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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

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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-

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TABLE 1. Strains used in the assays with the Vircell SL kit

Strain	Source
Chlamydophila pneumoniae	ATCC VR-1356
Mycoplasma pneumoniae	ATCC 15531
Coxiella burnetii	ATCC VR-616
Legionella pneumophila serogroup 1	NCTC 11286
L. pneumophila serogroup 1	NCTC 11191
L. pneumophila serogroup 1	NCTC 11231
L. pneumophila serogroup 1	NCTC 11286
L. pneumophila serogroup 1	NCTC 11424
L. pneumophila serogroup 1	NCTC 12006
L. pneumophila serogroup 1	NCTC 12007
L. pneumophila serogroup 1	NCTC 12008
L. pneumophila serogroup 1	NCTC 12009
L. pneumophila serogroup 1	NCTC 12024
L. pneumophila serogroup 1	NCTC 12098
L. pneumophila serogroup 2	ATCC 33154
L. pneumophila serogroup 3	ATCC 33155
L. pneumophila serogroup 4	ATCC 33156
L. pneumophila serogroup 5	ATCC 33215
L. pneumophila serogroup 6	ATCC 33216
L. pneumophila serogroup 7	NCTC 11984
L. pneumophila serogroup 8	NCTC 11985
L. pneumophila serogroup 9	NCTC 11986
L. pneumophila serogroup 10	NCTC 12000
L. pneumophila serogroup 11	NCTC 12179
L. pneumophila serogroup 12	NCTC 12180
L. pneumophila serogroup 13	NCTC 12181
L. pneumophila serogroup 14	NCTC 12174
Legionella bozemanii	NCTC 11368
Legionella dumoffii	NCTC 11370
Legionella gormanii	NCTC 11401
Legionella jordanis	NCTC 11533
Legionella longbeachae	NCTC 11477
Legionella micdadei	NCTC 11371
Haemophilus influenzae	Clinical isolate
Staphylococcus aureus	DSM 13661
Streptococcus pyogenes	Clinical isolate
Streptococcus agalactiae	Clinical isolate
Streptococcus pneumoniae	ATCC 49619
Streptococcus epidermidis	Clinical isolate
Streptococcus crista	Clinical isolate
Enterococcus faecalis	Clinical isolate
Acholeplasma oculi	Kindly provided by M. Duran (LCSA, Santa Fe, Granada)
Mycoplasma mycoides	Kindly provided by M. Duran (LCSA, Santa Fe, Granada)
Chlamydophila pecorum	Kindly provided by M. Duran (LCSA, Santa Fe, Granada)
Chlamydophila trachomatis	ATCC VR-398B
Chamydophila psittaci	ATCC VR-125
Bordetella pertussis	ATCC 8467
Bordetella parapertussis	NCTC 5952
Candida albicans	NCPF 3153
Aspergillus fumigatus	ATCC 204305
Escherichia coli	NCTC 50001
Mycobacterium tuberculosis	NCTC 13144
Listeria monocytogenes	NCTC 4885
Neisseria meningitis serogroup A	NCTC 10025
N. meningitis serogroup B	NCTC 10026
N. meningitis serogroup C	NCTC 8554

tion was determined spectrophotometrically at $A_{260\ nm}$, and DNA was considered to be pure at an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.8–2. Extraction was always performed in a biologically secure laboratory.

This method was also used to extract 50 ng of DNA from different microorganisms in order to verify the specificity of the kit. For this purpose, PCR was performed with DNA from the following pathogens (isolated in our hospital by reference techniques or from culture collections): *Haemophilus influenzae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus crista*, *Enterococcus faecalis*, *Acholeplasma oculi*, *Mycoplasma mycoides*, *Chlamydophila pecorum*, *Chlamydophila trachomatis*, *C. psittaci*, *Bordetella pertussis*, *Bordetella parapertussis*, *Candida albicans*, *Aspergillus fumigatus*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Neisseria meningitis* serogroups A, B, and C (Table 1).

Vircell SL prototype kit

Multiplex PCR. Five pairs of oligonucleotides (Table 2) were required to detect the agents of interest. A 383-bp fragment of the 16S rRNA gene was amplified to detect the *Legionella* genus, and a 289-bp fragment of *dnaJ* to detect *L. pneumophila* [21]. *Coxiella burnetii* was detected by amplifying a 438-bp fragment of the COM-1 gene [22]. Finally, for detection of *M. pneumoniae*, a 481-bp fragment of the P1 gene was amplified [23], and for detection of *C. pneumoniae*, a 350-bp fragment of the major outer membrane protein gene *ompA* was amplified [24]. After selection of the oligonucleotides, single PCRs were optimized for each microorganism. A pair of oligonucleotides for the amplification of an 817-bp fragment of the human mitochondrial cytochrome oxidase subunit III gene was included in the multiplex PCR as an extraction and amplification control.

