JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2008, p. 727–731 Vol. 46, No. 2
0095-1137/08/$08.00+0 doi:10.1128/JCM.01540-07

Simultaneous Detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* by Use of Molecular Beacons in a Duplex Real-Time PCR▽

Karolina Gullsby,[1] Martin Storm,[2] and Kåre Bondeson[2]*

Centre for Research & Development, Uppsala University/County Council of Gävleborg, 801 87 Gävle, Sweden,[1] and Uppsala University Hospital, Department of Medical Sciences, Virology, 751 85 Uppsala, Sweden[2]

Received 2 August 2007/Returned for modification 5 October 2007/Accepted 13 December 2007

A real-time PCR was designed for detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* such that each pathogen could be detected in a single tube and differentiated using molecular beacons marked with different fluorochromes. This duplex PCR, targeting the P1 adhesion gene for *M. pneumoniae* and the *ompA* gene for *C. pneumoniae*, was compared with two conventional PCR assays targeting the 16S rRNA gene and the *ompA* gene. A total of 120 clinical throat and nasopharyngeal swab samples were tested. DNA extraction was performed using an alkali denaturation/neutralization method, and real-time amplification, detection, and data analysis were performed using a Rotor-Gene 2000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Using conventional PCR as a reference in an analysis of 120 samples, 13 of 14 samples positive for *C. pneumoniae* were detected by the novel real-time PCR. In an analysis of *M. pneumoniae*, 22 samples were positive in the conventional PCR and the novel assay detected 24 positive samples. When using the conventional PCR as a reference, sensitivity and specificity were 93% and 100%, respectively, for *C. pneumoniae* and 100% and 98%, respectively, for *M. pneumoniae*. With an overall agreement of 98.8%, this suggests that performance of the new duplex real-time PCR is comparable to that of conventional PCR.

Chlamydophila pneumoniae and *Mycoplasma pneumoniae* are important and common causes of community-acquired pneumonia, where these pathogens are the etiological agent in 9 to 15% and 7 to 22% of cases, respectively (1, 14, 17, 19, 24, 29, 31). The highest incidence of *C. pneumoniae* and *M. pneumoniae* infections is among schoolchildren 5 to 14 years old (9, 16, 17, 23). Symptoms can be mild with nonproductive persistent cough, malaise, and fever, but more-severe illness occurs when the lower respiratory tracts are affected, giving rise to acute bronchitis and pneumonia (3, 10, 14). Cardiovascular disease and neurological diseases are serious nonrespiratory symptoms associated with *C. pneumoniae* and *M. pneumoniae* infections (4, 5, 11, 13, 28).

The agents causing respiratory infections are difficult to distinguish clinically, since many bacterial and viral infections often share clinical features, including symptoms (9, 22, 27). It is therefore important to find a sensitive and effective way of detecting these agents so that correct treatment will be offered and unnecessary use of antibiotic therapies avoided. *C. pneumoniae* and *M. pneumoniae* can show similar clinical features and share the characteristic of being difficult to diagnose using conventional methods, such as culture and serology. PCR has proven to be a fast and more-sensitive method and is therefore more widely used in clinical laboratories (6, 36). Recently several real-time PCR methods have been developed; these offer an even more advantageous way of detecting these pathogens (2, 15, 20, 25, 26, 30, 32, 35, 38). Real-time PCR has an advantage over conventional PCR because detection is performed in a closed system in real time, which minimizes the risk of contamination, and it is faster and requires less hands-on time.

In the present article, we describe a new duplex real-time PCR that can detect and distinguish both *C. pneumoniae* and *M. pneumoniae* in a single reaction tube. Detection is accomplished using two differently colored molecular-beacon probes that afford sensitive and specific detection.

MATERIALS AND METHODS

Bacterial strains and DNA. Bacterial strains used for controls and tests of the specificities of the real-time PCR methods are listed in Table 1. Quantified *C. pneumoniae* and *M. pneumoniae* DNA samples were obtained from Advanced Biotechnologies, Inc. (Columbia, MD).

