Viruses detected by systematic multiplex polymerase chain reaction in adults with suspected community-acquired pneumonia attending emergency departments in France

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Abstract

Infectious agents associated with community-acquired pneumonia (CAP) are under-studied. This study attempted to identify viruses from the upper respiratory tract in adults visiting emergency departments for clinically suspected CAP. Adults with suspected CAP enrolled in the ESCAPED study (impact of computed tomography on CAP diagnosis) had prospective nasopharyngeal (NP) samples studied by multiplex PCR (targeting 15 viruses and four intracellular bacteria). An adjudication committee composed of infectious disease specialists, pneumologists and radiologists blinded to PCR results reviewed patient records, including computed tomography and day 28 follow up, to categorize final diagnostic probability of CAP as definite, probable, possible, or excluded. Among the 254 patients enrolled, 78 (31%) had positive PCR, which detected viruses in 72/254 (28%) and intracellular bacteria in 8 (3%) patients. PCR was positive in 44/125 (35%) patients with definite CAP and 21/83 (25%) patients with excluded CAP. The most frequent organisms were influenza A/B virus in 27 (11%), rhinovirus in 20 (8%), coronavirus in seven (3%), respiratory syncytial virus in seven (3%) and *Mycoplasma pneumoniae* in eight (3%) of 254 patients. Proportion of rhinovirus was higher in patients with excluded CAP compared with other diagnostic categories (p = 0.01). No such difference was observed for influenza virus. Viruses seem common in adults attending emergency departments with suspected CAP. A concomitant clinical, radiological and biological analysis of the patient's chart can contribute to either confirm their role, or suggest upper respiratory tract infection or shedding. Their imputability and impact in early management of CAP deserve further studies.
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Introduction

Community-acquired pneumonia (CAP) is increasingly recognized as a leading cause of hospital admission and death among

adults in industrialized countries [1] and one of the most common infectious diseases encountered in emergency departments (ED). Its annual incidence is estimated at 600 cases per 100 000 inhabitants, which represents in France around 300 000 cases per year causing 3500−11 000 annual deaths, particularly in the elderly population.

Currently, the diagnosis of CAP is heavily dependent on clinical and radiological findings, some of which are largely non-specific and of unknown aetiology [2−5], and treatment remains in most cases empirical [6−8]. The conventional microbiological techniques, such as culture, serology, antigen detection, have practical challenges, notably, complex technology, poor sensitivity and prolonged turnaround time. Since the advent of PCR, the advances in these new molecular techniques have shown their applicability to improve the detection of agents causing various types of infections. The multiplex PCR method allows the potential amplification of many nucleic acid targets within a single reaction and has proven to be an attractive diagnostic method for multi-pathogen detection in respiratory tract infections [9,10].

In ED, timely and accurate diagnosis is crucial for treatment decisions and the outcome of patients; it has been advocated that the prognosis of CAP depends on the immediate initiation of specific treatment no later than 8 h after diagnosis [11]. We present here the identification of infectious agents using multiplex PCR in adult patients with suspected CAP visiting EDs in France within the framework of the ESCAPED study, a prospective, open-label, multicentre impact measurement study of thoracic computed tomography (CT) scans.

Material and methods

The primary objective of the present microbiological study was to determine the prevalence of infectious agents (viruses and bacteria) in nasopharyngeal (NP) swabs using multiplex PCR in adults presenting with clinically suspected non-severe CAP. The study also aimed to describe detected pathogens according to the probability of diagnosis of CAP.

Context and design
The ESCAPED study was conducted in four university hospitals (Bichat, Cochin, Pitié-Salpêtrière and Tenon) in Paris, France. The study was approved by the ethics committee (Comité de Protection des Personnes—CPP 1, Paris, France) and patients gave written informed consent. Consecutive adult patients (≥18 years) presenting to the hospital EDs with a clinical suspicion of non-severe CAP (classes 1 and 2 on the CRB65 score) were prospectively enrolled. Clinical suspicion of CAP was

based on investigator's own judgement and had to fulfil the following criteria: new onset of systemic features (at least one among sweat, fever, chills, aches and pain, temperature ≥38°C) and symptoms of an acute lower respiratory tract illness (at least one among: cough, sputum production, dyspnoea, chest pain, altered breathing sounds).

