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Development and Evaluation of Chlamylege, a New Commercial Test Allowing Simultaneous Detection and Identification of *Legionella*, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae* in Clinical Respiratory Specimens by Multiplex PCR

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This study describes the development and evaluation of a new commercial test, Chlamylege (Argene Inc.), which allows the simultaneous detection in respiratory samples of *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and most *Legionella* species, as well as PCR inhibitors, by using a multiplex PCR and microplate hybridization. The sensitivities of Chlamylege were 1×10^{-3} IFU, 5×10^{-2} color-changing units, and 1 CFU per reaction tube for *C. pneumoniae*, *M. pneumoniae*, and *Legionella pneumophila*, respectively. A cohort of 154 clinical samples from patients with documented respiratory infections was analyzed by the kit, including 2 samples from patients with *C. pneumoniae* infection, 9 samples from patients with *M. pneumoniae* infection, 19 samples from patients with *Legionella* species infection, and 114 samples that tested negative for the three pathogens. All the positive specimens were correctly detected and identified by the Chlamylege kit, and no false-positive result was observed with the negative samples. The kit was then evaluated in a pediatric prospective study that included 220 endotracheal aspirates, and the results were compared with those obtained by three single in-house PCR assays. Four specimens were found to be positive for *C. pneumoniae* and six were found to be positive for *M. pneumoniae* by using both strategies. The Chlamylege kit detected two additional samples positive for *M. pneumoniae* and one additional sample positive for a *Legionella* species other than *L. pneumophila*; these three samples were shown to be true positive by other techniques. These overall results demonstrate that the Chlamylege assay is sensitive, specific, and convenient for the rapid detection and identification of atypical pathogens in clinical samples from patients with respiratory infections.

Atypical bacterial pathogens, including *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, and some other *Legionella* species, are involved in infections of the respiratory tract. The rates of respiratory infections in relation to these microbial agents are probably underestimated due to the difficulty in identifying them.

The diagnosis of infections caused by *C. pneumoniae* or *M. pneumoniae* is usually performed by serology. Actually, *C. pneumoniae* is an obligate intracellular bacterium that must be cultivated within eukaryotic host cells. This culture remains difficult, time-consuming, and insensitive (8). The culture of *M. pneumoniae* on enriched agar medium is fastidious, and 2 to 5 weeks may be required to obtain a visible colony (13). Usually, serological testing needs two consecutive serum specimens to document an increase in the immunoglobulin G (IgG) titer, thus providing a retrospective diagnosis of an acute infection. In addition, serological results related to *C. pneumoniae* and *M. pneumoniae* are often difficult to interpret due to the high prevalence of specific IgG antibodies in adults and the lack of IgM antibodies in some cases of primary or secondary infection (8, 28).

Legionella infections are routinely diagnosed by culture or direct immunofluorescence assay in respiratory samples or by serology. The culture of *Legionella* on charcoal medium requires 3 to 10 days and is relatively insensitive. The detection of *Legionella* by using direct immunofluorescence assay with monoclonal antibodies is rapid and highly specific but lacks of sensitivity (11, 29). With serological testing, many false-positive cross-reactions may occur in patients infected with other bacteria (9), whereas an absence of seroconversion in culture-proven infections has also been described (29). The urinary antigen test is rapid and exhibits a good sensitivity, but it allows the detection only of *L. pneumophila* serogroup 1.

Nucleic acid amplification techniques are suitable for the rapid detection of *C. pneumoniae*, *M. pneumoniae*, and *Legionella* spp. directly in respiratory samples. Over the last 15 years, several molecular assays have been described for the detection of *C. pneumoniae* (4, 10, 16), *M. pneumoniae* (6, 14, 15, 23) and *Legionella* spp. (11, 13, 21, 24) in single assays. More recently, PCR assays have been described that allow the detection of at least one of these three pathogens in multiplex reactions with the following combinations: *C. pneumoniae* and *M. pneumoniae* (5); *C. pneumoniae*, *Chlamydia psittaci*, and *M. pneumoniae* (26); *C. pneumoniae*, *C. psittaci*, and *Chlamydia tracho-*

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matis (16); and *C. pneumoniae*, *M. pneumoniae*, and *L. pneumophila*. These multiplex reactions use two duplex PCR assays (30) or a single multiplex assay (18, 19).

Here we describe the development and evaluation of Chlamylege, a commercially available test that allows the simultaneous detection of *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila* and most of the other *Legionella* species in respiratory samples by combining a multiplex PCR assay and a microplate hybridization assay with generic and specific probes.