TABLE 2. Oligonucleotides used for amplification by PCR and detection by line blot of a specific fragment from the microorganisms included in the test and the internal control

	Oligonucleotides for PCR		Oligonucleotides for the generation of specific probes	
Microorganism	Oligonucleotide name	Sequence	Oligonucleotide name	Sequence
Legionella genus	Lsp-F	5′-AGCATKGTCTAGCTTGCTAG-3′	Lsp-int-F	5′-GGCGAGTGGCGAACGG-3′
	Lsp-R	5′-ACCTCCCCACTGAAAGTG-3′	Lsp-int-R	5′-CAGGCCTTCTTCACACAC-3′
Legionella pneumophila	LPN-F	5′-CAGGTGGTTTTGGCGGATTTG-3′	LPN-int-F	5′-TTTGGTGACGTTTTTGAAG-3′
	LPN-R	5′-TGTTGAATTCTGACTTGCCC-3′	LPN-int-R	5′-CCTGGCATGTTTCACAAG-3′
Mycoplasma pneumoniae	MPN-F	5′-ACTCGGAGGACAATGGTCAG-3′	MPN-int-F	5′-TGATCTCGCCAACGCTC-3′
	MPN-R	5′-AACCCGGTCTTTTCGTTATCC-3′	MPN-int-R	5′-ACCGTCTGCCCGCCATC-3′
Chlamydophila pneumoniae	CPN-F	5′-CACTAATGCAGGCTTCATTGCC-3′	CPN-int-F	5′-CATTTGGGATCGCTTTGATG-3′
	CPN-R	5′-TGTTTACAGAGAATTGCGATACG-3′	CPN-int-R	5′-CAGATCACATTAAGTTCTTC-3′
Coxiella burnetii	CBU-F	5′-CGCAACAAGAAGAACACGCTC-3′	CBU-int-F	5′-CGACCCTGCATCACCAG-3′
	CBU-R	5′-CTAATTGGAAGTTATCACGCAG-3′	CBU-int-R	5′-GGATAGCAGGATTATCC-3′
Internal control	IC-F	5′-CCACCAATCACATGCCTATCA-3′	IC-int-F	5′-CCTCTCAGCCCTCCTAATG-3′
	IC-R	5′-TAATTGGAAGTTAACGGTACTA-3′	IC-int-R	5′-AAATGCCAGTATCAGGCGG-3′

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To avoid loss of sensitivity in the multiplex PCR, each sample was simultaneously amplified with two multiplex PCR mixtures. The first contained oligonucleotides for the amplification of *M. pneumoniae*, *C. pneumoniae* and *Coxiella burnetii* at a final concentration in the PCR of 0.5 μM for each pair of oligonucleotides. The second multiplex PCR was optimized for *L. pneumophila*, *Legionella* genus and the human gene (internal control), with final oligonucleotide concentrations of 0.2, 1.2 and 0.25 μM, respectively. PCRs were optimized with respect to the concentration of oligonucleotides, dNTPs and $MgCl_2$, and the conditions of the PCR programme. The oligonucleotides were labelled with biotin at the 5′-end to be subsequently detectable by line blot hybridization. Each PCR was performed in a final volume of 25 μL, using 10 μL of sample. Final concentrations of dNTPs and $MgCl_2$ were 150 μM and 3 mM, respectively; 1 U of Taq polymerase (Bioline, S Main St Randolph, MA, USA) was used per PCR. PCR was carried out in an Eppendorf gradient thermocycler (Eppendorf, Madrid, Spain) under the following conditions: a first denaturation step at 92°C for 2 min, 40 amplification cycles (92°C, 20 s; 55°C, 30 s; 72°C, 30 s), and a final extension step at 72°C for 3 min.

