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Two Quality Control Exercises Involving Nucleic Acid Amplification Methods for Detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* and Carried Out 2 Years Apart (in 2002 and 2004)

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The quality performance of laboratories for the detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* by two quality control (QC) exercises with a 2-year interval was investigated. For the 2002 QC exercise, specimens were spiked with *M. pneumoniae* at concentrations of 5,000, 500, 50, and 0 color-changing units (CCU)/100 μl. The limit of detectability was 50 CCU/100 μl. Therefore, this concentration was omitted from the 2004 panel and was excluded from the analysis. In 2002, 2 out of 12 participants obtained 100% correct results, 2 out of 12 produced false-positive results, and 10 out of 12 had between 0 out of 9 and 8 out of 9 correct positive results. In 2004, correct results were obtained in 15 out of 18 tests, and no false-positive results were reported. In 2002, specimens were spiked with *C. pneumoniae* at concentrations of 490, 49, 4.9, and 0 inclusion-forming units/100 μl (IFU/100 μl). In the 2004 panel, samples spiked with a lower dilution of 0.49 IFU/100 μl were added to the panel. For the *C. pneumoniae* QC, correct results were produced in 12 out of 16 and 13 out of 18 tests in 2002 and in 2004, respectively. Both multiplex PCR and nucleic acid sequence-based amplification (NASBA) formats scored a smaller number of samples positive than the monoplex reactions.

A multitude of nucleic acid amplification tests (NAATs) have been described for the detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in respiratory specimens (6, 17). In addition to in-house PCR tests, commercial kits are becoming available, such as the LCx for *C. pneumoniae* (Abbott Laboratories) (8) and the NucliSens Basic kit (bioMérieux), for which the primers and the target-specific biotinylated capture probe are to be synthesized for each target by the user (15).

Although all tests aim to be rapid, sensitive, specific, and easy to perform, results of NAATs may be unreliable because of cross-contamination, inappropriate treatment of the clinical samples leading to loss of target nucleic acid, or the presence of inhibitors (2, 5, 12, 14, 26, 27).

To date, only one study compared the results of different NAATs for the detection of *C. pneumoniae* in respiratory specimens in different centers (8), and four studies compared the results of amplification methods performed in different centers for the detection of *C. pneumoniae* in atheroma specimens (2, 3, 11, 21). To our knowledge, no such studies have been published for *M. pneumoniae*.

The aim of this study was to assess the quality performance of laboratories for the detection of *M. pneumoniae* and *C. pneumoniae* by two quality control (QC) exercises with a 2-year interval.

MATERIALS AND METHODS

Participating laboratories. The participating laboratories are as follows, in alphabetical order: Academisch Ziekenhuis Vrije Universiteit Brussel, Brussels, Belgium; Algemeen Ziekenhuis Sint Augustinus, Wilrijk, Belgium; Algemeen Ziekenhuis Sint Jan, Brugges, Belgium; bioMérieux, Boxtel, The Netherlands; Centre Hospitalier Régional de la Citadelle, Liège, Belgium; Cliniques Universitaires Université Catholique de Louvain de Mont-Godinne, Yvoir, Belgium; Groupement des Centres Hospitaliers de Jolimont-Lobbes et de Tubize-Nivelles, La Louvière, Belgium; Institut Jules Bordet, Brussels, Belgium; Institution de Pathologie et de Génétique-Loverval, Loverval, Belgium; Leids Universitair Medisch Centrum, Leiden, The Netherlands; Onze Lieve Vrouwe Ziekenhuis, Aalst, Belgium; Ospedale Maggiore di Milano, Milan, Italy; Public Health Laboratory Friesland, Leeuwarden, The Netherlands; Université Libre de Bruxelles-Erasme, Brussels, Belgium; University Medical Center-Brugmann, Brussels, Belgium; University of Antwerp, Wilrijk, Belgium; Universitair Ziekenhuis Katholieke Universiteit Leuven, Leuven, Belgium; University Hospital Antwerp, Edegem, Belgium; Virga Jesse Ziekenhuis, Hasselt, Belgium; and Ziekenhuizen Noord Antwerpen, Antwerp, Belgium.