Clinical samples. A selection of 120 clinical samples was investigated. The samples had previously been analyzed with respect to either *C. pneumoniae* or *M. pneumoniae* or both, using two previously established PCR methods (7, 18). Selection and reanalysis of the samples were initiated to investigate several cases of possible false positives with respect to *C. pneumoniae*. Twenty-two samples previously defined as positive for *M. pneumoniae* and 66 samples previously defined as positive for *C. pneumoniae* were included, together with 32 samples previously defined as negative for both *C. pneumoniae* and *M. pneumoniae* when both PCR methods were used. Nasopharyngeal and throat swab samples from patients with respiratory tract infections were collected in 1997 and 1998 and in 2001 and 2002 at Gävle County Hospital.

The swab samples were transported in 2 ml sucrose phosphate buffer and were stored at −20°C until use. DNA extraction was performed on the suspension after the swabs were shaken vigorously in the buffer.

An additional 200 consecutive nasopharyngeal and throat swab samples collected in 2003 at Gävle County Hospital and shown to be negative for *C. pneumoniae* and *M. pneumoniae* when analyzed using the new real-time PCR were used to evaluate the rate and extent of PCR inhibition.

DNA extraction. The Amplicor respiratory specimen preparation kit (Roche Diagnostics, Basel, Switzerland) was used as described previously (12), although in the present study, the wash procedure was simplified by adding 200 μl wash reagent directly to the 200-μl sample, which was mixed before it was centrifuged

* Corresponding author. Mailing address: Uppsala University Hospital, Department of Medical Sciences, Virology, Dag Hammarskjoldsv. 17, 751 85 Uppsala, Sweden. Phone: 46-18-6115592. Fax: 49-18-551012. E-mail: kare.bondeson@medsci.uu.se.

▽ Published ahead of print on 19 December 2007.

TABLE 1. Species and strains used for specificity control

Species	Strain or source[a]
Chlamydophila pneumoniae	IOL 207
Mycoplasma pneumoniae	ATCC 15531
Chlamydia psittaci	Clinical isolate
Chlamydia trachomatis	Bu434
Mycoplasma genitalium	ATCC 33530
Bordetella parapertussis	CCUG 413
Bordetella pertussis	CCUG 33616
Candida albicans	CCUG 32723
Enterobacter aerogenes	CCUG 1429
Enterococcus faecalis	ATCC 29212
Enterococcus faecium	CCUG 542
Escherichia coli	ATCC 25922
Haemophilus influenzae	CCUG 23946
Haemophilus parainfluenzae	CCUG 12836
Legionella pneumophila	CCUG 9568
Moraxella catarrhalis	CCUG 18283
Neisseria meningitidis	CCUG 3269
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 29213
Staphylococcus epidermidis	CCUG 18000
Stenotrophomonas maltophilia	CCUG 5866
Streptococcus constellatus	CCUG 24889
Streptococcus pneumoniae	CCUG 33638
Streptococcus pyogenes	CCUG 4207

[a] ATCC, American Type Culture Collection; CCUG, Culture Collection University of Gothenburg, Gothenburg, Sweden.

once, resulting in one rinse less than what is recommended by the manufacturer. Positive and negative controls were included in every extraction run. The extracted DNA was stored at −20°C until PCR was performed.

Conventional PCR. Two conventional PCR procedures, used as routine methods at the laboratory, were compared with the novel assay. For detection of *C. pneumoniae*, a nested PCR, targeting the *ompA* gene, was used essentially as described by Tong and Sillis (34). The first reaction mixture contained 25 μl PCR Master kit (Roche Applied Science, Basel, Switzerland), 200 nM of each primer, and 10 μl template, giving a total volume of 50 μl. The second reaction mixture contained 25 μl PCR Master kit (Roche Applied Science, Basel, Switzerland), 400 nM of each primer, an additional 1.5 mM $MgCl_2$, and 10 μl of a diluted template originating with the first reaction (1/10 and 1/500), giving a total volume of 50 μl. Samples were run in single reactions, and the cycling conditions for the first PCR in the nested method were 10 min at 94°C, a touchdown PCR of 30 s at 94°C, and 30 s of hybridization at 65°C, down to 55°C in steps of 1°C at every cycle and 60 s at 72°C. After the touchdown PCR, an additional 20 cycles were run for 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C, with a final step of 5 min at 72°C. The cycling conditions for the nested PCR were 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 50°C, 60 s at 72°C, and one final step for 5 min at 72°C.