MATERIALS AND METHODS

Infectious agents. If not stated otherwise, the experiments were done with the following strains: *C. pneumoniae* IOL207, *M. pneumoniae* FH (ATCC 15531), and *L. pneumophila* Philadelphia (ATCC 33152). The strain collection also included 8 clinical isolates of *M. pneumoniae*; 64 different reference or environmental strains of *Legionella*, including the 15 serogroups of *Legionella pneumophila*; and 38 *Legionella* species other than *L. pneumophila* (Table 1). Six *Candida* yeast species, 2 fungi, and 16 bacterial strains possibly found in respiratory specimens or phylogenetically related to one of the three pathogens tested were used for the specificity testing (Table 1). *C. pneumoniae* was cultured by infection of HEp-2 cells and incubation for 72 h at 37°C under 5% CO_2, as described previously (22). *M. pneumoniae* was cultured successively in Hayflick biphasic and agar media for up to 5 weeks at 37°C under 5% CO_2. *Legionella* strains were grown on buffered charcoal yeast extract agar medium for 2 to 10 days at 37°C. The other bacteria, yeast, and fungi used for specificity testing were cultured on the appropriate media.

DNA extraction, multiplex PCR, and microplate hybridization by Chlamylege assay. For bacterial strains grown on agar medium, one colony was resuspended in 200 μl of phosphate-buffered saline (PBS) before extraction. For bacterial strains grown in liquid medium, 200 μl of liquid culture was used for extraction. Throat and nasal swab specimens were resuspended in 500 μl of PBS. Liquid respiratory specimens, including nasopharyngeal aspirates, endotracheal aspirates, sputum, and bronchoalveolar lavage (BAL) fluids, were extracted without any pretreatment.

Two hundred microliters of each sample was used for DNA extraction by using the QIAamp DNA blood extraction system (QIAGEN, Courtaboeuf, France) included in the Chlamylege kit (Argene Inc., North Massapequa, N.Y.), according to the manufacturer's instructions. DNA was recovered in 200 μl of elution buffer.

Three sets of primers, each set of which was specific for *C. pneumoniae*, *M. pneumoniae*, and *Legionella* spp., were designed to develop this test. The primers specific for *C. pneumoniae* enclosed a 170-bp fragment in the *omp-2* gene, located at positions 367 to 537 of the sequence available under GenBank accession number AF111201. The primers specific for *M. pneumoniae* enclosed a 298-bp fragment located at positions 2262 to 2560 of the cytadhesin *P1* gene (GenBank accession number AF290002) (7). The primers specific for *Legionella* enclosed a 300-bp fragment located at positions 34 to 334 of the 23S-5S rRNA intergenic region (GenBank accession number Z30341) (21). Hybridization probes were designed to detect the PCR product amplified by each set of primers.

For each sample, two amplifications were performed. The first reaction tube was dedicated to the amplification of the template DNA, and the second one was dedicated to the detection of PCR inhibitors. For the latter reaction, a composite control plasmid included in the PCR mixture was used at a low concentration. This plasmid contained an internal fragment that exhibited the same size and thermodynamic properties as the template DNA; it was amplified with the same primers described above but was detected by use of a different probe. With the exception of the plasmid, the contents of the PCR mixtures, provided in the kit, were the same in the two reaction tubes. Each reaction tube contained 35 μl of PCR mixture, 5 μl of diluted HotstarTaq DNA polymerase (QIAGEN) to a final concentration of 1.5 units per reaction, and 10 μl of DNA template. The amplification step was performed on a Geneamp 2700 PCR system (Applied Biosystems, Foster City, Calif.) by using the same program for the reactions in the two tubes: 15 min at 95°C for the enzyme activation, followed by 10 cycles of PCR amplification (95°C for 30 s, 57°C for 90 s, and 72°C for 30 s) and 30 cycles of additional PCR amplification (95°C for 30 s, 53°C for 90 s, and 72°C for 30 s). The amplified products from the first reaction tube were analyzed by microplate hybridization by using a generic probe that allows the detection of the three pathogens in a single well. Those from the second tube, which contained the control plasmid, were hybridized with a specific probe to allow the detection of PCR inhibitors. For samples found to be positive with the generic probe, the PCR product was then hybridized by using four probes specific for *C. pneumoniae*, *M. pneumoniae*, *Legionella* genus, and *L. pneumophila*, respectively. Briefly, the amplified product was denatured chemically and coated passively in a 96-well microplate for 1 h at 37°C. The hybridization with the corresponding biotinylated probe was then performed for 30 min at 37°C. After five washing steps, a streptavidin-peroxidase conjugate was added for 15 min at room temperature. After five more washing steps, the reaction was revealed by treatment with tetramethylbenzidine for 30 min at room temperature in the dark and by reading the optical density (OD) at 450 nm versus 620 nm after the reaction was stopped. The cutoff (CO) was calculated by using the formula CO = OD (blank) + 0.075; the coefficient 0.075 corresponds to three times the mean OD values obtained with the negative respiratory samples. Samples giving an OD of the CO value ±10% were extracted and tested again; they were then considered positive when the OD was greater than the CO and negative when the OD was less than or equal to the CO. The sensitivity of the Chlamylege kit was determined by assaying 10-fold serial dilutions of the strains of *C. pneumoniae*, *M. pneumoniae*, and *L. pneumophila* mentioned above.