An expert adjudication committee comprising a senior infectious disease specialist, pneumologist and radiologist reviewed patient records using all clinical data including day 28 follow up, radiological (thoracic X-ray and CT scan performed at ED admission), and laboratory findings to assess the diagnosis of CAP *a posteriori*. They jointly established the diagnosis of CAP and a final diagnostic probability according to four categories—definite, probable, possible, and excluded CAP. The committee was blinded to PCR results.

Microbiological samples
Systematic NP swabs were collected at enrolment from the nose, and also the throat in a sub-population of patients, and placed in a Middle Virocult MWE (Sigma, St Louis, MO, USA) transport medium. Samples were kept at room temperature and sent to the virology laboratory of Bichat-Claude Bernard Hospital as soon as possible after collection. The NP samples used in this study were not frozen and thawed. The other samples such as blood, urine and sputum were collected for bacteriological examinations within the scope of routine care as indicated by the attending physician.

Multiplex PCR
The RespiFinder-19 assay (Pathofinder, Maastricht, the Netherlands) was used to detect 15 respiratory viruses—coronaviruses 229E, NL63, OC43; human metapneumovirus; influenza A, A(H1N1)pdm2009 and B viruses, parainfluenza viruses 1, 2, 3 and 4; respiratory syncytial viruses A and B; rhinovirus; adenovirus; and four intracellular bacteria—*Bordetella pertussis*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, *Mycoplasma pneumoniae*—in one reaction. The RespiFinder-19 analysed the amplified PCR products by capillary electrophoresis using a DNA analyser (ABI 3130; Applied Biosystems, Darmstadt, Germany) and provided diagnosis within 6 h. The multiplex PCR method used in the present study was validated by the virology lab that participates every year to the European quality control program for each respiratory virus.

Statistical analysis
Data collected included demographic variables such as gender and age, clinical signs of CAP, coexisting conditions, severity score, as assessed by Fine score (12) and biological data, in particular inflammatory biomarkers.

Continuous data were compared using Student *t* test or analysis of variance. The Mann-Whitney *U* test or Kruskal-Wallis method were used for non-parametric comparisons. For categorical variables, proportions were compared using chi-squared or Fisher's exact test. Statistical analyses were performed using Stata software, version 11.2 (StataCorp, College Station, TX, USA).

Results

Patients' characteristics

In the ESCAPED study, 319 adult patients with suspected CAP were enrolled between November 2011 and December 2012. Of these, 254 (80%) patients with NP swabs available were included in the study, of whom, 51% were female, and with a mean (SD) age of 65 ± 19 years. The baseline characteristics of the 254 PCR study patients are shown in Table 1. One third (34%) of patients belonged to the Class IV or Class V of the FINE score (12). The baseline clinical characteristics of the 65 patients without swab (Non PCR Group) were not statistically different from those of patients who underwent PCR assay (PCR Study Group) except headache (more frequent in patients in PCR Study Group) and impaired consciousness (more frequent in patients in Non PCR Group).

Multiplex PCR findings

Among the 254 patients, 78 (31%) patients had at least one infectious agent detected (Fig. 1). The baseline characteristics of the 78 patients were not different from those with negative PCR results (Table 1) except neoplastic disease was more frequent in the negative PCR group.

The vast majority of patients (*n* = 75, 96%) had a single infectious agent, with a total of 81 pathogens detected in these 78 patients. Of the 254 study patients, 72 (28%), had a virus detected (Table 2). In the 78 patients with positive PCR, the most prevalent viral pathogens were influenza A/B virus in 35%, rhinovirus in 26%, respiratory syncytial virus A/B in 9% and coronavirus 229E/NL63/OC43 in 9% of patients. Other viruses identified were human metapneumovirus, parainfluenza viruses 3/4 and adenovirus. The only bacterium detected was *M. pneumoniae* in eight, 3% of patients. The seasonal distribution of the detected pathogens is shown in Fig. 2.

