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Development and evaluation of a multiplex test for the detection of atypical bacterial DNA in community-acquired pneumonia during childhood

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Abstract

An incorrect or late diagnosis can lead to an increase in the morbidity and mortality caused by pneumonia, and the availability of a rapid and accurate microbiological test to verify the aetiology is imperative. This study evaluated a molecular test for the identification of the bacterial cause of atypical community-acquired pneumonia (ACAP). Fifty-four children with pneumonia were studied using bacteriological cultures, *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Chlamydophila pneumoniae* and *Legionella* spp. serology, and *Streptococcus pneumoniae* and *Legionella* antigens. Simultaneously, the presence of bacterial and fungal DNA was tested for in respiratory secretion samples using the Vircell SL kit, including multiplex PCR and amplicon detection by means of line blots. There were 14 cases of ACAP caused by *M. pneumoniae*, with positive kit results for 13 of them, and two cases of Q-fever, with negative kit results for *Coxiella burnetii*. The test was negative in the remaining 38 cases (one staphylococcal pneumonia, 20 *Streptococcus pneumoniae* pneumonias, and 17 probable viral pneumonias). The sensitivity of the test for the detection of *M. pneumoniae* was 92.8% and the specificity was 100%. The Vircell SL kit allows detection of *M. pneumoniae* DNA in respiratory secretion samples from children with ACAP.

Keywords: *Chlamydophila pneumoniae*, *Coxiella burnetii*, *Legionella* spp., *Mycoplasma pneumoniae*, pneumonia
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Introduction

Community-acquired pneumonia (CAP) is associated with a high morbidity. Its initial treatment, which is currently empirical, has a decisive influence on the prognosis. The incidence and the clinical relevance of the microorganisms considered to be responsible for the disease, and designated 'atypical' (*Mycoplasma pneumoniae*, *Coxiella burnetii*, *Chlamydophila pneumoniae*, and *Legionella* spp.), are controversial [1–3].

Primary infection with *C. pneumoniae* is frequent in children, increases in frequency with age, and is considered to represent *c.*10% of all CAPs [4]. The detection of the disease is hampered by the high prevalence of anti-*C. pneumoniae* IgG in adults, repeated asymptomatic infections, and the absence of IgM in some cases of primary infection [5]. The diagnosis of *M. pneumoniae* infection is similarly problematic: the culture is not reliable [6], there is a high prevalence of anti-*M. pneumoniae* IgG in adults, and IgM can be absent in some primary infection cases [7].

Most cases of *Legionella* pneumonia (85%) are caused by *Legionella pneumophila* [8], which is diagnosed by culture, immunofluorescence, or serology. Culture requires approximately 2 weeks, and false-negative results are frequent. Immunofluorescence on sputum is fast and specific but has low sensitivity [9]. Serological tests have a sensitivity of 80%, but the diagnosis is delayed, and false-positive results are obtained when there is infection with other bacteria [10]. Moreover, seroconversion can be absent in *Legionella* infections diagnosed by other methods [11]. Other shortcomings are that a negative serology result in the acute phase does not rule out infection, that a positive result in two consecutive sera without seroconversion is not a reliable diagnostic criterion, and that the sensitivity is limited if serum is obtained too early in the convalescence phase [12].

Available tests for the detection of *Legionella* antigen in urine have a sensitivity of 60–85%, but mainly reveal

infections with *L. pneumophila* of serogroup 1 [13]. Finally, antigenuria can persist for many months, and must therefore be interpreted with caution, especially in patients with a possible history of infection with this pathogen [12].

Coxiella burnetii causes Q-fever and chronic endocarditis. Rapid diagnosis of infection with this bacterium can markedly reduce its severity [14]. Serological tests are not useful for detecting infection in an early phase, when antibodies cannot be detected, and it is difficult to discriminate between current and past infections, owing to the presence of the corresponding IgG in the healthy population [15].

The emergence of new molecular techniques has considerably enhanced the possibility of accurate diagnosis of CAP [16–18]. Vircell SL (Santa Fe, Granada, Spain) has developed a kit (not yet commercially available) to detect *C. pneumoniae*, *M. pneumoniae*, *Coxiella burnetii*, the genus *Legionella* and *L. pneumophila* in respiratory tract samples using PCR and line blot. This study prospectively analysed the diagnostic capacity of this procedure in clinical samples from children with pneumonia and contaminated respiratory secretions. The aim of the study was to test the performance of the kit.