Preparation of proficiency panels. A stock suspension of *M. pneumoniae* strain ATCC 29085 was quantitated by incubation of 10-fold dilutions in triplicate in SP4 medium at 37°C. The cultures were monitored for 2 months, and the titer was expressed as color-changing units (CCU) per milliliter; 1 CCU corresponds to 10 to 100 cells (4).

A stock suspension of *C. pneumoniae* strain ATCC VR-1355 was quantified by incubation of five replicates of 10-fold dilutions on confluent layers of Hep-2 cells. The vials were centrifuged at 3,500 rpm at 25°C for 60 min and incubated at 37°C. After 1 h, the medium was replaced by fresh culture medium containing 1 mg/liter cycloheximide. After 3 days, cells were fixed with 96% ethanol and stained by the fluorescent antibody technique with specific mouse monoclonal antibodies and fluorescein-labeled rabbit anti-mouse immunoglobulin (both from Dako A/S, Glostrup, Denmark). The titer was expressed as inclusion-forming units (IFU) per milliliter.

Bronchoalveolar lavage (BAL) specimens were collected from patients in the University Hospital of Antwerp and stored at −20°C. BAL pools were negative for *M. pneumoniae* and *C. pneumoniae* as tested by nucleic acid sequence-based amplification (NASBA) (16, 21) and by in-house-developed PCR (12, 27).

The 2002 proficiency panel consisted of parts A and B: part A was prepared in sterile physiologic saline, and part B was prepared in BAL. The negative samples were prepared in a laminar flow cabinet in a separate room prior to the preparation of the positive samples. Materials and pipettes had never been used for *M. pneumoniae*- or *C. pneumoniae*-related work previously.

M. pneumoniae parts A and B each contained four negative samples and three

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TABLE 1. Procedures used for detection of *M. pneumoniae* in 2002[a]

Lab no.	NA extraction method	NAAT	Detection method	Target	Monitoring inhibition	Monitoring of the procedure		dUTP-UDG	Sample volume (μl)	
						Neg. contr.	Pos. contr.		Extraction	Amplification
1	NucliSens Basic kit	In-house NASBA	ECL	16S rRNA	Y	Prep + Amp	Prep + Amp	N	100	5
1	NucliSens Basic kit	In-house NASBA	Real time	16S rRNA	Y	Prep + Amp	Prep + Amp	N	100	5
2	Roche Amplicor sputum	In-house PCR	Real time	P1 gene	Y	None	None	Y	50	5
3	QIAamp DNA minikit	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Prep + Amp	Y	200	10
4	Roche High Pure PCR Template Preparation kit	Com. PCR	Real time	P1 gene	Y	Prep + Amp	Amp	N	200	10
5	Boiling	Single-step PCR	Agarose gel	P1 gene	N	Prep + Amp	Prep + Amp	Y	50	5
6	QIAamp DNA blood	Single-step PCR	Agarose gel	P1 gene	Y	Prep + Amp	Amp	N	250	6
7	QIAamp DNA minikit	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Prep + Amp	Y	200	5
8	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	ATPase gene	N	Prep + Amp	Prep + Amp	Y	200	5
9	QIAamp DNA blood	In-house PCR	Real time	P1 gene	N	Amp	Amp	Y	200	5
10	QIAamp stool	In-house PCR	Real time	P1 gene	N	Amp	None	Y	100	1.5
11	QIAamp DNA blood	In-house PCR	Real time	P1 gene	N	Prep + Amp	Prep + Amp	Y	200	10
12	Roche High Pure PCR Template Preparation kit	In-house nested PCR	Agarose gel	ATPase gene	N	Prep + Amp	Prep + Amp	N	200	200