The conventional method for detection of *M. pneumoniae*, targeting the 16S rRNA, was performed essentially as described by van Kuppeveld et al. (37). The reaction mixture contained 2.5 μl PCR buffer (GE Healthcare Bio-Science AB, Uppsala, Sweden), 2 μl deoxynucleoside triphosphate (GE Healthcare Bio-Science AB, Uppsala, Sweden), 0.1 μl *Taq* polymerase (GE Healthcare Bio-Science AB, Uppsala, Sweden), an optical density at 260 nm of 0.625 for each primer, and 5 μl of template (undiluted and diluted 1/10), giving a total volume of 25 μl. The cycling conditions were 40 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C.

Amplification of both pathogens was performed using a Perkin-Elmer GeneAmp 9600 thermal cycler (Applied Biosystems, Foster City, CA). The PCR products (8 μl) were stained with ethidium bromide and visualized using UV light after gel electrophoresis on a 1.2% agarose gel.

Real-time PCR. The novel real-time PCR methods were designed for a duplex PCR such that both pathogens can be detected and distinguished in one reaction tube using two sets of primers and two differently colored molecular beacon probes. The molecular beacons were designed using BioEdit, version 5.0.1 (http://www.ctu.edu.vn/~dvxe/Bioinformatic/Software/BioEdit.htm), for sequence alignments and the Mfold DNA folding program (39). Accession numbers for sequences used were AF290002, M18639, AF290001, AF290000, M29372, M31431, and U39698 and AF184214, AF131230, AF131229, AF131889, S50607, M64064, and L04982 for *Mycoplasma* P1 adhesin genes and *Chlamydophila ompA* genes, respectively.

A BLAST search was performed to check the specificities of the DNA sequences for primers and probes (http://www.ncbi.nlm.nih.gov/BLAST, version 2.2.17). All oligonucleotides were synthesized by Thermo Electron Corporation (Ulm, Germany) and are shown in Table 2.

The reaction volume for real-time PCR was 25 μl, containing 12.5 μl HotstarTaq master mix (Qiagen, Hilden, Germany), 600 nM each of the CPR, MPR, and MPF primers, 200 nM of the CPF primer and each probe, and 5 μl of the DNA template. Clinical samples were run as duplicates, and the cycling conditions were 10 min at 95°C, followed by 50 cycles of 30 s at 95°C, 45 s at 55°C, and 20 s at 72°C. Amplification, detection, and data analysis were performed using a Rotor-Gene 2000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Samples were regarded as positive if the two reactions or the majority of the reactions after reanalysis exceeded the fixed threshold of 0.01 normalized fluorescence units and showed an amplification curve.

Analytical performance of the real-time PCR. Analytical sensitivity of the real-time PCR was determined by serially diluting quantified *C. pneumoniae* and *M. pneumoniae* DNA in a mixture of lysis and neutralization reagents (1:1) belonging to the DNA extraction kit. The concentrations tested were 100, 200, 250, 500, and 1,000 Geq/ml. The results of eight repeats of each concentration were then used to calculate the 95% probability of detection using PROBIT analysis. To evaluate whether coamplification and detection of *C. pneumoniae* and *M. pneumoniae*, respectively, would significantly lower the analytical sensitivity, mixtures of the two organisms were used as a template in the same reaction tube. A quantified DNA control for each organism at concentrations of 10 and 10,000 copies, respectively, per reaction was amplified together with 0, 10, and 10,000 copies per reaction of the other organism. The samples were run in triplicate, and the mean cycle-to-threshold values (C_T values) and standard deviations were calculated. The rate and extent of PCR inhibition were estimated by spiking the clinical samples defined as negative. One hundred samples were spiked with the same amounts of the *C. pneumoniae* and *M. pneumoniae* DNA controls, respectively. A spiked sample found to be negative or with a C_T value higher than 2 standard deviations above the mean C_T value for all determinations was regarded as inhibitory to the PCR.