Probe synthesis. For detection of the microorganisms, specific probes were generated by single PCR using the internal oligonucleotide pair of each one of the fragments amplified in the multiple PCRs (Table 2). For these PCRs, the six internal fragments were cloned in a pGEM-T vector (Promega, Seville, Spain). Each clone was used as template DNA for amplification of the corresponding probe. A final volume of 50 μL containing 5 ng of plasmid was used for each PCR. Final concentrations of dNTPs and $MgCl_2$ were 200 and 3 mM, respectively; 1 U of Taq polymerase (Bioline) was used per PCR. The PCR was carried out in an Eppendorf gradient thermocycler (Eppendorf) under the following conditions: a first denaturation step at 92°C for 1 min, 40 amplification cycles (92°C, 20 s; 55°C, 30 s; 72°C, 30 s), and a final extension step at 72°C for 3 min. The six PCR products were used as probes to draw lines on a nylon membrane.

The probes were denatured with NaCl (1.5 M) and NaOH (1.5 M) for 10 min, neutralized with Tris-HCl (1 M) and NaCl (1.5 M) (pH 7.4), and fixed on the membrane with UV light for 2 min.

Line blot hybridization. PCR-generated amplicons were visualized by line blot hybridization. The line blot was formed by a nylon membrane strip onto which six DNA probes (Table 2) were placed: five probes were DNA fragments specific for each pathogen under study, and the other probe was a fragment specific for the amplified human DNA fragment (extraction and PCR control). The line blot also included a control line to react with the conjugate that was subsequently used in the colorimetric detection. The seven probes were dosed (0.3 μL/cm) using a Bio-Dot line dispenser, model ZX1000 (Bio-Dot, Irvine, CA, USA).

The strip with biotinylated PCR product was hybridized in a specially designed tray. The two biotinylated PCR products for each sample were each denatured by adding 20 μL of solution (NaOH (0.25 M), NaCl (0.75 M)) to the PCR tube and incubating them for 10 min at room temperature. Then, 1 mL of hybridization solution was added (*N*-lauryl sarcosine (2.4 g/L), SDS (0.02%), 6 × SSC, salmon sperm DNA (100 mg/L), skimmed milk powder (2%), Tris-HCl (0.2 M), NaCl (0.3 M) (pH 7.4)) and preheated in the hybridization tray with the strip and two denatured PCR products. This mixture was incubated for 1 h at 50°C under agitation (80 r.p.m.) in a hybridization oven. Then, the strip was washed twice for 10 min at 50°C with a stringent wash solution (0.1 × SSC, SDS (1%)). Finally, colorimetric detection was performed by incubating with 1 mL of conjugate (streptavidin-peroxidase (Caltag, San Francisco, CA, USA)) dissolved (1/4000) in conjugate stabilizer (Vircell SL) for 10 min under agitation (40 r.p.m.). After two washes with washing solution (phosphate-buffered saline at pH 7.2, Tween-20 (0.05%)), the strip was incubated with TMB (Neogen Corp., Lansing, MI, USA) for 5 min to visualize the signal. After washing of the strip with water, results were interpreted using an interpretation card.

Results

In preliminary assays using the Vircell SL kit with a multiplex PCR, a loss of sensitivity was found with a mix of the six pairs of primers. Therefore, the multiplex PCR was optimized by testing different combinations and concentrations of primers and different PCR conditions to avoid this loss of sensitivity. The assay results suggested the preparation of two PCR mixes, one for *M. pneumoniae*, *C. pneumoniae* and *Coxiella burnetii*, and the other for *L. pneumophila*, genus *Legionella* and the internal amplification control, at the concentrations shown in Materials and Methods. With these combinations of oligonucleotides, no loss of sensitivity was detected with respect to the individual PCRs.

Among the 54 children in the study, 14 (26%) had ACAP due to *M. pneumoniae*, based on serology, with two showing IgM in the first serum sample. In the other 12 cases, a second serum sample was required to observe seroconversion of IgM and IgG, delaying the diagnosis. A further two children (3.7%) had Q-fever, based on serology, one (1.9%) had

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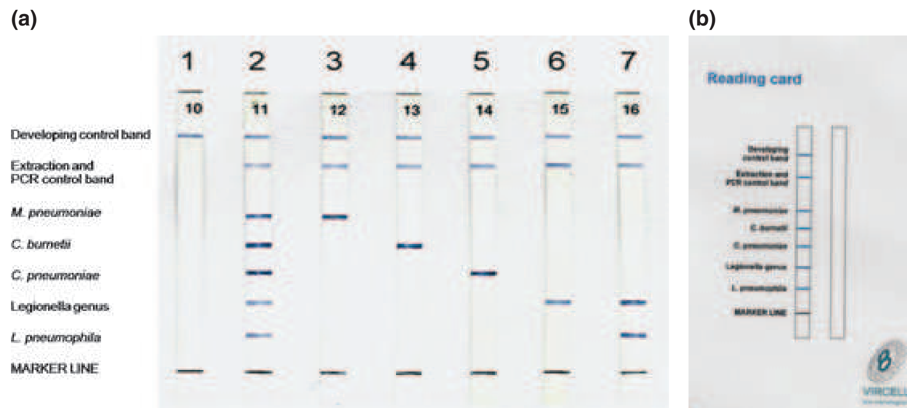