[a] Amp, amplification; com. PCR, commercial assay from Minerva Biolabs (Berlin, Germany); dUTP-UDG, deoxyuridine triphosphate–uracyl-*N*-glycosylase (dUTP replaces dTTP in PCR reactions, and the enzyme excises uracyl from any contaminating PCR product to prevent false positives); ECL, electrochemiluminescent detection; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; N, no; prep, preparation; Y, yes; NA, nucleic acid; neg., negative; pos., positive; contr., control.

samples with 500 CCU/100 μl of solution (see Table 5); part A also contained three samples with 50 CCU/100 μl, and part B contained three samples with 5,000 CCU/100 μl. For *C. pneumoniae* (see Table 7), parts A and B contained four negative samples each and three samples with 49 IFU/100 μl; furthermore, part A contained three samples with 4.9 IFU/100 μl, and part B contained three samples with 490 IFU/100 μl.

For the 2004 proficiency tests, all samples were prepared in BAL. Each panel contained four negative samples, prepared as described above. Three different BAL pools were used: pool 1 was fluid, pool 2 was more viscous, and pool 3 was blood stained. Three samples of the *M. pneumoniae* panel (see Table 6) were spiked with 5,000 CCU/100 μl, and three samples were spiked with 500 CCU/100 μl. The *C. pneumoniae* proficiency panel (see Table 8) included one sample with 490 IFU/100 μl, one sample with 49 IFU/100 μl, one sample with 4.9 IFU/100 μl, and three samples with 0.49 IFU/100 μl BAL.

The proficiency panels were coded and sent refrigerated on the same day or were stored at −80°C until they were shipped frozen to the participants. The samples were to be tested as routine specimens, and the results were returned within 4 weeks together with a questionnaire collecting information on the procedures applied.

TABLE 2. Procedures used for detection of *M. pneumoniae* in 2004[a]

Lab no.	NA extraction method	NAAT	Detection method	Target	Monitoring inhibition	Monitoring of the procedure		dUTP-UDG	Sample volume (μl)		
						Neg. contr.	Pos. contr.		Input extract	Elution	Amplification
1	NucliSens Basic kit	In-house NASBA	Real time	16S rRNA	N	Prep + Amp	Prep + Amp	N	100	50	5
1	NucliSens Basic kit	In-house MX NASBA	Real time	16S rRNA	N	Prep + Amp	Prep + Amp	N	100	50	5
2	Roche Amplicor sputum	In-house PCR	Real time	P1 gene	Y	None	None	Y	300	60	5
3	QIAamp DNA minikit	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Prep + Amp	Y	200	100	10
4	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Prep + Amp	Y	400	100	10
5	Puregene genomic DNA for gram^{+} bacteria	In-house PCR	Real time	P1 gene	Y	Amp	Amp	Y	400	50	10
6	QIAamp DNA minikit	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Amp	N	200	100	4
8	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	ATPase gene	N	Prep + Amp	Amp	N	200	100	5
9	QIAamp DNA	In-house PCR	Real time	P1 gene	Y	Amp	Amp	N	200	200	5
11	QIAamp DNA blood minikit	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Prep + Amp	N	200	100	10
11	QIAamp DNA blood minikit	In-house MX PCR	Real time	P1 gene	Y	Prep + Amp	Prep + Amp	N	200	100	10
14	QIAamp DNA tissue	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Amp	Y	400	200	5
16	QIAamp DNA minikit	In-house PCR	Real time	P1 gene	Y	Amp	Prep + Amp	Y	200	100	NS
17	QIAamp DNA blood	In-house PCR	Real time	P1 gene	Y	Prep + Amp	Amp	Y	200	100	5
18	Amplicor HCV	In-house PCR	EIA	16S rRNA gene	Y	Prep + Amp	Prep + Amp	N	100	30	4.5
18	Amplicor HCV	In-house MX PCR	EIA	16S rRNA gene	Y	Prep + Amp	Prep + Amp	N	100	30	4.5
19	Nucleospin tissue	com MX-PCR	EIA	NS	Y	Prep + Amp	Prep + Amp	N	400	100	10
20	MagnaPure	In-house PCR	Real-time	P1 gene	Y	Prep + Amp	Prep + Amp	Y	200	100	5