TABLE 2. Primers and probes used for real-time detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*

Primer or probe[a]	Target gene	Primer or probe sequence[b]	Amplicon size (bp)
CPF	*ompA*	AGGCGTTGCTTTCCCCTTGCC	105
CPR	*ompA*	GATAGAGAGGCTCCTACTTGCCAT	105
CPP	*ompA*	FAM-GCGCTGGCTACTGGAACAAAGTCTGCGA CCATCCAGCGC-Dabcyl	105
MPF	*P1 adhesion*	GCAGACGGTCGCGGATAACG	158
MPR	P1 adhesion	CGAACCAGGTGAGGTTGCCAATG	158
MPP1	P1 adhesion	JOE-GCGCTGTCGGCCCCGATCGCCCTCCCGCA GCGC-Dabcyl	158

[a] CP and MP indicate *C. pneumoniae* and *M. pneumoniae*, respectively; F and R indicate forward and reverse.
[b] The underlined nucleotide sequence corresponds to the stem structure of the molecular beacon.

TABLE 3. Sensitivity of duplex real-time PCR when coamplification was performed

Coamplification of DNA	Detection (C_T value) of DNA from:			
	C. pneumoniae		*M. pneumoniae*	
	10 copies	10^4 copies	10 copies	10^4 copies
None	33.9 ± 0.7	24.3 ± 0.08		
M. pneumoniae (10 copies)	33.9 ± 0.7	24.5 ± 0.3		
M. pneumoniae (10^4 copies)	32.2 ± 0.5	23.7 ± 0.02		
None			30.2 ± 0.3	20.8 ± 0.3
C. pneumoniae (10 copies)			30.7 ± 0.2	20.5 ± 0.5
C. pneumoniae (10^4 copies)			29.9 ± 0.2	20.8 ± 0.05

RESULTS

Analytical performance. Specificity was confirmed when no signs of amplification were found when the organisms shown in Table 1 were tested using the real-time PCR. The analytical sensitivity was determined by PROBIT analysis of eight replicates in each concentration. The limits of detection, expressed as 95% probability of detection and 95% confidence limits, were 4.3 Geq/reaction (3.0 to 10.1) and 2.8 Geq/reaction (2.0 to 9.5) for *C. pneumoniae* and *M. pneumoniae*, respectively.

Both *C. pneumoniae* and *M. pneumoniae* were detected in reactions containing both pathogens. The C_T values were not affected significantly when the reaction contained the other pathogen at a 1,000-fold excess compared to results when the detected pathogen was present in the tube alone, as shown in Table 3.

Diagnostic performance. All of the 100 samples spiked with *C. pneumoniae* were found to be positive and thus showed no sign of total inhibition. The mean C_T value was 34.3 cycles. One of the samples had a C_T value higher than 2 standard deviations above the mean C_T value (>36.8 cycles), suggesting that it contained a factor that gives rise to partial inhibition of the PCR. There was no case of total inhibition in the 100 samples spiked with *M. pneumoniae*, although 4 samples showed signs of a partial inhibitory effect. The mean C_T value for *M. pneumoniae* was 31.3 cycles, and samples were regarded as containing inhibitory factors if they exceeded 35 cycles. When the samples were diluted 20-fold, the inhibitory effect was no longer detectable.