The multiplex PCR detected viruses in both NP samples in 72 (92% concordant results) of the 78 patients with both NP swabs taken. The central virology laboratory received and processed 91% (232/254) of NP swabs within 24 h. The probability of PCR positivity was not influenced by the time intervals between NP swab collection and PCR testing. The PCR positivity rate for viral infection and the agents found were comparable between NP (20/78) and only nasal (58/176) samples.

TABLE 1. Baseline characteristics of the 254 patients enrolled in PCR study according to PCR results

	PCR study group (254 patients), n (%)	PCR-positive (78 patients), n (%)	PCR-negative (176 patients), (%)	p Value (PCR-pos. versus PCR-neg.)
Demographic characteristics				
Sex, female	130 (51)	37 (47)	93 (53)	0.43
Age, years, mean (SD)	65 (19)	60 (21)	66 (18)	0.06
Clinical features				
Temperature >38 or <36°C	88 (35)	28 (36)	60 (34)	0.44
Cough	191 (75)	72 (92)	119 (68)	**<0.001**
Chest pain	88 (35)	19 (24)	69 (39)	**0.03**
Sputum production	120 (47)	48 (62)	72 (41)	**0.001**
Dyspnoea	182 (72)	58 (74)	124 (71)	0.31
Chills	83 (33)	24 (31)	59 (34)	0.34
Headache	47 (19)	20 (26)	27 (15)	**0.03**
Aches and pain	50 (20)	24 (31)	26 (15)	**0.001**
Crepitation (unilateral)	87 (34)	32 (41)	55 (31)	0.17
Heart rate >90/min	172 (68)	54 (69)	118 (67)	0.42
Respiratory rate >20/min	121 (60)	43 (68)	78 (56)	0.07
Coexisting conditions				
At least one comorbidity	114 (45)	32 (41)	82 (47)	0.41
Neoplastic disease	28 (11)	3 (4)	25 (14)	**0.02**
Congestive cardiac disease	28 (11)	8 (10)	20 (11)	I
Chronic lung disease	71 (28)	21 (27)	50 (28)	0.88
Before ED characteristics				
Duration of symptoms before ED,(hours, mean (SD)	173.3 (240.5)	171.6 (245.8)	174.0 (238.8)	0.9
Antibiotics before ED	89 (35)	34 (44)	55 (31)	**0.04**
FINE classification[a]				
Class I	38 (15)	17 (22)	21 (12)	
Class II	73 (29)	22 (28)	51 (29)	
Class III	56 (22)	21 (27)	35 (20)	0.06
Class IV	68 (27)	15 (19)	53 (30)	
Class V	19 (7)	3 (4)	16 (9)	

