96)	54 (83)	15 (79)	70 (81)	162 (84)	0.190
Expectoration	16 (62)	38(58)	15 (75)	44(51)	113 (57)	0.407
Pleuritic chest pain	9 (38)	37 (56)	8 (40)	45 (52)	99 (51)	0.211
Dyspnea	17 (68)	45 (68)	10 (50)	61 (71)	133 (68)	0.329
Radiographic patterns						
Alveolar pattern	25 (96)	60 (93)	19 (100)	78 (93)	176 (93)	0.897
Interstitial pattern	1 (4)	6 (7)	0 (0)	9 (9)	16 (7)	
Pleural effusion	1 (4)	9 (14)	0 (0)	9 (10)	19 (10)	0.147
Evolution						
ICU admission	4 (15%)	6 (9)	5 (25)	2 (2)	17 (9)	0.150
Days of stay*	6 (4–8)	8 (5–10)	7 (5–10)	5 (3–7)	6 (4–9)	0.208
Any complication	2 (8)	14 (21)	1 (6)	6 (7)	23 (12)	0.224
Mechanical ventilation	2 (8)	3 (5)	1 (6)	0 (0)	6 (3)	0.840
Renal failure	0 (0)	12 (16)	2 (2)	5 (5)	19 (10)	0.074
Shock	1 (4)	8 (12)	1 (6)	1 (1)	11 (6)	0.573
Death	1 (4)	2 (3)	0 (0)	3 (3)	6 (3)	0.663

*Quantitative characteristics are described with median and interquartile range (IQR), and the rest as number (percentage). *P*-values refer to the comparison among viral community-aquired pneumonia (CAP), bacterial CAP and mixed CAP. ICU, intensive care unit.

represented the second cause of CAP. Influenza virus type A was the most common virus found followed by adenovirus and rhinovirus. We did not find cases of CAP due to influenza virus type B, probably because the patients studied corresponded to the 2003–2004 influenza season, in which influenza type A viruses (H3N2) were exclusively detected throughout Spain. A high proportion (43%) of viral CAP cases had mixed infections with at least one bacterium. Identification of more than one aetiological agent may reflect the role of one organism in leading to infection with a second agent [23]. Previous studies have shown that viral infections of the respiratory tract predispose to bacterial superinfections [24]. Structural and functional disruption of the respiratory mucosal epithelium appear to be a major contributor to the synergistic effects of superinfection. More convincing data have been reported in experimental human infections with influenza type A. In one study, 15% of influenza-infected individuals became nasopharyngeally colonized with *S. pneumoniae* 6 days after viral challenge, whereas at the start of the study none were colonized with this bacterium [25]. These data suggest that a proportion of CAP due to *S. pneumoniae* may be preceded by a viral infection and that at the time of diagnosis this virus may not be detected in the nasapharynx because of viral clearance in the first days [26]. These facts support that achieving a diagnosis of upper respiratory viral infection as well as CAP of viral origin as early as possible would allow to administer

Table 3. Aetiology of community acquired pneumonia in the adult

Pathogen	Patients, *n* (112)
Viruses (group 1)	**26**
Influenza A	11
Influenza A + coronavirus 229E	1
Respiratory syncytial virus (RSV) type A	3
RSV type A + coronavirus OC43	1
Adenovirus	3
Parainfluenza virus type 1	1
Parainfluenza virus type 2	2
Parainfluenza virus type 3	1
Coronavirus 229E	1
Rhinovirus	2
Bacteria (group 2)	**66**
Streptococcus pneumoniae	41
S. pneumoniae + *Coxiella burnetti*	1
S. pneumoniae + *Pseudomonas aeruginosa*	1
Escherichia coli	1
Haemophilus influenzae	7
Staphylococcus aureus	3
S.aureus + *Coxiella burnetti*	1
Pseudomonas aeruginosa	1
Mycoplasma pneumoniae	3
Chlamydophila pneumoniae	1
Legionella pneumophila	6
Viruses and bacteria (group 3)	**20**
S. pneumoniae + influenza A	2
S. pneumoniae + adenovirus	3
S. pneumoniae + *S. aureus* + adenovirus	1
S. pneumoniae + RSV type A	1
S. pneumoniae + parainfluenza virus type 1	2
S. pneumoniae + coronavirus 229E + herpes simplex 1	1
S. pneumoniae + rhinovirus	3
S. pneumoniae + *Haemophilus influenzae* + rhinovirus	1
S. pneumoniae + herpes simplex 1 + enterovirus	1
Haemophilus influenzae + rhinovirus	2
S. aureus + influenza A	1
Escherichia coli + adenovirus	1
Haemophilus influenzae + influenza A	1