Of the 120 clinical specimens, 13 samples were positive for *C. pneumoniae* and 22 samples were positive for *M. pneumoniae* when both the real-time duplex PCR and the conventional PCR were used. Retesting was performed with 10 out of 120 samples (6 *M. pneumoniae* samples and 5 *C. pneumoniae* samples) when only 1 positive reaction was found in the initial testing. After retesting, one of these samples was subsequently found to be positive using majority rule as described above (one positive for *M. pneumoniae*). Furthermore, one sample was positive for *C. pneumoniae* when the nested conventional PCR was used, and two additional samples were positive for *M. pneumoniae* when the real-time PCR was used. All three discrepant samples were sent for analysis using a third set of methods in an outside laboratory (Department of Clinical Microbiology, Capio Diagnostik AB, Kärnsjukhuset, Skövde, Sweden) (15, 20). Only one of the two samples positive for *M. pneumoniae* was confirmed. The sensitivity and specificity, when using the conventional PCR as a reference, were 93% and 100%, respectively, for *C. pneumoniae.* For *M. pneumoniae*, the sensitivity was 100% and the specificity was 98%. When the results from the discrepant samples were included, after analysis by the third set of methods, the sensitivity and specificity were 93% and 100%, respectively, for *C. pneumoniae.* For *M. pneumoniae*, the sensitivity was 100% and the specificity was 99%.

DISCUSSION

In the present study, we describe a newly constructed duplex real-time PCR that has the advantage of detecting *C. pneumoniae* and *M. pneumoniae* from a clinical sample using a single reaction tube. Using differently colored molecular beacon probes, the duplex detection is performed with high sensitivity and specificity. Both pathogens were detected with high sensitivity, and no cross-reaction with other tested species was observed.

The use of molecular-beacon probes enables specific detection, owing to the thermodynamics of the binding mechanisms. Such probes are also useful in multiplex reactions, since they show a low background signal. In a search for pathogens that give rise to similar clinical features, the multiplex approach is advantageous because it saves time, reagents, and use of equipment (21). The drawback of multiplex PCR assays is the phenomenon of competition between simultaneous amplification reactions of nucleic acid templates from several different pathogens in the same tube. This could affect detection of a dual infection, especially if one of the pathogens is found in a larger quantity than the other (21). Coinfection of *C. pneumoniae* and *M. pneumoniae* is rare, but it has been described (22, 38). From the 120 clinical samples tested in the present study, none tested positive for both *C. pneumoniae* and *M. pneumoniae* when real-time PCR or conventional PCR was used. Based on the current rates of positive samples seen at our laboratories, coinfection can be expected in 1 of 1,800 samples. Although the influence on analytical performance in dual detection of both pathogens was investigated, no significant effect on C_T values was revealed when pathogens were present in a 1,000-fold excess compared to C_T values achieved when only 1 of the pathogens was present. The multiple target amplification did not reduce the analytical sensitivity of the real-time PCR method, which is consistent with the results of Welti et al. (38). Another drawback of multiplex PCR is that its analytical sensitivity could be lower than that of monoplex PCR, owing to the complexity of optimizing the multiple variables to achieve optimal conditions for all included PCRs (33). The analytical sensitivity achieved for both pathogens using this duplex real-time PCR method was comparable to the high sensitivity described for other real-time PCR methods (20, 25, 32, 38).

In the present study, the Amplicor preparation kit was used as the DNA extraction method because it has previously been shown to be appropriate for this type of material and pathogens (12; data not shown). The choice of DNA extraction can influence PCR sensitivity and rates of inhibition, which can easily be monitored using real-time PCR. None of the 200

swab samples spiked with a small amount of *C. pneumoniae* or *M. pneumoniae* was inhibited for detection, although 2.5% of the samples showed partial inhibition.

A comparison between this duplex real-time PCR and two previously described conventional PCR methods (34, 37) showed an overall agreement of 98.8% when 120 clinical swab samples were tested. Of these 120 samples, 13 were positive for *C. pneumoniae* when both methods were used and 1 additional sample was positive when the conventional nested touchdown PCR was used, suggesting that the nested PCR is more sensitive than is the real-time PCR. The same target gene, *ompA*, is used in both methods, and nested PCR is generally recognized to have high sensitivity (8). However, it is important to consider that nested PCR entails a high risk of carryover contaminations, which cannot be completely eliminated even when appropriate precautions are taken.