Abbreviations: ED, emergency departments; Neg., negative; Pos., positive. Numbers in bold are p values <0.05.
The p value was calculated with the use of *t* test, otherwise chi-squared test was used.
[a]A prediction rule to assess the severity of community-acquired pneumonia [12].
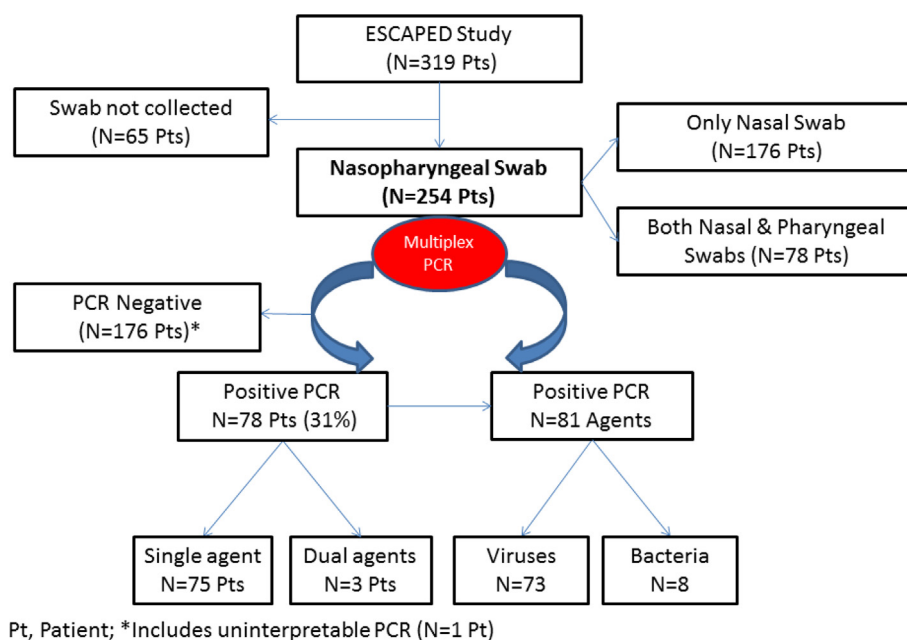
FIG. 1. Flow chart of detection of infectious agents in nasopharyngeal swabs by multiplex PCR in the 319 patients included in the ESCAPED study.

Pt, Patient; *Includes uninterpretable PCR (N=1 Pt)

Probability of diagnosis of CAP and PCR results

Among the 254 patients, 49% were classified as definite CAP, 7% as probable, 11% as possible and 33% were excluded by the adjudication committee (Table 3). Although the PCR findings were not different according to the diagnosis probability, among the 125 patients with definite CAP, 44 (35%) had positive PCR results and 81 (65%) were PCR negative. Among the 83 patients with excluded CAP, 21 (25%) were PCR positive. The probability of PCR detection of rhinoviruses was significantly higher in excluded CAP than in the other three groups combined (10/21 (48%) versus 10/57 (18%), respectively, p 0.01), whereas there was no difference in detection of influenza viruses (9/21 (43%) versus 18/57 (32%), respectively). Referring to the category of diagnosis, the sensitivity and specificity of

multiplex PCR were 35% and 74%, and the positive and negative predictive values were 56% and 54%, respectively.

Routine bacteriological findings

Routine bacteriological examinations were available for 190 (75%) of the 254 patients. Bacterial agents were detected in 28 patients, of whom 11 (39%) had a probably non-pulmonary infection, and 17 (61%) had detection of bacteria with 'lung tropism', possibly associated with CAP (Table 4). They were: *Streptococcus pneumoniae* in nine patients; *Legionella pneumophila* and other intracellular agents in three patients; and *Haemophilus influenzae*, *Branhamella catarrhalis*, a Gram-positive coccus, *Mycobacterium tuberculosis*, and an Enterobacteriaceae, each in one patient. Of the 17 patients with bacteria with "lung tropism", 11 had definite and two had probable or possible CAP according to the experts.

A bacterial−viral co-infection was present in seven (3.6%) of the 190 patients. The proportion of bacterial documentation was not different according to antibiotic administration before admission to ED. Overall, 95 (37%) CAP patients had detection of a viral or bacterial agent that was possibly responsible for the CAP.

Discussion

The present study found that approximately one-third of adult ED patients presenting with suspected CAP had PCR detection of viruses in the upper respiratory tract, mainly influenza A/B

TABLE 2. Multiplex PCR detection of virus and bacteria in nasopharyngeal swabs collected from 78 of the 254 adult patients enrolled in the PCR study