specific antiviral therapy that would contribute to reduce the risk of bacterial infection [27,28]. This would be particularly important in patients with any comorbidity, because they represent more than 80% of the population studied in our study.

It has been previously shown that any clinical, laboratory, or radiographic findings are poor indicators of aetiological diagnosis in CAP [2,3,29]. This was also the case in our study. Despite the use of an extensive database for each patient, we hardly found any difference among the aetiological groups studied. Seasonality has been considered a distinctive feature of viral CAP due to a greater incidence of some respiratory viruses in the community [30]. This holds true for influenza virus and RSV, but not for others such as parainfluenza and adenovirus that may be present during the whole year. In our study, viral CAP was characterized by significantly less leukocytes compared with bacterial CAP. The high levels of CRP found in patients with viral pneumonia are puzzling; a potential interpretation may be that these patients actually had a secondary but not always documented bacterial infection [31,32].

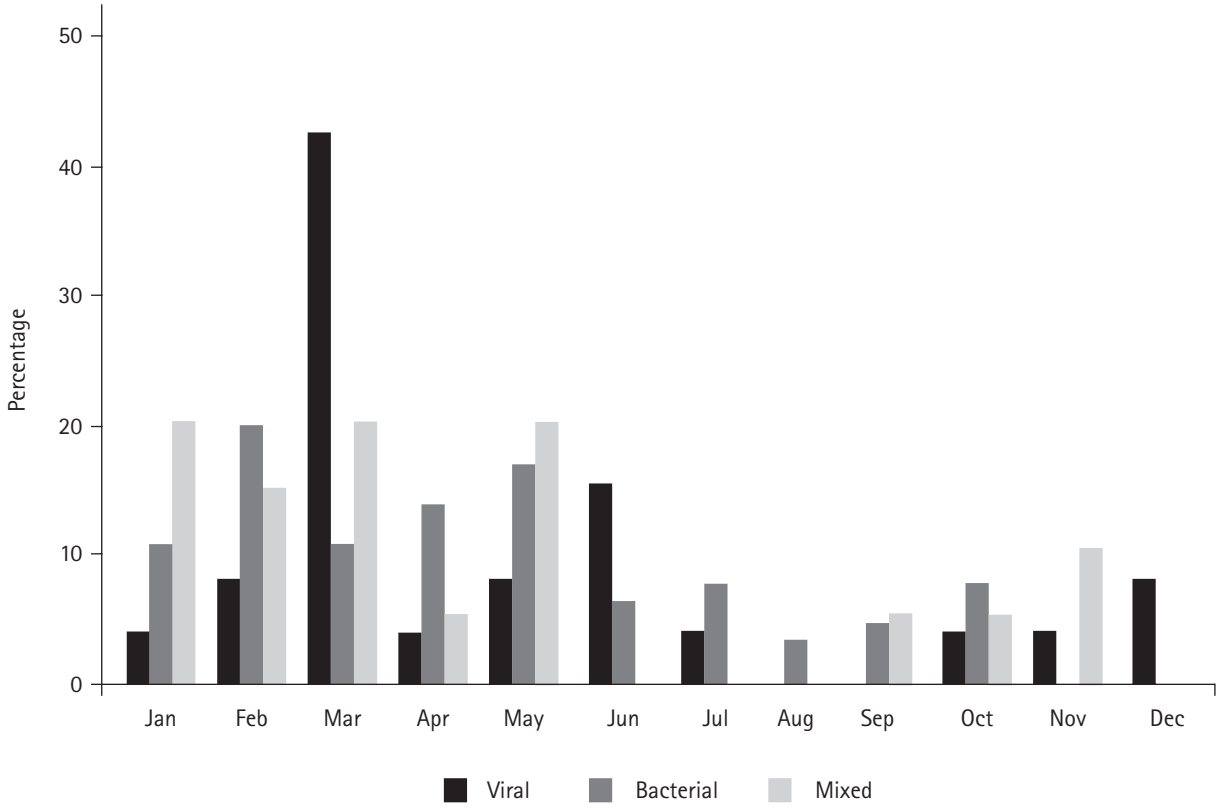
Until very recently, the true incidence of respiratory viruses as a cause of CAP has been underestimated, fundamentally because of the limitations of the diagnostic methods used. Although viral culture has been the 'gold standard' for the diagnosis of respiratory viruses, it shows important limitations. Its results are usually not available for several days and its yield is closely related to the amount and viability of the viruses harboured in the clinical samples, similar to what occurs with direct antigen detection techniques (for example, IFA). In adults, the viral nasopharyngeal load appears lower in CAP than in upper respiratory tract illnesses [6]. In a study on upper respiratory infections performed in our laboratory, the yield of cell culture was greater than that found in present study on CAP, and similar to that found with RT-PCR techniques [33]. In the present study, cell culture allowed diagnosis only of approximately one-third of the respiratory viruses. Serological tests are not helpful in the early management of CAP since serocoversion is required between acute and convalescent serum before adscribing the cause of the disease to a specific pathogen, which may delay the diagnosis up to 2–3 weeks. In addition, the difficulties for obtaining covalescent serum in a high proportion of patients should be recognized. Adequate paired serology samples were obtained only in one third of the patients in our study. Of six cases of viral CAP diagnosed by serology in our study, five were also confirmed by RT-PCR and in the remaining case the patient had presented with clinical manifestations during more than 1 week. In our study we used nested RT-PCR, which is capable of detecting simultaneously several respiratory viruses with high sensitivity [17,18]. Moreover, this assay includes a rapid single method of genomic extraction with guanidinium thiocyanate acid buffer for the isolation of either RNA or DNA, which allows the freezing of the genomic product extracted and makes it possible to perform posterior RNA and DNA virus and even atypical bacteria studies.