Different target genes were used in the real-time PCR assay and the conventional assay for *M. pneumoniae*: the P1 adhesion gene and the 16S rRNA gene, respectively. A total of 22 samples were positive for *M. pneumoniae* with both assays, and an additional 2 samples were positive when the real-time PCR was used but negative when conventional PCR was used, suggesting that the real-time PCR could be a more sensitive method. However, it was possible to reproduce only one of these discrepant results using a separate set of methods in an independent laboratory (15, 20).

In conclusion, this new duplex real-time PCR can specifically detect and distinguish *C. pneumoniae* and *M. pneumoniae* in clinical samples with high sensitivity. Molecular-beacon probes are successfully used in this duplex setting to detect both pathogens in the same sample. This is of great clinical value, since the symptoms of *C. pneumoniae* and *M. pneumoniae* are often difficult to distinguish.

ACKNOWLEDGMENTS

We thank Hans Högberg at the Centre for Research & Development, Uppsala University/County Council of Gävleborg, for helping us with statistical calculations. We also thank Helena Enroth and colleges at the Department of Clinical Microbiology, Capio Diagnostik AB, Kärnsjukhuset, Skövde, for assistance in validation of the method.

The work described in this report was supported by grants from the Uppsala and Gävleborg County Council (to K.B. and K.G.).