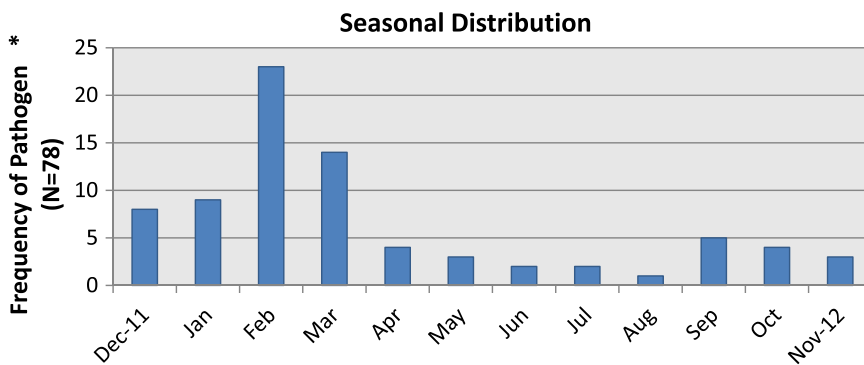| Type	Patients with positive PCR (n = 78) n (%)
Single agent	**75 (96)**
Influenza A/B	27 (35)
Rhinovirus	20 (26)
Respiratory syncytial virus A/B	7 (9)
Coronavirus 229E/NL63/OC43	7 (9)
Mycoplasma pneumoniae	6 (8)
Parainfluenza virus 3/4	4 (5)
Human metapneumovirus (hMPV)	3 (4)
Adenovirus	1 (1)
Multiple agents	**3 (4)**
Co229E—*Mycoplasma pneumoniae*	1 (1)
Coronavirus OC43—hMPV	1 (1)
Rhinovirus—*Mycoplasma pneumoniae*	1 (1)

FIG. 2. Seasonal distribution of detected pathogens in nasopharyngeal swabs by multiplex PCR in the 319 patients included in the ESCAPED study. * As compared with the total number of samples.

virus and rhinoviruses. For bacteria, multiplex PCR detected only *M. pneumoniae*, although one patient was diagnosed with CAP due to *L. pneumophila* by antigenuria detection.

To our knowledge, this is the first prospective clinical trial in ED describing the viruses associated with suspected CAP in adult patients taking into account an adjudication committee diagnosis of CAP as 'reference' (due to the inadequate accuracy of conventional diagnostic tests). Of note, the adjudication committee based its judgement not only on follow-up data, but also on systematic day 0 thoracic CT scan, which is unique. The value of 35% PCR-positive patients with definite CAP is in agreement with several studies without an adjudication

TABLE 3. PCR findings according to the adjudication committee diagnostic probability of community-acquired pneumonia (CAP) in the 254 adult patients enrolled in the PCR study

Adjudication committee diagnosis of CAP	PCR results, n (%)			
	Positive		Negative	Total
	Types of agents	(n)		
Definite	44 (35)[a]		81 (65)	125
	Influenza A/B	12		
	Rhinovirus	6		
	Respiratory syncytial virus A/B	5		
	Coronavirus 229E/NL63/OC43	4		
	Mycoplasma pneumonia[a]	6		
	Parainfluenza virus 3/4	4		
	Human metapneumovirus	3		
	Adenovirus	1		
	Multiple agents[a]	3		
Probable	4 (22)		14 (78)	18
	Influenza A/B	1		
	Rhinovirus	1		
	Respiratory syncytial virus A/B	1		
	Coronavirus 229E/NL63/OC43	1		
Possible	9 (32)		19 (68)	28
	Influenza A/B	5		
	Rhinovirus	3		
	Coronavirus 229E/NL63/OC43	1		
Excluded	21 (25)		62 (75)	83
	Influenza A/B	9		
	Rhinovirus	10		
	Respiratory syncytial virus A/B	1		
	Coronavirus 229E/NL63/OC43	1		
Total	78 (31)		176 (69)	254

[a]Includes the eight patients with positive PCR for *Mycoplasma pneumoniae*.