The results of our study suggest that RT-PCR represents an important improvement over the other methods available, diagnosing 94% of the 48 respiratory viruses compared with the 44% diagnosed with

Table 4. Yield of different laboratory techniques for the diagnosis of respiratory viruses

	Respiratory syncitial virus (*n*=5)	Influenza virus type A (*n*=16)	Adenovirus (*n*=8)	Parainfluenza viruses (*n*=6)	Total (*n*=35)
Immunofluorescence (IFA)	0	0	0	0	0
Viral culture (VC)	0	2	0	0	2
Serology	0	0	1	0	1
PCR	1	5	5	4	15
PCR + IFA	2	0	0	0	2
PCR + VC	0	3	1	0	4
PCR + Serology	0	4	0	1	5
PCR + VC + IFA	2	2	1	1	6

Figure 1. Monthly distribution of CAP cases according to the aetiology



conventional methods. Twenty-eight viruses were detected exclusively by RT-PCR. We believe that these represented true positive results because all the patients included had CAP and because the presence of respiratory viruses in swabs denotes a recent and not a latent infection [26], although our study did not include non-symptomatic controls.

We found a higher sensitivity of RT-PCR for respiratory viruses compared with conventional methods largely because PCR is able to detect the virus genome when it is present at low titres or when the virus is not replication-competent [8,9,34,35]. In addition, RT-PCR is able to detect certain virus that are hardly detected with conventional methods. Nevertheless, we found two cases with culture-positive influenza type A virus in which RT-PCR had been negative. Although the presence of an inhibitor in specimens may cause false-negative results, when our samples were tested no inhibitor was detected. Alterations in the genome that result in the virus being undetectable by the assay are a potential problem for all molecular assays. These data suggest that in the study of CAP, RT-PCR and cell

cultures may be complementary methods of diagnosis. In addition, conventional cell culture continues to be important for selected uses that are not yet amenable to RT-PCR (for example, antigenic characterization and influenza vaccine strain selection).

