Single-Run, Parallel Detection of DNA from Three Pneumonia-Producing Bacteria by Real-Time Polymerase Chain Reaction

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A molecular assay for parallel detection of three bacteria, *Chlamydia* (*C.*) *pneumoniae*, *Legionella* (*L.*) spp., and *Mycoplasma* (*M.*) *pneumoniae*, in clinical specimens by a set of real-time polymerase chain reactions (PCRs) in a single run was evaluated. Bacterial DNAs were extracted by an automated DNA extraction protocol on the MagNA Pure LC System. Amplification and detection were done by real-time PCR on the LightCycler (LC) instrument. For amplification, specific oligonucleotides derived from the 16s rRNA genes of *C. pneumoniae*, *L. spp.*, and *M. pneumoniae* were used. The three assays were complemented with an internal control (IC), a specially designed DNA fragment which contains the specific primer binding sites for the three PCRs. The IC was added to the samples, co-extracted, and co-amplified. Primers and hybridization probes were designed to suit one LC PCR program. LC PCRs were established, detection limits were determined, and clinical samples were tested. The detection limits were found between 5.0 and 0.5 IFU/CFU per PCR reaction for each of the bacteria. A total number of 100 clinical specimens were tested for validation of the molecular assay. Tested samples included 63 bronchoalveolar lavages (BALs) and 37 induced sputa specimens. The internal control was detected in all negative and low-positive samples; no inhibition was found throughout the whole study. Additionally, samples underwent testing by culture for *L. spp.*, and *M. pneumoniae*; for *C. pneumoniae*, the serological microimmunofluorescence (MIF) test was used. In conclusion, the developed set of LC PCR assays permits parallel detection of *C. pneumoniae*, *L. spp.*, and *M. pneumoniae* in a single LC run. This molecular assay may lead to accurate and early diagnosis of pneumonia produced by these three types of bacteria. The assay proved to be suitable for the high-throughput routine diagnostic laboratory. (*J Mol Diagn* 2005, 7:133–138)

Chlamydiae (*C.*) *pneumoniae*, *Legionella* (*L.*) *pneumophila*, and *Mycoplasma* (*M.*) *pneumoniae* are responsible for respiratory tract infections, particularly pneumonia.^{1,2} *C. pneumoniae* infection, frequently asymptomatic, can be responsible for long-lasting disease, which may be severe in elderly patients. Furthermore, *C. pneumoniae* is the microorganism most often found associated with inflammation seen in arteriosclerosis.³ Seroprevalence among adults is 40 to 70%, indicating that most people are exposed at least once during their lifetime and re-infections are common.⁴ Mostly, detection of *C. pneumoniae* infection is based on serology. The microimmunofluorescence (MIF) test is the serological testing method of choice for diagnosis of acute *C. pneumoniae* infection.⁵ This method relies on antibody detection, which means that *C. pneumoniae* may not be present when the test is positive. Cultivation of the organism is difficult and slow, showing poor sensitivity.⁶ Immunohistochemistry is a method successfully used for detection of *C. pneumoniae* in paraffin-embedded, formalin-fixed tissue.^{7,8} Recently, nucleic acid amplification techniques have been introduced. They permit rapid and sensitive diagnosis of *C. pneumoniae* infections.^{5,9,10}

The species *L. pneumophila*, an aerobic gram-negative bacterium from the family Legionellaceae, is known as the causative agent of Legionnaires' disease, which is associated with severe pneumonia, especially in elderly individuals. In immunocompromised patients, pneumonia produced by legionellae can result in fatal outcome in up to 50% cases.¹¹ Isolation of legionellae from various samples by culture is the method usually preferred but shows limitations. Problems with culture include fastidious growth requirements, extended incubation periods, overgrowth by other bacteria, and the presence of viable but non-culturable legionellae.^{12,13} Direct detection of organisms out of clinical specimens, usually performed with