committee, which reported detection of viruses in a substantial proportion of patients with CAP. Jennings et al. and Johansson et al. both reported a viral diagnosis in 29% of hospitalized adult patients with CAP of similar age group (61−70 years) by PCR, whereas Liu et al. detected 36% viruses in adult outpatients with CAP and Templeton et al. detected viruses in 56% of adult patients from outpatient and inpatient departments, of whom half of the patients were <60 years old [2−4,13]. The most frequently detected viruses in our study were influenza A/B virus and rhinoviruses, as reported in previous studies [14]. Like our study, these reports addressed patients with an approximately similar rate, 28−32%, of coexisting conditions. Paediatric studies identified viral infections by real-time PCR in 28% of hospitalized patients, increasing to 50% in those with asthma [14,15]. The frequency and distribution of viruses detected were slightly different in two recent studies of patients in intensive care units and of elderly adults with moderate to severe illness [16,17]. These differences may partly be due to differences in viruses circulating in the community during the study period or to the sensitivity and specificity of diagnostic technology used. The seasonal distribution of detected pathogens in our study was similar to that observed in the community French surveillance network of patients with influenza-like illness in 2012−2013 (V Enouf, Institut Pasteur, personal communication). All of these findings confirm the major changes induced by molecular techniques in describing CAP-associated microorganisms; in comparison, a review of 26 prospective studies in ten European countries (approximately 6000 patients), published in 1998 before the wide use of PCR, found respiratory viruses accounted for only 8% of patients with CAP [18].

In patients with a definite CAP diagnosis, the 35% rate of PCR-positive results has to be balanced with the still high rate of negative results, which could be explained by other viral, bacterial or fungal agents that are not included as targets in the RespiFinder-19 assay. The fact that NP samples are only a proxy of the infectious agents present deeper in the lung has to be considered. Whether lower respiratory samples would have

TABLE 4. Positive bacteriological data from routine assessment in 28 of the 254 adult patients with suspected CAP enrolled in of the study

| PCR detection of viruses | Type of bacteriologial pathogen | Modality of detection | | | | Diagnosis of CAP[a] |
		Respiratory sample	Blood culture	Urine antigen	Others	
PCR positive						
Influenza A virus	*Streptococcus pneumoniae*		Blood culture			Definite
Influenza A virus	*Escherichia coli; Staphylococcus* coagulase negative				Ascitic fluid	Excluded
Influenza A virus	Gram-positive cocci	Sputum				Possible
Influenza A virus	Group B streptococcus				Urine culture	Definite
Rhino virus	*Branhamella catarrhalis*	Sputum				Excluded
Rhino virus	*Escherichia coli*				Urine culture	Definite
Rhino virus	*Streptococcus pneumoniae* (sputum and urine antigen); *Escherichia coli* (sputum culture)	Sputum		Urine antigen		Definite
Rhino virus	*Streptococcus pneumoniae*	Sputum				Excluded
PCR negative						
Negative	*Streptococcus pneumoniae*		Blood culture	Urine antigen		Definite
Negative	Other intracellular				Serology	Definite
Negative	Other intracellular				Serology	Probable
Negative	*Mycobacterium tuberculosis*	Sputum				Excluded
Negative	*Streptococcus pneumoniae*			Urine antigen		Definite
Negative	*Escherichia coli*				Vertebral puncture culture	Excluded
Negative	*Legionella pneumophila*			Urine antigen		Definite
Negative	Other pyogen		Blood culture			Possible
Negative	Enterobacteriaceae	Sputum				Excluded
Negative	Enterobacteriaceae				Urine culture	Definite
Negative	Other pyogen				Urine culture	Excluded
Negative	Enterobacteriaceae		Blood culture		Urine culture	Excluded
Negative	*Enteroccoccus* sp.; *Citrobacter*			Urine culture		Excluded
Negative	*Streptococcus pneumoniae*	Sputum		Urine antigen		Definite
Negative	*Haemophilus influenzae*		Blood culture			Definite
Negative	*Streptococcus pyogenes* (blood); *Escherichia coli* (urine)		Blood culture		Urine culture	Possible
Negative	Enterobacteriaceae		Blood culture			Excluded
Negative	*Streptococcus pneumoniae*			Urine antigen		Definite
Negative	*Streptococcus pneumoniae*			Urine antigen		Definite
	Streptococcus pneumoniae		Blood culture	Urine antigen		Definite

[a]Adjudication committee diagnosis of CAP.

facilitated imputability and given fewer negative results has to be studied in patients with more severe conditions that allow for invasive diagnostic investigations [16]. It is important to admit that multiplex PCR may have different analytical sensitivities for different viruses, which would affect the diagnostic yield. It is interesting to underline that the eight patients with PCR-detected *M. pneumoniae* had definite CAP, reinforcing the interest of this test in the context of *M. pneumoniae* epidemics.