In-house PCR assays targeting *C. pneumoniae*, *M. pneumoniae*, and *Legionella*. *C. pneumoniae* was detected by real-time PCR with primers specific for sequences located in the *omp-1* gene (primers ForC [CGCAGAAGAATCTCCT ACTCAT] and RevC [TCTTCGGCTTCCTAACAAT]). *M. pneumoniae* was detected by real-time PCR with primers specific for sequences located in the *P1* gene (6). The beta-globin gene amplification, used as a control for PCR inhibition, was performed in the same reaction tube by using primers ForB (GGGC AGGTTGTTATCAAGG) and RevB (AGCCAGGCCATCACTAAA). *Legionella* was detected by real-time PCR with primers specific for sequences located in the 16S rRNA gene (13). All the assays were performed by using the QuantiTect SYBR Green PCR kit (QIAGEN). For the three tests, the $MgCl_2$ and primer concentrations were 2.5 mM and 0.5 μM, respectively, in all assays with the exception of the beta-globin assay, in which the primer concentration was 0.05 μM. All these assays were performed on a LightCycler thermocycler (Roche Diagnostics, Meylan, France) by using the following protocol: 15 min at 95°C for *Taq* polymerase activation, 50 cycles of PCR amplification (95°C for 15 s, 55°C for 25 s, and 72°C for 25 s), and generation of a final melting curve. For the detection of *C. pneumoniae*, the annealing temperature was 50°C instead of 55°C. The positive signals were detected by the use of SYBR Green fluorescence and analysis of the melting curves.

Clinical specimens used to evaluate the performance of the Chlamylege kit. Thirty specimens (12 endotracheal aspirates, 7 sputum samples, 7 BAL fluid specimens, 3 nasopharyngeal aspirates, and 1 nasal swab specimen) from 18 patients with evidence of infection by one of these three pathogens by culture, urinary antigen assay, in-house PCR, and/or serology were analyzed (Table 2). The bacterial cultures were performed as described above for the bacterial strains. The Now *Legionella* urinary antigen test (Binax Inc., Portland, Maine) was used for the detection of *L. pneumophila* serogroup 1 antigen in urine specimens. Serological tests were performed by using the following reagents, according to the manufacturers' instructions: Sero CP IgM kit and Sero CP IgG kit (Savyon Diagnostic Ltd., Ashdod, Israel) for *C. pneumoniae*, MP Immunocard *Mycoplasma* IgM kit (Meridian Bioscience Inc., Cincinnati, Ohio) and Serodia-Myco kit (Fujirebio Inc., Tokyo, Japan) for *M. pneumoniae*, and *L. pneumophila* 1-6 IFA kit (Meridian Bioscience) for *L. pneumophila* serogroups 1 to 6. The serological diagnosis was considered positive when IgM antibodies were detected or when a minimum of a fourfold increase in the IgG antibody titer was observed for two successive serum specimens.

In addition, 86 nasopharyngeal aspirates collected from the pediatric unit of the University Hospital of Caen (France) and 28 endotracheal aspirates collected from the intensive care unit of the University Hospital of Saint-Etienne (France), all of which tested negative for the three pathogens by the in-house PCR assays, were used to check the specificity of the Chlamylege technique.

To evaluate whether the Chlamylege assay was able to detect more than one pathogen in specimens from coinfected patients, six samples found to be positive for each of the three testable pathogens were selected, and mixtures of equal amounts of samples containing two different pathogens were tested with the kit.