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immunofluorescence methods, is more rapid than culture. Sensitivity of these methods, however, has been reported to be poor.^{14–16} A variety of techniques, including radioimmunoassay and latex agglutination, can be used to detect a soluble polysaccharide antigen of *L. pneumophila* (serogroup 1 only) in urine with reported sensitivities up to 90%.^{16–21} Serological methods are highly sensitive but their utility is generally limited to epidemiological studies because of the serological window.^{11,16} New methods for detection of legionellae in various samples by molecular techniques including polymerase chain reaction (PCR) have recently been developed and have overcome the limitations of culture methods.^{22–27}

M. pneumoniae is a frequent causative agent of tracheobronchitis and pneumonia in children.²⁸ Moreover, *M. pneumoniae* is one of the most common causes of community-acquired pneumonia in children and young adults, ranging from mild to life-threatening infections.^{29,30} Methods for routine diagnosis of infections produced by *M. pneumoniae* include culture, serology, and molecular assays. Culture requires up to 3 weeks generating results and is relatively insensitive.³¹ Serological methods are usually not helpful during the early stages of the disease; thus, accurate diagnosis is often made, only retrospectively, after the onset of therapy.^{31–33} Recently developed molecular assays have shown to be superior to serological diagnosis with respect to speed, sensitivity, and specificity.^{31,34–36}

In the present study, the development and primary evaluation of a molecular method for parallel detection and identification of the three most common bacterial causes of pneumonia, *C. pneumoniae*, *L. spp.*, and *M. pneumoniae* in BALs and induced sputa specimens by a set of real-time PCRs in a single run is described. Automated DNA extraction was based on the MagNA Pure LC System (Roche Applied Science, Mannheim, Germany). Real-time PCR used the LightCycler instrument (Roche). A specially designed DNA fragment, which served as a *Chlamydia-Legionella-Mycoplasma*-specific multiple internal control (IC) was included in this assay and co-extracted and co-amplified with the specimen. After determination of detection limits, clinical specimens were investigated. BALs and induced sputa were additionally tested by culture for *L. spp.* and *M. pneumoniae*. The serological MIF Test was used, testing sera for *C. pneumoniae*.

Materials and Methods

Study Design

In the first step, detection limits of the three molecular assays were determined. BALs and induced sputa samples were obtained from patients without clinical presentations compatible with pneumonia. Aliquots were spiked with standards (5000, 500, 50, 5, and 0.5 IFU or CFU per PCR reaction) prior DNA extraction. Standards had been prepared from strains *C. pneumoniae* ATCC 1227980, *L. pneumophila* serogroup 1 ATCC 33152, and *M. pneu-*

Figure 1. DNA fragment including the specific forward and reverse primer sequences of *Chlamydia pneumoniae* (CP)-, *Legionella pneumophila* (LP)-, and *Mycoplasma pneumoniae* (MP)-specific real-time PCRs. The center box represents the heterologous DNA derived from the neomycin phosphotransferase gene (neo), which is detected with one pair of fluorescence resonance energy transfer hybridization probes. The composite primers for the preparative PCRs are shown as horizontal lines from bottom up in the order of their use. Primer sequences are listed in Table 1.

moniae ATCC 49894. Aliquots containing 1×10^5 infection-forming units (IFU)/ml or colony-forming units (CFU)/ml were taken and serial dilutions were prepared to yield standards ranging from 5000 IFU or CFU per PCR reaction to 0.5 IFU or CFU per PCR reaction corresponding to 10^6 to 10^2 IFU/CFU/ml. Experiments were done five times in repeat tests on different days.

In the second step, a total of 100 clinical specimens from patients presenting clinical symptoms compatible with pneumonia were tested. Clinical specimens tested included 63 BALs and 37 induced sputa derived from 59 adults and 41 children, respectively. All runs included two different aliquots of the three bacterial standards each, serving as positive controls (high-positive and low-positive), and sterile PCR-grade water as a negative control.