The 25% PCR-positive patients among those with an excluded diagnosis of CAP might be explained by the fact that the infection was localized in the bronchi and the upper respiratory tract and did not affect the pulmonary parenchyma. This could also be explained by the PCR detection of viral shedding in patients with other diseases mimicking CAP symptoms [19]. In our study, the higher proportion of rhino-viruses in patients with excluded CAP suggests that at least part of these rhinoviruses were either viral shedding or could be associated with limited upper respiratory tract infection, as suggested previously in symptomatic and asymptomatic children and adults [20–22]. The seasonal distribution of influenza A/B virus detection and the similar proportion in excluded and non-excluded CAP suggest that pneumonia is not the primary manifestation of influenza, as already known [23]. Another hypothesis for this high rate of positive PCR in excluded CAP

may be that some patients with minimal or early lesions of CAP could present with a normal CT scan, as has been known for a long time for chest X-rays.

The epidemiology of CAP in adults has probably changed in recent years because of the indirect impact of pneumococcal conjugate vaccines in children [24]. However, establishing the aetiology of CAP remains difficult and the imputability of pathogens detected in the upper respiratory tract remains questionable. As in the present study, no cause is determined in half or more of the patients. Even considering the limit related to the routine non-standardized sampling for bacterial investigations in the study, *Streptococcus pneumoniae* was the most commonly identified bacteria associated with CAP, as is generally recognized. The rate of detection of other bacteria, and atypical agents was similar to that reported in a recent review [24]. Respiratory viruses, particularly influenza virus and respiratory syncytial virus, are commonly detected in patients with CAP as described here [14]. However, it remains challenging to differentiate viral CAP from mixed-infection or bacterial CAP. It is a hypothesis that the increasing recognition (15–54%) of viruses as a cause of CAP in adults may reflect advances in viral detection by PCR-based testing. Better ways to diagnose viral CAP would increase the use of targeted antiviral therapies. Specifically, detection of influenza virus should lead

to consideration of antiviral therapy within the 48 h after onset of symptoms, which has been shown to improve prognosis [23]. It can be useful to establish the virological diagnosis for epidemiological purposes in hospitalized patients who may require infection control measures to prevent nosocomial transmission, especially in the case of a new and/or contagious virus outbreak, such as the recent Middle East respiratory syndrome coronavirus outbreak [25]. Whether the rapid viral diagnosis is likely to change individual antibiotic management and reduce antibiotic overuse has to be studied [24,26]. Nevertheless, the relatively low sensitivity of the test and the high cost issues need to be addressed before widespread use of the multiplex PCR method.

The current study had several limitations. The multiplex PCR (RespiFinder-19) assay was not performed in real time, a limitation that may have underestimated identification of viral agents. However, this assay is generally considered to be as sensitive as monoplex PCR, which suggests that such a delay is not a major determinant of false-negative results [27]. One major issue is that the PCR test did not include *S. pneumoniae*, the leading organism of CAP, as a target [3,24,28,29], which precludes comments on the respective roles of *S. pneumoniae* and viruses as CAP agents in our study. Using sputum samples in adult patients attending ED, Yang et al. found that PCR assay might be a useful diagnostic adjunct for clinicians to detect a pneumococcal pneumonia [30].

In summary, viruses in nasopharyngeal samples seem common in adults attending ED with suspected CAP. A concomitant clinical, radiological and biological analysis of the patient's chart can contribute to either confirm their role in CAP, or suggest upper respiratory tract infection or shedding. Their imputability and possible impact in early management of CAP deserve further studies.

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