Prospective comparative study with specimens from children. A prospective study was performed with 220 nasopharyngeal aspirates collected from children with respiratory tract infections seen at the pediatric unit of the University Hospital of Saint-Etienne during the 2003-2004 winter season. The detection of *C. pneumoniae*, *M. pneumoniae*, and *Legionella* species was done by in-house PCR assays; and the Chlamylege test was performed by different experimenters in a blinded fashion. The specimens were also investigated for the presence of viruses and other bacteria. Viruses were detected by direct immunofluorescence

TABLE 1. Bacterial strains, yeast, and fungi used for evaluation of the Chlamylege kit

Species (serogroup)	Strain or source	Result with:				
		Generic probe	*C. pneumoniae*-specific probe	*M. pneumoniae*-specific probe	*Legionella* genus-specific probe	*L. pneumophila*-specific probe
Chlamydophila pneumoniae IOL207	Clinical isolate	+	+	−	−	−
Mycoplasma pneumoniae FH type 2	ATCC 15531	+	−	+	−	−
M. pneumoniae type 1	Seven clinical isolates	+	−	+	−	−
M. pneumoniae type 2	One clinical isolate	+	−	+	−	−
Legionella pneumophila (1)[a]	ATCC 33152	+	−	−	+	+
Legionella pneumophila (2)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (3)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (4)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (5)[a]	ATCC 33737	+	−	−	+	+
Legionella pneumophila (5)[a]	ATCC 33216	+	−	−	+	+
Legionella pneumophila (5)[a]	ATCC 33235	+	−	−	+	+
Legionella pneumophila (6)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (7)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (8)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (9)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (10)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (11)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (12)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (13)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (14)[a]	Environmental isolate	+	−	−	+	+
Legionella pneumophila (15)[a]	ATCC 33251	+	−	−	+	+
Legionella adelaidensis	ATCC 49625	+	−	−	+	−
Legionella anisa[a]	ATCC 35292	+	−	−	+	−
Legionella anisa[a]	ATCC 35291	+	−	−	+	−
Legionella beliardensis	ATCC 700512	+	−	−	+	−
Legionella beliardensis	ATCC 700760	+	−	−	+	−
Legionella birminghamensis[a]	ATCC 43702	+	−	−	+	−
Legionella bozemaniae (1)[a]	ATCC 33217	+	−	−	+	−
Legionella bozemaniae (2)[a]	ATCC 35545	+	−	−	+	−
Legionella brunensis	ATCC 43878	+	−	−	+	−
Legionella cherrii	ATCC 35252	+	−	−	+	−
Legionella cincinnatiensis[a]	ATCC 43753	+	−	−	+	−
Legionella dumoffii[a]	ATCC 33279	+	−	−	+	−
Legionella erythra (1)	ATCC 35303	+	−	−	+	−
Legionella erythra (2)[a]	NCTC 11977	+	−	−	+	−
Legionella feeleii (1)[a]	ATCC 35072	+	−	−	+	−
Legionella feeleii (2)[a]	ATCC 35849	+	−	−	+	−
Legionella geestiana	ATCC 49504	+	−	−	+	−
Legionella genomospecies	ATCC 51913	+	−	−	+	−
Legionella gormanii[a]	ATCC 33297	+	−	−	+	−
Legionella gratiana	ATCC 49413	+	−	−	+	−
Legionella gresiliensis	ATCC 700509	+	−	−	+	−
Legionella hackeliae (1)[a]	ATCC 35250	+	−	−	+	−
Legionella hackeliae (2)[a]	ATCC 35999	+	−	−	+	−
Legionella israelensis[a]	ATCC 43119	+	−	−	+	−
Legionella jamestowniensis[a]	ATCC 35298	+	−	−	+	−
Legionella jordanis[a]	ATCC 33623	+	−	−	+	−
Legionella lansingensis[a]	ATCC 49751	+	−	−	+	−
Legionella londiniensis (1)[a]	ATCC 49505	+	−	−	+	−
Legionella londiniensis (2)[a]	ATCC 700510	+	−	−	+	−
Legionella longbeachae (2)[a]	ATCC 33484	+	−	−	+	−
Legionella maceachernii[a]	ATCC 35300	+	−	−	+	−
Legionella micdadei[a]	ATCC 33218	+	−	−	+	−
Legionella nautarum	ATCC 49506	+	−	−	+	−
Legionella oakridgensis[a]	ATCC 33761	+	−	−	+	−
Legionella parisiensis[a]	ATCC 700174	+	−	−	+	−
Legionella quateirensis	ATCC 49507	+	−	−	+	−
Legionella quinlivanii (1)	ATCC 43830	+	−	−	+	−
Legionella quinlivanii (2)	ATCC BAA-538	+	−	−	+	−
Legionella rubrilucens	ATCC 35304	+	−	−	+	−
Legionella sainthelensi (1)[a]	ATCC 35248	+	−	−	+	−
Legionella sainthelensi (2)[a]	ATCC 49322	+	−	−	+	−
Legionella shakespearei[a]	ATCC 49655	+	−	−	+	−