Multiple Internal Control (IC)

For the set of three assays, a single IC DNA-molecule was constructed by using composite primers in preparative PCRs as recently described.^{37,38} The IC contained a stretch of the bacterial neomycin phosphotransferase gene (neo) (GenBank Accession No. V00618, bp 452–603), which was flanked in 5' and 3' by the three forward and reverse primer binding sites specific for each PCR of the set (Figure 1). Briefly, the IC construct was generated by three consecutive conventional PCRs in which for each following PCR, the products of the previous PCR were used as template (Table 1, Figure 1). The obtained construct (292 bp) was cloned into the plasmid vector PCR II-TOPO by using the TOPO TA Cloning kit (Invitrogen Ltd, Paisley, UK). Plasmid DNA was purified from transformed bacteria by using the Quantum Plasmid Miniprep kit (Bio-Rad, Richmond, CA). The IC was subjected to sequence analysis to confirm the expected DNA sequence. The IC plasmid was linearized with *HindIII* restriction followed by purification with the High-Pure Viral Nucleic Acid kit (Roche). To confirm the PCR product sizes, the IC DNA was subjected to the set of real-time PCRs (see below), and the products were also analyzed by agarose gel electrophoresis followed by visualization with ethidium bromide staining and UV illumination. This revealed the expected product sizes of 227 bp, 292 bp, and 228 bp for the *Chlamydia*-, *Legionella*-, and *Mycoplasma*-specific PCRs, respectively.

Table 1. Composite Primers Used for the Generation of the Multiple Internal Control DNA in Preparative PCR

1st PCR
Forward: MP ^a -Neo (5'-CGCCAGCTTGTAAGAGTGAAGC-CTCCTGTCATCTCACCTTGC-3')
Reverse: Neo-CP ^b (5'-AGTGTCTCAGTCCCAGTGTGGC-ACCGGCTTCCATCCGA-3')
2nd PCR
Forward: CP ^b -MP ^a (5'-AACTTGGGAATAACGGTTGGAAAC-CGCCAGCTTGTAAGAGTGAAGC-3')
Reverse: CP ^b -MP ^a (5'-TAGCAACACGTTTTTAAATATTACC-AGTGTCTCAGTCCCAGTGTGGC-3')
3rd PCR
Forward: LP ^c -CP ^b (5'-AGGGTTGATAGGTTAAGAGC- AACTTGGGAATAACGGTTGGAAAC-3')
Reverse: MP ^a -LP ^c (5'-CCAACAGCTAGTTGACATCG- TAGCAACACGTTTTTAAATATTACC-3')

^aMP-, ^bCP-, ^cLP-specific primer sequences used in real-time PCRs.

DNA Extraction

Before the start of the automated DNA extraction on the MagNA Pure DNA Extraction Automate (Roche), 130 μ l bacteria lysis buffer, 6 μ l IC, and 20 μ l proteinase K were added to 100 μ l sample material, followed by a 10-minute incubation at 65°C. After this, the MagNA Pure LC Microbiology M^{GRADE} kit (Roche) with the standard protocol according to the manufacturer's instruction was used. The elution volume was set to 100 μ l.

Primers and Probes

Oligonucleotides deduced from the published sequences of the 16S rRNA genes of the *Chlamydia*, *Legionella*, and *Mycoplasma* genomes were used (Table 2). Specificity studies have been published elsewhere.^{24,36,39} For the detection of the target sequences, hybridization probes (TIB MOLBIOL, Berlin, Germany) were labeled with LC Red 640 at the 5' end and with fluorescein at the 3' end (Table 2). For the detection of the IC, hybridization probes (TIB MOLBIOL) were labeled with LC Red 705 at the 5' end and with fluorescein at the 3' end (Table 2).