REFERENCES

1. **Almirall, J., I. Morato, F. Riera, A. Verdaguer, R. Priu, P. Coll, J. Vidal, L. Murgui, F. Valls, F. Catalan, et al.** 1993. Incidence of community-acquired pneumonia and Chlamydia pneumoniae infection: a prospective multicentre study. Eur. Respir. J. **6:**14–18.
2. **Apfalter, P., W. Barousch, M. Nehr, A. Makristathis, B. Willinger, M. Rotter, and A. M. Hirschl.** 2003. Comparison of a new quantitative *ompA*-based real-time PCR TaqMan assay for detection of *Chlamydia pneumoniae* DNA in respiratory specimens with four conventional PCR assays. J. Clin. Microbiol. **41:**592–600.
3. **Atmar, R. L., and S. B. Greenberg.** 1989. Pneumonia caused by Mycoplasma pneumoniae and the TWAR agent. Semin. Respir. Infect. **4:**19–31.
4. **Bitnun, A., E. L. Ford-Jones, M. Petric, D. MacGregor, H. Heurter, S. Nelson, G. Johnson, and S. Richardson.** 2001. Acute childhood encephalitis and Mycoplasma pneumoniae. Clin. Infect. Dis. **32:**1674–1684.
5. **Boelen, E., H. W. Steinbusch, A. J. van der Ven, G. Grauls, C. A. Bruggeman, and F. R. Stassen.** 2007. Chlamydia pneumoniae infection of brain cells: an in vitro study. Neurobiol. Aging **28:**524–532.
6. **Boman, J., A. Allard, K. Persson, M. Lundborg, P. Juto, and G. Wadell.** 1997. Rapid diagnosis of respiratory Chlamydia pneumoniae infection by nested touchdown polymerase chain reaction compared with culture and antigen detection by EIA. J. Infect. Dis. **175:**1523–1526.
7. **Campbell, L. A., M. Perez Melgosa, D. J. Hamilton, C. C. Kuo, and J. T. Grayston.** 1992. Detection of *Chlamydia pneumoniae* by polymerase chain reaction. J. Clin. Microbiol. **30:**434–439.
8. **Dowell, S. F., R. W. Peeling, J. Boman, G. M. Carlone, B. S. Fields, J. Guarner, M. R. Hammerschlag, L. A. Jackson, C. C. Kuo, M. Maass, T. O. Messmer, D. F. Talkington, M. L. Tondella, and S. R. Zaki.** 2001. Standardizing Chlamydia pneumoniae assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). Clin. Infect. Dis. **33:**492–503.
9. **Foy, H. M.** 1993. Infections caused by Mycoplasma pneumoniae and possible carrier state in different populations of patients. Clin. Infect. Dis. **17**(Suppl. 1)**:** S37–S46.
10. **Gaillat, J., A. Flahault, B. deBarbeyrac, J. Orfila, H. Portier, J. P. Ducroix, C. Bebear, and C. Mayaud.** 2005. Community epidemiology of Chlamydia and Mycoplasma pneumoniae in LRTI in France over 29 months. Eur. J. Epidemiol. **20:**643–651.
11. **Gnarpe, H., J. Gnarpe, B. Gastrin, and H. Hallander.** 1997. Chlamydia pneumoniae and myocarditis. Scand. J. Infect. Dis. Suppl. **104:**50–52.
12. **Gnarpe, J., and K. Eriksson.** 1995. Sample preparation for Chlamydia pneumoniae PCR. APMIS **103:**307–308.
13. **Grayston, J. T.** 2000. Background and current knowledge of Chlamydia pneumoniae and atherosclerosis. J. Infect. Dis. **181**(Suppl. 3)**:**S402–S410.
14. **Grayston, J. T., L. A. Campbell, C. C. Kuo, C. H. Mordhorst, P. Saikku, D. H. Thom, and S. P. Wang.** 1990. A new respiratory tract pathogen: Chlamydia pneumoniae strain TWAR. J. Infect. Dis. **161:**618–625.
15. **Hardegger, D., D. Nadal, W. Bossart, M. Altwegg, and F. Dutly.** 2000. Rapid detection of Mycoplasma pneumoniae in clinical samples by real-time PCR. J. Microbiol. Methods **41:**45–51.
16. **Heath, P. T.** 2000. Epidemiology and bacteriology of bacterial pneumonias. Paediatr. Respir. Rev. **1:**4–7.
17. **Heiskanen-Kosma, T., M. Korppi, A. Laurila, C. Jokinen, M. Kleemola, and P. Saikku.** 1999. Chlamydia pneumoniae is an important cause of community-acquired pneumonia in school-aged children: serological results of a prospective, population-based study. Scand. J. Infect. Dis. **31:**255–259.
18. **Jensen, J. S., J. Sondergard-Andersen, S. A. Uldum, and K. Lind.** 1989. Detection of Mycoplasma pneumoniae in simulated clinical samples by polymerase chain reaction. Brief report. APMIS **97:**1046–1048.
19. **Korppi, M., T. Heiskanen-Kosma, and M. Kleemola.** 2004. Incidence of community-acquired pneumonia in children caused by Mycoplasma pneumoniae: serological results of a prospective, population-based study in primary health care. Respirology **9:**109–114.
20. **Kuoppa, Y., J. Boman, L. Scott, U. Kumlin, I. Eriksson, and A. Allard.** 2002. Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. J. Clin. Microbiol. **40:**2273–2274.
21. **Markoulatos, P., N. Siafakas, and M. Moncany.** 2002. Multiplex polymerase chain reaction: a practical approach. J. Clin. Lab. Anal. **16:**47–51.
22. **Marrie, T. J., R. W. Peeling, M. J. Fine, D. E. Singer, C. M. Coley, and W. N. Kapoor.** 1996. Ambulatory patients with community-acquired pneumonia: the frequency of atypical agents and clinical course. Am. J. Med. **101:**508–515.
23. **McIntosh, K.** 2002. Community-acquired pneumonia in children. N. Engl. J. Med. **346:**429–437.
24. **Michelow, I. C., K. Olsen, J. Lozano, N. K. Rollins, L. B. Duffy, T. Ziegler, J. Kauppila, M. Leinonen, and G. H. McCracken, Jr.** 2004. Epidemiology and clinical characteristics of community-acquired pneumonia in hospitalized children. Pediatrics **113:**701–707.
25. **Morozumi, M., E. Nakayama, S. Iwata, Y. Aoki, K. Hasegawa, R. Kobayashi, N. Chiba, T. Tajima, and K. Ubukata.** 2006. Simultaneous detection of pathogens in clinical samples from patients with community-acquired pneumonia by real-time PCR with pathogen-specific molecular beacon probes. J. Clin. Microbiol. **44:**1440–1446.
26. **Raggam, R. B., E. Leitner, J. Berg, G. Muhlbauer, E. Marth, and H. H. Kessler.** 2005. Single-run, parallel detection of DNA from three pneumonia-producing bacteria by real-time polymerase chain reaction. J. Mol. Diagn. **7:**133–138.
27. **Saikku, P.** 1997. Atypical respiratory pathogens. Clin. Microbiol. Infect. **3:**599–604.
28. **Saikku, P.** 1996. Chlamydia pneumoniae and cardiovascular diseases. Clin. Microbiol. Infect. **1**(Suppl. 1)**:**S19–S22.
29. **Sundelof, B., J. Gnarpe, H. Gnarpe, L. Grillner, and S. Darougar.** 1993. Chlamydia pneumoniae in Swedish patients. Scand. J. Infect. Dis. **25:**429–433.
30. **Templeton, K. E., S. A. Scheltinga, A. W. Graffelman, J. M. Van Schie, J. W. Crielaard, P. Sillekens, P. J. Van Den Broek, H. Goossens, M. F. Beersma, and E. C. Claas.** 2003. Comparison and evaluation of real-time PCR, real-time nucleic acid sequence-based amplification, conventional PCR, and serology for diagnosis of *Mycoplasma pneumoniae*. J. Clin. Microbiol. **41:**4366–4371.
31. **Thom, D. H., J. T. Grayston, S. P. Wang, C. C. Kuo, and J. Altman.** 1990. Chlamydia pneumoniae strain TWAR, Mycoplasma pneumoniae, and viral infections in acute respiratory disease in a university student health clinic population. Am. J. Epidemiol. **132:**248–256.
32. **Tondella, M. L., D. F. Talkington, B. P. Holloway, S. F. Dowell, K. Cowley, M. Soriano-Gabarro, M. S. Elkind, and B. S. Fields.** 2002. Development and