Continued on following page

TABLE 1—*Continued*

Species (serogroup)	Strain or source	Result with:				
		Generic probe	*C. pneumoniae*-specific probe	*M. pneumoniae*-specific probe	*Legionella* genus-specific probe	*L. pneumophila*-specific probe
Legionella spiritensis (2)[a]	ATCC BAA-537	+	−	−	+	−
Legionella taurinensis[a]	ATCC 700508	+	−	−	+	−
Legionella tucsonensis[a]	ATCC 49180	+	−	−	+	−
Legionella wadsworthii[a]	ATCC 33877	+	−	−	+	−
Legionella waltersii[a]	ATCC 51914	+	−	−	+	−
Acinetobacter baumannii	Clinical isolate	−	−	−	−	−
Bordetella pertussis	Clinical isolate	−	−	−	−	−
Chlamydia trachomatis	Clinical isolate	−	−	−	−	−
Enterococcus faecalis	ATCC 29212	−	−	−	−	−
Haemophilus influenzae	Clinical isolate	−	−	−	−	−
Klebsiella oxytoca	ATCC 700324	−	−	−	−	−
Klebsiella pneumoniae	Clinical isolate	−	−	−	−	−
Mycoplasma hominis	Clinical isolate	−	−	−	−	−
Pseudomonas aeruginosa	ATCC 27853	−	−	−	−	−
Serratia marcescens	Clinical isolate	−	−	−	−	−
Staphylococcus aureus	ATCC 29213	−	−	−	−	−
Streptococcus agalactiae	ATCC 9242	−	−	−	−	−
Streptococcus pyogenes	Clinical isolate	−	−	−	−	−
Streptococcus mitis	Clinical isolate	−	−	−	−	−
Streptococcus orale	Clinical isolate	−	−	−	−	−
Streptococcus pneumoniae	Clinical isolate	−	−	−	−	−
Ureaplasma urealyticum	Clinical isolate	−	−	−	−	−
Candida albicans	ATCC 90028	−	−	−	−	−
Candida glabrata	Clinical isolate	−	−	−	−	−
Candida inconspicua	Clinical isolate	−	−	−	−	−
Candida krusei	ATCC 6258	−	−	−	−	−
Candida parapsilosis	ATCC 6602	−	−	−	−	−
Candida tropicalis	ATCC 2219	−	−	−	−	−
Aspergillus fumigatus	Clinical isolate	−	−	−	−	−
Pneumocytis carinii	Clinical isolate	−	−	−	−	−

[a] Species of *Legionella* involved in human diseases.

and/or culture in two cell lines, according to standard protocols; conventional bacteria were detected by culture on classical agar media. For 10 patients shown to be infected by one of the three atypical bacteria, a throat swab specimen, available in addition to the nasopharyngeal aspirate, was also tested both by the in-house PCR assays and with the Chlamylege kit.

RESULTS

Development and performance of the Chlamylege kit. When the three sets of selected primers were used in the multiplex assay, they specifically amplified the DNA of the three pathogens and provided bands of the expected sizes, with no nonspecific bands (Fig. 1). As shown in Table 1, the Chlamylege kit was found to be able to recognize and correctly identify the *C. pneumoniae* IOL207 strain, the reference strain and the 8 clinical isolates of *M. pneumoniae*, and all the 64 *Legionella* reference or environmental strains tested, including the 20 species previously involved in human infections. No cross-reaction was observed between the PCR product specific for a pathogen and the other selected probes.

In parallel, 30 respiratory specimens sampled from 18 patients found to be infected by one of the three pathogens, as evidenced by other bacterial tests, were assayed with the Chlamylege kit. Each of them was found to be positive with the generic probe and was correctly identified by use of the corresponding specific probe (Table 2).

The analytical sensitivities assessed by serial dilutions of the three reference strains were approximately 1.1×10^{-3} IFU per reaction tube for *C. pneumoniae*, 5×10^{-2} color-changing units per reaction tube for *M. pneumoniae*, and 1 CFU per reaction tube for *L. pneumophila*.

No DNA sample from other bacteria, yeast, or fungi genetically linked to one of the three pathogens or potentially present in the respiratory tract tested positive with the Chlamylege kit (Table 1). In addition, 114 respiratory specimens that tested negative by in-house PCR assays for the three pathogens also tested negative with the Chlamylege kit, including 86 samples from hospitalized children with pneumonia and 28 samples from adult patients from an intensive care unit. For all 114 samples that tested negative with the kit, no inhibitor was detected; indeed, for these negative reactions, the corresponding reaction containing the control plasmid exhibited a positive signal.

Six additional samples containing a mixture of two specimens, each of which was positive for one of the three test pathogens, were tested. As shown in Table 3, both pathogens were detected in five of the six mixed samples, and only one pathogen was detected in the sixth mixed sample.