Real-Time PCR on the LC Instrument

The real-time PCR was performed on the LC instrument (Roche Diagnostics). For the present study, each of the three PCRs was assigned to its own capillary. All samples were tested by the LC Fast Start DNA Master Hybridization Probes kit according to the manufacturer's recommendations (Roche Applied Systems). PCR reactions contained 15 μ l of Master mix and 5 μ l of template DNA. The final reaction mixture contained: 4 mmol/L/L MgCl₂, 0.5 mmol/L/L forward and reverse primers each, 0.2 mmol/L/L of the bacteria-specific fluorescein and LC Red 640-labeled hybridization probes each, 0.2 mmol/L/L of LC Red 705- and fluorescein-labeled neo-hybridization probes each, and 1X LC Fast Start Master DNA Hybridization Probes buffer.

Cycling conditions included one cycle of 95°C for 7 minutes followed by 60 cycles consisting of denaturation for 3 seconds at 95°C, annealing for 10 seconds at 60°C, and elongation for 10 seconds at 72°C. After the final cycle, the capillaries were cooled for 2 seconds at 40°C. Then, the melting curve was started at 50°C and the temperature was slowly raised to 82°C (0.2°C/s). Fluorescence curves were analyzed with the LC software (ver-

Table 2. Oligonucleotides Used for Real-Time PCR Assay

Primers	Length (nucleotides)	Melting temperature (°C)
LP Forward (5'-AGGGTTGATAGGTTAAGAGC) (24*)	20	49.8
LP Reverse (5'-CCAACAGCTAGTTGACATCG) (24*)	20	53.4
MP Forward (5'-CGCCAGCTTGTAAGAGTGAAGC) (36*)	21	59.4
MP Reverse (5'-TAGCAACACGTTTTTAAATATTACC) (36*)	25	53.0
CP Forward (5'-AACTTGGGAATAACGGTTGGAAAC) (39*)	24	59.3
CP Reverse (5'-AGTGTCTCAGTCCCAGTGTGGC) (39*)	23	64.2
Hybridization probes		
LP fluorescein (5'-GTGGCGAAGGCGGCTACCT-FL)	19	64.4
LP LCRed640 (5'-LC Red640-TACTGACACTGAGGCACGAAAGCGT)	25	65.3
MP fluorescein (5'-CGGTGAATACGTTCTCGGGTCTTGTAC)	27	64.9
MP LCRed640 (5'-LCRed640-CACCGCCCGTCAAACCTATGAAAGC)	24	66.4
CP fluorescein (5'-CCAACAAGCTGATATCGCATAAACTCTTCC)	30	65.4
CP LCRed640 (5'-LCRed640-CAACCGAAAGGTCCGAAGATCCCT)	25	66.3
Neo fluorescein (5'-GCTGCATACGCTTGATCCGGCT-FL)	22	66.0
Neo LCRed705 (5'-LC Red705-CCTGCCCATTCGACCACCAAGC)	22	68.6

CP, *Chlamydia pneumoniae*; GenBank Accession No. Z49873.

LP, *Legionella pneumophila*; GenBank Accession No. M159157.

MP, *Mycoplasma pneumoniae*; GenBank Accession No. M29061.

Neo, *Neomycin phosphotransferase gene*; GenBank Accession No. V00618 516-537, V00618 539-560.

*, Indicates the appropriate reference.

sion 3.5.3). Channel F2 back F1 was selected for the calculation of crossing points of the target-derived products. The calculation of crossing points was automatically done by the second derivative maximum method of the LC software (version 3.5.3.). For the detection of the IC-derived products, channel F3 back F1 was selected.

Culture

BAL and induced sputa specimens were centrifuged for 15 minutes at $1200 \times g$ and, if necessary, the induced sputa specimens were suspended with 10 ml of sterile water. In a next step, supernatant was removed and the remaining cell suspension was used for culture.

Culture for *Legionella* spp. was performed on buffered charcoal-yeast extract agar (BCYE α), and plates were incubated at 37°C for up to 14 days. Organisms from characteristic colonies were gram-stained and identified to the species level using a commercially available panel of fluorescein isothiocyanate (FITC)-labeled antibodies (SciMedX, Denville, NJ).