Materials and Methods

Human samples

Between January 2006 and April 2007, we studied 54 children (<1–13 years old; 32 males, 22 females) referred to the San Cecilio University Hospital in Granada on the basis of clinical and radiological suspicion of CAP (characterized by the presence of newly evident infiltrates on chest X-rays with suggestive clinical and analytical data, cough, purulent expectoration (in older patients), fever, pleuritic chest pain and/or leukocytosis). Patients with possible nosocomial pneumonia and immunodepressed patients were excluded. Patients diagnosed with CAP had their complete clinical history recorded, and underwent physical examination, including chest X-rays; basic laboratory tests were performed. Antibiotic treatment was given according to criteria established in consensus documents [19,20]. Age, sex and associated morbidity factors were systematically recorded for all patients, following appropriate clinical, analytical, functional and pathological criteria. Radiological signs were categorized as lobular or multilobular, and the presence of pleural effusion was recorded. In hospitalized patients, studies included blood cultures, Gram staining and culture of sputum (when there was expectoration), culture of pleural liquid (when there was effusion), and a search for antigens of *Streptococcus pneumoniae* and *Legionella* in urine.

CAP aetiology was attributed to atypical bacteria if the corresponding IgG titres increased four-fold between serological determination in the acute phase and a second determination in the convalescence phase (separated by an average interval of 2 weeks) and if specific IgM or *Legionella* spp. antigen was detected in urine in the acute phase (Binax NOW, Leti, Spain). ELISA (Vircell SL) was used to detect anti-*M. pneumoniae* IgG and IgM, and indirect immunofluorescence (Vircell SL) to detect anti-*Coxiella*, anti-*C. pneumoniae*, anti-*Chlamydophila psittaci* and anti-*Legionella* IgG and IgM, following the manufacturers' instructions.

Viral pneumonia was suspected when no bacteria responsible for atypical CAP (ACAP) were isolated and no antibodies induced by these bacteria were detected in patients with compatible clinical findings (lymphocytosis and/or monocytosis, C-reactive protein <7 μg/L) who failed to respond to antibiotic treatment in the first 48 h [19,20]. Viral cultures were not performed.

Nasopharyngeal swabs or aspirates of secretions were obtained from all patients for the detection of *C. pneumoniae*, *M. pneumoniae*, *Coxiella burnetii*, *Legionella* genus and *L. pneumophila* DNA by using the Vircell SL kit. Fifty-five throat swabs from asymptomatic adults were also analysed. Samples were taken using a sterile swab, resuspended in transport medium for *Chlamydia* (Vircell SL), and kept at −20°C until DNA extraction.

DNA extraction

The QIAamp DNA blood mini kit (Qiagen, Turn berry Lane Valencia, CA, USA) was used to extract DNA from all respiratory secretions, and 200-μL samples were processed according to the manufacturer's instructions.

DNA was also extracted from bacterial suspensions of *C. pneumoniae*, *M. pneumoniae*, *Coxiella burnetii* and *Legionella* spp., including *L. pneumophila* (all serotypes), *Legionella dumoffii*, *Legionella longbeachae*, *Legionella jordanis*, *Legionella gormanii*, *Legionella micdadei* and *Legionella bozemanii* (Table 1), all of which may cause ACAP.

Ten serial dilutions of 5 ng of this DNA were used to spike the pharyngeal secretion samples from the 55 asymptomatic individuals. Bacterial suspensions (1.28×10^6 CFU/mL for *Legionella* species and 5.59×10^6 CFU/mL for *M. pneumoniae*) were diluted into 800 μL of Tris (0.2 M)/EDTA (0.1 M) (pH 8). Then, SDS (final concentration 0.5%) and proteinase K (final concentration 0.1 g/L) were added, and the solution was incubated at 37°C for 4 h. Two phenol extraction steps were performed with one volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and one volume of chloroform/isoamyl alcohol (24 : 1); DNA was precipitated with two volumes of ethanol and 5 M NaCl. DNA concentra-