Prospective comparative study with specimens from children. A total of 220 nasopharyngeal aspirates were tested prospectively for the presence of the DNA of *C. pneumoniae*, *M. pneumoniae*, and *Legionella* by using the Chlamylege kit and in-house real-time PCR assays targeting each of the three

TABLE 2. Detection of *C. pneumoniae*, *M. pneumoniae*, and *Legionella* by the Chlamylege kit in samples from patients previously diagnosed with infections with the pathogens[a]

Patient no./ sex/age (yr)	Bacterial diagnosis		Specimen	Chlamylege kit result with probe specific for:			
	Agent	Positive method(s)		*Chlamydophila pneumoniae*	*Mycoplasma pneumoniae*	*Legionella* genus	*Legionella pneumophila*
1/F/48.4	*L. pneumophila* 2	Serology, PCR	BAL fluid	−	−	+	+
			Sputum	−	−	+	+
			ETA	−	−	+	+
			ETA	−	−	+	+
			ETA	−	−	+	+
2/M/38.9	*L. pneumophila* 1	Urinary antigen, PCR	Sputum	−	−	+	+
			Sputum	−	−	+	+
3/M/84.1	*L. pneumophila* 1	Culture, PCR	BAL fluid	−	−	+	+
4/M/49.0	*L. pneumophila* 1	Urinary antigen, PCR	ETA	−	−	+	+
			ETA	−	−	+	+
			ETA	−	−	+	+
5/M/49.7	*L. pneumophila*	Urinary antigen, PCR	Sputum	−	−	+	+
6/M/72.8	*L. pneumophila* 1	Urinary antigen, PCR	ETA	−	−	+	+
7/M/69.6	*L. pneumophila* 3	Culture, serology, PCR	BAL fluid left lung	−	−	+	+
		PCR	BAL fluid right lung	−	−	+	+
8/M/32.1	*Legionella*	PCR	Sputum	−	−	+	−
			Sputum	−	−	+	−
9/M/52.7	*L. pneumophila* 1	Urinary antigen, PCR	ETA	−	−	+	+
			BAL fluid	−	−	+	+
10/M/3.9	*C. pneumoniae*	PCR	NPA	+	−	−	−
11/M/0,5	*C. pneumoniae*	PCR	ETA	+	−	−	−
12/F/12.4	*M. pneumoniae*	PCR	NPA	−	+	−	−
13/F/5.8	*M. pneumoniae*	PCR	NPA	−	+	−	−
14/F/2.7	*M. pneumoniae*	PCR	BAL fluid	−	+	−	−
15/M/11.8	*M. pneumoniae*	PCR	Nasal swab	−	+	−	−
16/M/44.4	*M. pneumoniae*	Serology, PCR	ETA	−	+	−	−
			BAL fluid	−	+	−	−
			Sputum	−	+	−	−
17/F/9.1	*M. pneumoniae*	Serology, PCR	ETA	−	+	−	−
18/F/0.5	*M. pneumoniae*	Serology, PCR	ETA	−	+	−	−

[a] Abbreviations: F, female; M, male; ETA, endotracheal aspirate; NPA, nasopharyngeal aspirate.

pathogens. The global agreement between the two diagnostic approaches was 98.64%. None of the negative specimens showed inhibition, as determined by checking for the presence of a signal with the housekeeping gene for the in-house PCR assays and with the control plasmid for the Chlamylege kit.

As illustrated in Table 4, four specimens were positive for *C. pneumoniae* and six specimens were positive for *M. pneumoniae* both by the in-house tests and with the Chlamylege kit. By comparison with the results of the in-house assays, the Chlamylege kit detected two additional samples that were positive for *M. pneumoniae* and one additional sample that was positive for *Legionella*; the latter sample was found to react

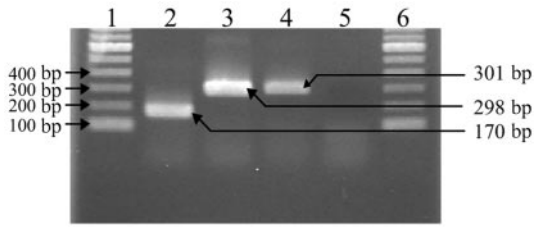

FIG. 1. Migration of the PCR products obtained with the Chlamylege kit by electrophoresis on a 1.5% agarose gel. Lanes 1 and 6, molecular size markers; lane 2, *C. pneumoniae*; lane 3, *M. pneumoniae*; lane 4, *L. pneumophila*; lane 5, water negative control. bp, base pairs.

with the *Legionella* genus-specific probe but not with the *L. pneumophila*-specific probe. Whereas all the concordant positive samples exhibited high OD values (>3) by the Chlamylege assay, the three samples with discrepant results had lower OD values, ranging from 0.39 to 2.67. Since no serological data were available to document the two discrepant results obtained for *M. pneumoniae*, they were checked by using a nested PCR assay with primers targeting the 16S ribosomal gene and by sequencing (31). An *M. pneumoniae* sequence was obtained in both cases by the latter technique (data not shown). In order to control the discordant result obtained for *Legionella*, the PCR product was sequenced. The phylogenetic analysis of the product gave a percentage of homology that indicated that it was closely related to the sequences of non-*L. pneumophila Legionella* species (data not shown). According to these complementary data, the resolved sensitivity was 100% for the Chlamylege kit.