M. pneumoniae culture was carried out with the Pneumofast kit (International Mycoplasma, Signes, France) according to the manufacturer's recommendations. The kit contains both reagents for the preparation of solid agar plates and Pneumofast trays for broth culture. The trays contain 10 separate wells, allowing semi-quantitative determination of colony counts, biochemical identification of growing organisms, and antimicrobial resistance testing. The plates and trays were cultured at 37°C for 12 days and were examined daily for the presence of colonies with a granular and/or a fried egg appearance or a color change in the tray wells. Positive cultures resistant to ampicillin (40 mg/ml), sulfa-trimethoprim (4 mg/ml), and lincomycin (1 mg/ml) but sensitive to erythromycin (8 mg/ml) were identified as *M. pneumoniae*.

Microimmunofluorescence (MIF) Test

C. pneumoniae MIF IgG (IgG), IgA, and IgM, (Lab-systems, Helsinki, Finland), a species-specific test where *C. pneumoniae* elementary bodies are used as the antigen, was used according to the manufacturer's instruction. Seropositivity was defined as IgA ≥ 32 , IgG ≥ 64 , and IgM ≥ 32 respectively. The serum samples were analyzed twice on 2 different days by the same technician and the same equipment.

Results

When BALs and induced sputa spiked with 10-fold dilutions of the standards of *C. pneumoniae*, *L. pneumophila*, and *M. pneumoniae* were tested, the detection limits for the molecular assays were found between 5.0 and 0.5 IFU/CFU per PCR reaction for these three bacteria. With the dilutions containing 0.5 IFU/CFU per PCR reaction, the assay produced inconsistent negative and positive results (Table 3). The IC could be consistently detected in all negative and low-positive samples. In high-positive

Table 3. Determination of Detection Limits

IFU or CFU/PCR reaction	CP	Positives (n)	MP
		LP	
5000	10 (10)	10 (10)	10 (10)
500	10 (10)	10 (10)	10 (10)
50	10 (10)	10 (10)	10 (10)
5	10 (10)	10 (10)	10 (10)
0.5	4 (10)	6 (10)	7 (10)

CP, *Chlamydia pneumoniae*; LP, *Legionella pneumophila*; MP, *Mycoplasma pneumoniae*.

samples, amplification of the IC was depressed because of competitive PCR between the target and the IC (Figure 2B).

Sample Testing

From a total of 63 BAL samples, four were found to be positive for *C. pneumoniae*, two tested positive for *L. spp.*, and another seven were found to be positive for *M. pneumoniae* by the molecular assay. The remaining 50 BAL samples tested negative with the assay. A total number of 37 induced sputa samples were tested. Three gave positive results for *C. pneumoniae*, none of the samples showed a positive result for *L. spp.*, and five of them tested positive for *M. pneumoniae*. The remaining 29 induced sputa samples tested negative with the assay. The IC could always be detected in negative and low-positive samples.

BALs and induced sputa were also tested by culture for *L. spp.* and *M. pneumoniae*. All specimens that were PCR-positive for *L. spp.* and *M. pneumoniae* were also culture positive. In addition, sera of patients were investigated by the MIF test for *C. pneumoniae*. When four positive results for *C. pneumoniae* in BALs were obtained by the molecular assay, only two of them could be confirmed by using the MIF test. On repetition, both BAL samples tested positive once more with the molecular assay but the sera remained negative in MIF testing. When calculating crossing points, those obtained for the MIF-negative/PCR-positive specimens were distinctly lower than those obtained for the MIF-positive/PCR-positive specimens indicating a lower concentration of bacterial DNA in MIF-negative/PCR-positive specimens. For extraction of 32 clinical specimens, the time required was 120 minutes with the MagNA Pure LC System. This included a 15-minute set-up time for the instrument and 15 minutes for the manual steps before automated DNA extraction. The following real-time PCR on the LightCycler instrument took another 60 minutes for eight clinical specimens. This run included PCRs for each of the three bacteria (24 capillaries), corresponding high-positive and low-positive controls (six capillaries), and two negative controls (two capillaries). Results of *Legionella* cultures could be obtained within 5 to 14 days. *M. pneumoniae* culture results took 7 to 12 days. MIF test results were available on the same day performed.