Despite the improvement in the diagnosis of CAP in our study, a substantial proportion of patients still remained without any aetiological diagnosis. However, a valuable genomic product has been kept and it will allow us to address the role of other respiratory viruses and atypical bacteria potentially involved in CAP [36].

In summary, PCR revealed that viruses represent a common aetiology of CAP. The incorporation of RT-PCR into the routine aetiological diagnosis of CAP seems justified to achieve an early and accurate diagnosis of viral CAP and thereby to allow adequate therapy to be administered, to prevent the nosocomial spread of the disease and to alert physicians as to the possible onset of epidemics [37,38].

Acknowledgements

This work was supported by grants from Sociedad Española de Neumología y Cirugía Torácica (SEPAR), from the Spanish Ministry of Health (FIS PI020305) and from red Respira ISCII v-2003-red 11B-O.

References

1. File TM. Community-acquired pneumonia. *Lancet* 2003; **362:**1991–2001.
2. Ruiz M, Ewig S, Marcos MA, *et al.* Etiology of community acquired pneumonia in hospitalized patients: impact of age, comorbidity and severity. *Am J Resp Crit Care Med* 1999; **160:**397–405.
3. Jokinen C, Heiskanen L, Juvonen H, *et al.* Microbial etiology of community-acquired pneumonia in the adult population of 4 municipalities in eastern finland. *Clin Infect Dis* 2001; **32:**1141–1154.
4. Chien JW, Johnson JL. Viral pneumonias: multifaceted approach to an elusive diagnosis. Postgraduate Medicine 2000; **107:**67–72.
5. Flamaing J, Engelmann I, Joosten E, *et al.* Viral lower respiratory tract infection in the elderly: a prospective in-hospital study. *Eur J Clin Microbiol Infect Dis* 2003; **22:**720–725.
6. Casiano-Colon AE, Hulbert BB, Mayer TK, Walsh EE, Falsey AR. Lack of sensitivity of rapid antigen tests for the diagnosis of respiratory syncytial virus infection in adults. *J Clin Virol* 2003; **28:**169–174.
7. De Roux A, Marcos MA, Garcia E, *et al.* Viral community-acquired pneumonia in nonimmunocompromised adults. *Chest* 2004; **125:**1343–1351.
8. Lolios L, Jenney A, Spelman D, *et al.* Comparison of a multiplex reverse transcription-PCR-enzyme hybridisation assay with conventional viral culture and immunofluorescence techniques for the detection of seven viral respiratory pathogens. *J Clin Microbiol* 2001; **39:**2779–2783.
9. Van Elden LJ, van Kraaij MG, Nijhuis M, *et al.* Polymerase chain reaction is more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia. *Clin Infect Dis* 2002; **34:**177–183.
10. Carrigan DR. Adenovirus infectious in immunocompromised patients. *Am J Med* 1997; **102:**71–74
11. Martino R, Rámila E, Rabella N, *et al.* Respiratory virus infections in adults with hematologic malignancies: a prospective study. *Clin Infect Dis* 2003; **36:**1–8.
12. Henrickson KJ. Viral pneumonia in children. *Semin Pediat Infect Dis* 1998; **9:**217–233.
13. Weinberg A, Zamora MR, Li S, Torres F, Hodges TN. The value pf polymerase chain reaction for the diagnosis of viral respiratory tract infections in lung transplant recipients. *J Clin Virol* 2002; **25:**171–175.
14. Ewig S, Ruiz M, Mensa J, *et al.* Severe community-acquired pneumonia:assessment of severity criteria. *Am J Resp Crit Care Med* 1998; **158:**1102–1108.
15. Marcos MA, Jiménez de Anta MT, de la Bellacasa JP, *et al.* Rapid urinary antigen test for diagnosis of pneumococcal community-acquired pneumonia in adults. *Eur Resp J* 2003; **21:**209–214.
16. Garcia-Vazquez E, Marcos MA, Mensa J, *et al.* Assessment of the usefulness of sputum culture for diagnosis of community-acquired pneumonia using the PORT predictive scoring system. *Arch Intern Med* 2004; **164:**1807–1811.
17. Casas I, Powell L, Klapper PE, Cleator GM. New method for the extraction of viral RNA and DNA from cerebrospinal fluid for use in the polymerase chain reaction. *J Virol Methods* 1995; **53:**25–36.
18. Coiras MT, Perez-Breña P, Garcia ML, Casas I. Simultaneous detection of influenza A, B, and C viruses, respiratory syncityal virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. *J Med Virol* 2003; **69:**132–144.
19. Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Breña P. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. *J Med Virol* 2004; **72:**484–495.
20. Motulsky H. *Intuitive Biostatistics*. New York: Oxford University Press, Inc.; 1995, pp. 129–139.
21. Murray PR, Washington JA. Microscopic and bacteriologic analysis of expectorated sputum. *Mayo Clin Procced* 1975; **50:**339–344.
22. Howard LSGE, Sillis M, Pasteur MC, Kamath AV, Harrison BDW. Microbiological profile of conmunity-acquired pneumonia in adults over the last 20 years. *J Infect* 2005; **50:**107–113.
23. Schwarzmann SW, Adler JL, Sullivan RJ. Bacterial pneumonia during the Hong Kong influenza epidemicof 1968–1969. *Arch Intern Med* 1971; **127:**1037–1041.
24. Bakaletz LO. Viral potentiation of bacterial superinfeccion of the respiratory tract. *Trends Microbiol* 1995; **3:**110–114
25. Doyle WJ, Skoner DP, Hayden F, *et al.* Nasal and otologic effects of experimental influenza A virus infection. *Ann Otol Rhinol Laryngol* 1994; **103:**59–69.
26. Hall CB. Respiratory syncytial virus and parainfluenza virus. *N Eng J Med* 2001; **344:**1917–1928.
27 Glezen WP,Greenberg SB, Atmar RL. Impact of respiratory virus infections on persons with chronic underlying conditions. *J Am Med Assoc* 2000; **283:**499–505.
28. Gross PA, Hermogenes AW, Sacks HS, *et al.* The efficacy of influenza vaccine in elderly persons: a meta-analysis and review of the literature. *Ann Intern Med* 1995; **123:**518–527.
29. Garcia Garcia ML, Ordobas Gabin M, Calvo Reya C, *et al.* Viral infection of the lower respiratory tract in hospitalized infants: etiology, clinical features, and risk factors. *An Esp Pediat* 2001; **55:**101–107.
30. Glezen WP. Serious morbidity and mortality associated with influenza epidemics. *Epidemiologic Rev* 1982; **4:**25–44.
31. Garcia-Vazquez E, Martinez JA, Mensa J, *et al.* C-reactive protein levels in community-acquired pneumonia. *Eur Respir J* 2003; **21:**702–705.