This clinical study was also used to test the specificity of the Chlamylege kit, since one or more other infectious agents were isolated from a large number of the nasopharyngeal aspirates. Actually, viruses were detected in 102 samples by culture and/or immunofluorescence (respiratory syncytial virus, 81 samples; influenza virus type A, 11 samples; adenovirus, 8 samples; enterovirus, 2 samples; herpes simplex virus type 1, 1 sample), and bacteria were detected at a concentration of more than 10^3 CFU/ml in 160 samples (*Streptococcus pneumoniae*, 93 samples; *Haemophilus influenzae*, 87 samples; *Branhamella catarrhalis*, 51 samples; *Staphylococcus aureus*, 8 samples; *Streptococcus pyogenes*, 8 samples; *Escherichia coli*, 3 samples; *Streptococcus agalactiae*, 1 sample; *Klebsiella oxytoca*, 1 sample; *Enterobacter cloacae*, 1 sample; *Acinetobacter baumannii*, 1 sample). No cross-reaction with any of these agents was observed by use of the Chlamylege test.

TABLE 3. Evaluation of the Chlamylege kit with mixtures resulting from the combination of two positive specimens in equal amounts

Mixed samples[a]	Chlamylege kit result for:			
	C. pneumoniae	*M. pneumoniae*	*Legionella* spp.	*L. pneumophila*
A + B	−	+	−	−
A + C	+	−	+	+
B + C	−	+	+	+
D + E	+	+	−	−
D + F	+	−	+	+
E + F	−	+	+	+

[a] Samples A and D were positive for *C. pneumoniae*, samples B and E were positive for *M. pneumoniae*, and samples C and F were positive for *L. pneumophila*.

TABLE 4. Comparative evaluation of the Chlamylege kit and of the in-house PCR assays with 220 nasopharyngeal aspirates tested prospectively from children of the University Hospital of Saint-Etienne, France, during the 2003–2004 winter season

Organism	Total no. (%) of specimens positive by:	
	Chlamylege	In-house PCR
None[a]	207 (94.1)	210 (95.5)
C. pneumoniae	4 (1.8)	4 (1.8)
M. pneumoniae	8 (3.6)	6 (2.7)
Legionella	1 (0.5)	0 (0)

[a] Negative for *C. pneumoniae*, *M. pneumoniae* and *Legionella* spp. by the corresponding PCR assays.

Ten throat swab specimens from patients whose nasopharyngeal aspirates had positive results (three for *C. pneumoniae* and seven for *M. pneumoniae*) were also tested by the in-house PCR assays and with the Chlamylege kit. By both methods, only six of the samples (two of which were positive for *C. pneumoniae* and four of which were positive for *M. pneumoniae*) were found to be positive for the same bacterium detected in the corresponding nasopharyngeal aspirate, suggesting that lower bacterial loads were present in the throat swab specimens.

DISCUSSION

By using conventional techniques, the numbers of respiratory infections due to *C. pneumoniae*, *M. pneumoniae*, and *Legionella* species are underestimated. The detection of these bacteria in respiratory samples by single or multiplex PCR has been described to be rapid, sensitive, and specific (1, 2, 11–14, 18, 19, 25). The Chlamylege assay described in this report allows the detection of the three pathogens within 6.5 h.

Each set of primers was designed (i) to be specific for the target pathogen, (ii) to be efficient when it was mixed with the two other sets of primers, and (iii) to allow the sensitive detection of each pathogen when detection with the primer was coupled with hybridization detection. Each probe was designed to be specific for the corresponding amplified DNA. The primers specific for *C. pneumoniae* were located in the *omp-2* gene, described to be highly conserved within the species (27). The primers specific for *M. pneumoniae* were located in a conserved part of the gene coding for the cytadhesin P1, which allows the recognition of the two major genetic variants observed within the species (7).