Figure 2. Fluorescence versus cycle number plots of 10-fold dilutions of a bronchoalveolar lavage specimen spiked with standards of the *Mycoplasma pneumoniae* strain (5000, 500, 50, and 5 CFU per PCR reaction) and repeat tested showing *Mycoplasma*-specific amplification products (channel F2/backF1; **a**) and the corresponding internal control-specific amplification products (channel F3/backF1; **b**).

Discussion

Molecular methods have shown to be useful for detection of fastidious, slow-growing organisms such as chlamydiae, legionellae, and mycoplasma. Moreover, recently introduced molecular assays turned out to be more sensitive as culture methods.^{5,23} The real-time PCR assay described in this study provides an effective way to detect and discriminate infections with *C. pneumoniae*, *L. spp.*, and *M. pneumoniae*. Furthermore, it checks for inhibition. Several approaches have been reported for direct detection by real-time PCR of these organisms; however, all of those assays were performed in separate runs with different protocols.^{8,26,31} In this study, a set of three LightCycler PCR assays specific to the detection of *C.*

pneumoniae, *L. spp.*, and *M. pneumoniae* was developed in such a way that all PCRs, while assigned to their own capillaries, can be performed in parallel within a single run of the LightCycler instrument. For this assay, oligonucleotides derived from the 16S rRNA genes of *C. pneumoniae*, *L. spp.*, and *M. pneumoniae* were used. Specificity of the primers, deduced from the 16S rRNA genes of the *Chlamydia*, *Legionella*, and *Mycoplasma* genomes used in the present study has recently been described.^{36,39,40} The different primers and hybridization probes were tailored to meet one cycling program. This resulted in reduced processing time and hence faster turn-around. Furthermore, the use of hybridization probes instead of a TaqMan probe offers the advantage to perform melting curve analysis to discriminate unspecific PCR products.

Samples as induced sputa or bronchoalveolar lavages may include inhibitors to PCR which could cause false-negative results. It is, therefore, obligatory to incorporate an IC in each molecular assay. The IC used in this study is a multiple internal control consisting of a single DNA fragment that represents all primer sequences used in the set of assays. The IC was co-extracted with the clinical samples and co-amplified with the same primers used for the target DNA. This ensures an accurate control of the whole molecular assay and, therefore, represents the state of the art for ICs. The *Chlamydia*-*Legionella*-*Mycoplasma*-specific IC gave positive results for all negative and low-positive samples throughout the whole study, indicating a successful remove of potential inhibitors by the applied automated DNA extraction protocol.

This molecular assay showed sufficient sensitivity. The detection limits were found between 5.0 and 0.5 IFU/CFU per PCR (1000 to 100 IFU/CFU/ml) reaction for each of the three bacteria. These results are in agreement with those previously reported, in which conventional and real-time nucleic acid amplification methods were described.^{5,10,36,41,42}

When 100 samples obtained from patients presenting clinical symptoms compatible with pneumonia were tested, the different specimen types gave positive and negative results. All samples that tested positive by this molecular assay were diagnosed as pneumonia by chest X-ray. Samples tested positive for *L. spp.* and *M. pneumoniae* could be confirmed by culture. Two patient's sera remained negative by the MIF test, while the corresponding BALs were tested repeatedly positive for *C. pneumoniae* by the molecular assay. These discrepant results may rely to the early stage of the disease, where antibody detection is an insensitive diagnostic tool.

All patients underwent appropriate antibiotic therapy in the very early onset of the disease, and were successfully treated. In conclusion, the described set of real-time PCR assays showed a good and clinically meaningful sensitivity, produces a fast turn-around time, and may provide a reduction in the risk of carryover contamination. The whole assay was usually completed within 3 hours and proved to be suitable for the high-throughput routine diagnostic laboratory.

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