FIG. 1. Hybridization results for samples positive for each microorganism of interest in the following order: 1, negative PCR control; 2, positive PCR control; 3, *Mycoplasma pneumoniae*; 4, *Coxiella burnetii*; 5, *Chlamydophila pneumoniae*; 6, *Legionella* sp; 7, *Legionella pneumophila*. (b) Reading card for the interpretation of results.

S. aureus pneumonia and 20 (37%) had *Streptococcus pneumoniae* pneumonia, based on culture and/or on antigen testing, and viral CAP was suspected in the remaining 17 patients (31%). Vircell SL kit results were interpreted using the reading card designed for the test (Fig. 1b).

Patient samples

For both children with Q-fever, the Vircell SL test result was negative for *Coxiella burnetii*, and for all of those with ACAP due to *M. pneumoniae*, except one, the Vircell SL test results were positive. The test result was negative in the remaining cases (one *S. aureus* pneumonia, 20 *Streptococcus pneumoniae* pneumonias and 17 viral CAPs). No positive results were obtained for *C. pneumoniae* and *Legionella* spp. using the Vircell SL kit. Globally, therefore, the kit yielded 13 (24%) positive results for *M. pneumoniae* in this population of children with CAP. Line blots were clearly visible and easily interpreted using the reading card. The absence of PCR inhibitors was verified in all cases, as an intense signal was obtained for the DNA extraction and PCR control in all samples. Finally, the test sensitivity for the detection of *M. pneumoniae* was 92.8% and the specificity was 100%.

DNA was also extracted from serum from the two patients who were positive for *Coxiella burnetii* by serology; Vircell SL test results were negative for *Coxiella burnetii* in both cases.

Samples from asymptomatic individuals

Test specificity was assessed by performing the Vircell SL test with the 55 respiratory tract samples from asymptomatic individuals. None of these samples yielded a positive signal.

Analytical sensitivity studies were performed with artificial samples generated with serially diluted purified DNA of the microorganisms to be detected. A specific hybridization was obtained for each, with no signal observed in the remaining lines. A single intense signal was seen in the reaction, extraction and PCR controls (Fig. 1a). The limit of detection was established by performing PCRs on replicas of dilutions in triplicate, with this limit being considered to be the lowest level at which all three dilutions gave a positive result. The limit of detection was 5 pg of DNA for *C. pneumoniae*, *Coxiella burnetii* and the genus *Legionella*, and 0.5 pg for *L. pneumophila* and *M. pneumoniae*. Because we selected highly conserved regions of the *mip* gene among different *L. pneumophila* serogroups and of the 16S rRNA gene in the genus *Legionella*, no differences in sensitivity were observed among the species.

The reproducibility of the test was evaluated by using three different serial dilutions of each microorganism in intra-assay and inter-assay experiments carried out by two examiners. Identical dilution limits were obtained in these experiments. The technique took 3.5 h to complete: 1.5 h for the PCR, and 2 h for the line blot development. With the primer combinations selected for the multiplex PCRs, their reproducibility was the same as found for the individual PCRs.

Other specificity studies

The absence of cross-reactions was assessed by performing the test with 50 ng of DNA of each of the 23 microorganisms described above. No false-positive results were obtained.

Discussion

This study evaluated a multiplex molecular test to determine the bacterial cause of ACAP in 54 children (14 due to *M. pneumoniae*, two cases of Q-fever, one staphylococcal

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pneumonia, 20 *Streptococcus pneumoniae* pneumonias, and 17 probable viral pneumonias). The test identified 13 cases of ACAP due to *M. pneumoniae*, and was negative in the remaining 41 cases.