32. Ruiz-Gonzalez A, Falguera M, Nogués A, Rubio-Caballero M. Is Streptococcus pneumoniae the leading cause of pneumonia of unknown etiology? A microbiologic study of lung aspirates in consecutive patients with community-acquired pneumonia. *Am J Med* 1999; **106:**385–390.

33. Pumarola T, Dominguez A, Marcos MA, *et al.* Vigilancia virológica de la gripe (2002–2003). *Vacunas* 2003; **4:**119–126.

34. Weinberg GA, Erdman DD, Edwards KM, *et al.* Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. *J Infect Dis* 2004; **189:**706–710.

35. Templeton KE, Scheltinga SA, van Eeden WCJFM, *et al.* Improved diagnosis of the etiology of community-acquired pneumonia with real-time polymerase chain reaction. *Clin Infect Dis* 2005; **41:**345–351.

36. Welti M, Jaton K, Altwegg M, Sahli R, Wenger A, Bille J. Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagnost Microb Infect Dis* 2003; **45:**85–95.

37. Cooper NJ, Sutton AJ, Abrams KR, Wailoo A, Turner DA, Nicholson KG. Effectiveness of neuraminidase inhibitors in treatment and prevention of influenza A and B: systematic review and meta-analyses of randomised controlled trials. *BMJ* 2003; **326:**1235–1239.

38. Englund JA, Anderson LJ, Rhame FS. Nosocomial transmission of respiratory syncytial virus in immunocompromised adults. *J Clin Microbiol* 1991; **29:**115–119.

Accepted for publication 25 January 2006