evaluation of real-time PCR-based fluorescence assays for detection of *Chlamydia pneumoniae*. J. Clin. Microbiol. **40:**575–583.
33. **Tong, C. Y., C. Donnelly, G. Harvey, and M. Sillis.** 1999. Multiplex polymerase chain reaction for the simultaneous detection of Mycoplasma pneumoniae, Chlamydia pneumoniae, and Chlamydia psittaci in respiratory samples. J. Clin. Pathol. **52:**257–263.
34. **Tong, C. Y., and M. Sillis.** 1993. Detection of Chlamydia pneumoniae and Chlamydia psittaci in sputum samples by PCR. J. Clin. Pathol. **46:**313–317.
35. **Ursi, D., K. Dirven, K. Loens, M. Ieven, and H. Goossens.** 2003. Detection of Mycoplasma pneumoniae in respiratory samples by real-time PCR using an inhibition control. J. Microbiol. Methods **55:**149–153.
36. **van Kuppeveld, F. J., K. E. Johansson, J. M. Galama, J. Kissing, G. Bolske, E. Hjelm, J. T. van der Logt, and W. J. Melchers.** 1994. 16S rRNA based polymerase chain reaction compared with culture and serological methods for diagnosis of Mycoplasma pneumoniae infection. Eur. J. Clin. Microbiol. Infect. Dis. **13:**401–405.
37. **van Kuppeveld, F. J., J. T. van der Logt, A. F. Angulo, M. J. van Zoest, W. G. Quint, H. G. Niesters, J. M. Galama, and W. J. Melchers.** 1992. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl. Environ. Microbiol. **58:**2606–2615.
38. **Welti, M., K. Jaton, M. Altwegg, R. Sahli, A. Wenger, and J. Bille.** 2003. Development of a multiplex real-time quantitative PCR assay to detect Chlamydia pneumoniae, Legionella pneumophila and Mycoplasma pneumoniae in respiratory tract secretions. Diagn. Microbiol. Infect. Dis. **45:** 85–95.
39. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31:**3406–3415.