Among earlier molecular biology approaches, DNA hybridization techniques with labelled probes and polymorphism analyses of restriction fragments were used to detect DNA of atypical respiratory tract bacteria [25]. However, techniques based on nucleic acid amplification should offer faster and improved pathogen detection, allowing earlier diagnosis and treatment. Over the past few years, numerous PCR tests have been optimized for the detection of different pathogens implicated in pneumonia, e.g. *C. pneumoniae* [26], *M. pneumoniae* [27], *Coxiella burnetii* [22], *L. pneumophila*, and the genus *Legionella* [21]. In the design of these PCRs, authors used primers for specific genes of the bacteria of interest, to avoid false-positive results. These are commonly designed for genes of membrane-specific antigens, e.g. the P1 gene for *M. pneumoniae* [23,24] or the *mip* gene for *L. pneumophila* [9]. Ribosomal RNA genes or intergenic spacers are also considered as PCR targets, mostly for the design of genus-specific primers [21].

Although there are numerous single PCR systems for microorganism detection, multiplex PCR protocols are less common, despite the savings that they offer in time and cost. Multiplex PCR systems are available that include some of the microorganisms investigated in the present study, e.g. *M. pneumoniae* and *C. pneumoniae* [28], *C. pneumoniae*, *C. psittaci* and *M. pneumoniae* [29], *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* [30], and *C. pneumoniae*, *M. pneumoniae* and genus *Legionella* [24]. Others include *M. pneumoniae* and *C. pneumoniae* with *Streptococcus pneumoniae* and *H. influenzae* [23]. In these systems, results are sometimes interpreted by using agarose gels or ELISA. The test evaluated in this article offers a direct visual reading, adding to its value in the clinical setting.

The proposed test showed excellent performance in the detection of ACAP caused by *M. pneumoniae*. In one patient with positive serology for *M. pneumoniae*, no signal was obtained with the Vircell SL kit, and no PCR inhibitors were detected in this sample. This false-negative result can be attributed to a more advanced phase of the disease. In this case, seroconversion was not accompanied by the presence of bacterial DNA in the respiratory tract sample. This factor should be taken into account in the clinical interpretation of results.

Serology gave positive results for *Coxiella burnetii* in the two samples studied, but a negative result was obtained with the Vircell SL kit in both respiratory tract samples. In general, *Coxiella burnetii* has been detected by PCR with DNA extracts of serum samples [22,31]. Therefore, PCR was also performed with serum samples that were positive for *Coxiella burnetii* by indirect immunofluorescence, yielding negative results in both cases. False-negative results are frequently reported in the detection of *Coxiella burnetii* using PCR with serum samples. Thus, Zhang *et al.* [22] obtained 14.8% false-negative results in a nested PCR with specific plasmids of *Coxiella burnetii* in serum samples, and Fenollar *et al.* [32] obtained only 64% sensitivity with sera of patients with endocarditis or vascular infection.

Fournier and Raoult [31] found the sensitivity to be low (26%) in samples collected during the first 2 weeks after onset of the disease, when antibodies could not yet be detected. The sensitivity was even lower (5%) in samples drawn after the first 2 weeks, when antibodies could be detected [30]. Low PCR sensitivity with serum samples is due to the scant amount of DNA present, limiting the detection of *Coxiella burnetii* by PCR.

Our serological study suggests that the two samples positive for *Coxiella burnetii* were from patients who had contracted the disease more than 2 weeks earlier, when DNA detection in serum has the lowest diagnostic value [31]. A larger number of *Coxiella burnetii*-positive serum samples are required to assess the validity of the technique. Nevertheless, it is possible that this test is not effective in Q-fever patients, who are often asymptomatic and are only diagnosed using serology. In these cases, it is possible that a higher diagnostic accuracy could be obtained at the onset of the disease by analysing peripheral blood or lower respiratory tract samples.

We believe that this new PCR kit offers a clear advantage over serological techniques for the diagnosis of ACAP caused by *M. pneumoniae*, cases in which there are frequent losses of the second serum sample, especially when there is clinical improvement, meaning that the causal agent often remains unknown. So, in adults, owing to the high seroprevalence of IgG [33], re-infection with any of the microorganisms produces elevated titres of IgG in the first sample, preventing demonstration of seroconversion. By contrast, direct diagnosis using the Vircell SL kit allows immediate and specific detection of the pathogen in the acute phase of the disease in a single sample.

In conclusion, a highly reproducible technique has been optimized. The multiplex PCR test, in conjunction with a line blot used in the Vircell SL kit, has high sensitivity for the detection of *M. pneumoniae* in upper respiratory tract secretions from patients with ACAP. There were no false-positive results when respiratory samples from asymptomatic individuals were analysed, and no cross-reactions were detected for the microorganisms studied.

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Transparency Declaration

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