The primers specific for the *Legionella* genus allowed the amplification of the 23S-5S rRNA intergenic region. This region was chosen because it combines the two following properties: (i) the rRNA sequences are highly conserved within the *Legionella* genus, which allows the detection of most of the *Legionella* species, and (ii) the intergenic region is variable from one species to another, which allows the design of probes that differentiate *L. pneumophila* from the other *Legionella* species. Actually, 38 of the 50 *Legionella* species were tested, including the 20 species that have been involved at least once in human infections, and all of them were correctly detected

and differentiated by the Chlamylege probes (Table 1). Human infections involving *Legionella* species other than *L. pneumophila* have been described anecdotally (9). However, their recognition as cases of legionellosis is probably underreported due to the poor conditions of their microbial diagnosis: these bacteria are not detected by the urinary antigen, only a few species can be detected by serology, some species are difficult to recover by classical agar culture, some species give an atypical morphology on charcoal medium, and other species are difficult to recognize due to their growth on conventional blood agar (9). Therefore, the Chlamylege kit should help to improve the diagnosis of infections due to *Legionella* other than *L. pneumophila*.

All 30 respiratory samples taken from patients with a documented infection by one of the three pathogens and kept frozen tested positive, and the 126 samples that tested negative by other techniques also tested negative with this kit. No false-positive result was observed for the 220 pediatric clinical specimens tested prospectively, even though the specimens were highly contaminated with other bacteria or viruses, since at least one bacterium or virus was detected in 85% of these samples. These results confirm the performance of the kit observed with the reference strains.

Although it is unusual, coinfection by two of the three pathogens assayed by the Chlamylege kit is possible, especially in immunocompromised patients. As the assay is based on a multiplex PCR concept, competition for the *Taq* polymerase should occur during the amplification of more than one specific DNA. By mixing positive specimens in equal amounts, the load of each bacterial pathogen corresponded roughly to that present in the clinical samples. Despite this competition, most of the dual infections were recognized, demonstrating the relative robustness of the technique in such an unusual clinical situation.

The absence of a "gold standard" for the quantification of intracellular pathogens makes comparison of the sensitivities of different assays difficult. Furthermore, the units used to quantify *M. pneumoniae* and *Legionella* may vary from one study to another, although the correspondence between different units is available for *M. pneumoniae* (3, 20). The Chlamylege kit was shown to have a sensitivity similar to or better than that reported for *C. pneumoniae* (2, 5, 16, 17, 25, 26, 30) or *M. pneumoniae* (1, 5, 14, 15, 23, 26, 30) in a single or a multiplex PCR. For *L. pneumophila*, the sensitivity of the Chlamylege kit was similar to that of the technique described by Jonas et al. (13), but it was less sensitive than the one described by Templeton et al. (24). Comparison of the sensitivity of the Chlamylege kit with the sensitivities of other studies for *Legionell*a detection was not possible because of the absence of standardized units.

The prospective study performed with respiratory specimens from 220 children with respiratory tract infections seen at the pediatric unit of the University Hospital of Saint Etienne during the 2003-2004 winter season reports prevalences of infections by *C. pneumoniae*, *M. pneumoniae*, and *Legionella* spp. of 1.8%, 3.6%, and 0.5%, respectively. The comparison of these results with those obtained in other studies was made difficult due to the heterogeneity of the epidemiological conditions, the study population, the clinical specimens, and the bacteriological methods used (1, 2, 11–14, 18, 19, 25).

The use of deep specimens, such as bronchoalveolar lavage fluid or endotracheal aspirates, is recommended for the detection of pathogens involved in lower respiratory tract infections; and these specimens were found to be adequate for the detection of the intracellular pathogens with the Chlamylege kit (Table 2). However, since these specimens are obtained by invasive methods and not available from patients sampled outside the hospital, nasopharyngeal aspirates and throat swabs have been proposed as alternative samples (4). None of the latter specimens was shown to contain PCR inhibitors, as assessed by the detection of the plasmid control in the Chlamylege kit and of the housekeeping gene in the in-house PCR. For the 10 patients from whom a throat swab specimen was also available, the nasopharyngeal aspirate was shown to be more sensitive, as four additional positive results were detected by using these samples.

From an economic point of view, the mean cost of detection with the Chlamylege kit was evaluated to be nearly twice the additive cost of the raw material for the three in-house PCRs (since three agents are tested for simultaneously).

This study reports on the development and the evaluation of a commercial test that allows the simultaneous detection of *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila*, and most of the other *Legionella* species in respiratory samples. The detection is performed in the same reaction tube by multiplex PCR, followed by microplate hybridization with specific probes. The test is provided as a kit that includes all the reagents useful for the extraction, the amplification (but not the enzyme), and the detection of the genomes of the three bacterial species. It also provides the specific controls used to validate the amplification step and the absence of inhibition of the PCR. Because of its high specificity and sensitivity with both bacterial strains and characterized clinical samples, the Chlamylege kit appears to be a useful tool for the rapid detection of atypical pathogens in respiratory specimens.

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