[a] Amp, amplification; com., commercial assay (Pneumoplex from Prodesse); dUTP-UDG, deoxyuridine triphosphate–uracyl-*N*-glycosylase (dUTP replaces dTTP in PCR reactions, and the enzyme excises uracyl from any contaminating PCR product to prevent false positives); EIA, enzyme immunoassay; MX, multiplex; N, no; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; NS, not specified; prep, preparation; Y, yes; NA, nucleic acid; neg., negative; pos., positive; contr., control.

TABLE 3. Procedures used for detection of *C. pneumoniae* in 2002

Lab no.	NA extraction method	NAAT	Detection method	Target	Monitoring inhibition	Monitoring of the procedure[a]		dUTP-UDG	Sample volume (μl)	
						Neg. contr.	Pos. contr.		Extraction	Amplification
1	NucliSens Basic kit	In-house NASBA	ECL	16S rRNA	Y	Prep + Amp	Prep + Amp	N	100	5
1	NucliSens Basic kit	In-house NASBA	Real time	16S rRNA	Y	Prep + Amp	Prep + Amp	N	100	5
2	Roche Amplicor sputum	In-house PCR	Real time	PstI fragment	Y	None	None	Y	NS	5
3	QIAamp DNA	In-house PCR	Real time	16S rRNA gene	Y	Prep + Amp	Prep + Amp	N	200	10
4	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	PmP4 gene	Y	Prep + Amp	Amp	Y	200	10
5	QIAamp DNA	In-house PCR	Real time	MOMP gene	N	Prep + Amp	Prep + Amp	Y	NS	NS
6	QIAamp DNA blood	In-house PCR	Agarose gel	PstI fragment	Y	Prep + Amp	None	N	120	6
8	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	MOMP gene	N	Prep + Amp	Prep + Amp	Y	200	5
9	QIAamp DNA blood	In-house PCR	Real time	Cytadhesin gene	N	Amp	Amp	Y	200	5
10	In-house	In-house PCR	Real time	16S rRNA gene	N	Amp	Amp	Y	200	2
11	QIAamp DNA blood	In-house PCR	Real time	16S rRNA gene	N	Prep + Amp	Prep + Amp	Y	200	200
12	Roche High Pure PCR Template Preparation kit	In-house nested PCR	Agarose gel	MOMP gene	N	Prep + Amp	Prep + Amp	N	200	200
13	QIAamp DNA tissue	In-house PCR	Real time	PstI fragment	N	Amp	Amp	Y	200	5
14	Boiling	In-house PCR	Real time	PstI fragment	N	NS	Amp	Y	100	5
15	NucliSens Basic kit	In-house NASBA	Real time	16S rRNA	N	None	None	N	100	5
15	NucliSens Basic kit	In-house NASBA	Real time multiplex	16S rRNA	N	None	None	N	100	5

[a] Amp, amplification; ECL, electrochemiluminescent detection; dUTP-UDG, deoxyuridine triphosphate–uracyl-*N*-glycosylase (dUTP replaces dTTP in PCR reactions, and the enzyme excises uracyl from any contaminating PCR product to prevent false positives); N, no; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; prep, preparation; Y, yes; NA, nucleic acid; neg., negative; pos., positive; contr., control; NS, not specified.

Quality control assurance. Fifteen serial dilutions of *M. pneumoniae* were tested by NASBA and PCR (12, 16) after one and two freeze-thaw cycles, mimicking the conditions of the preparation of the panels and on arrival at the participating laboratory. Similar tests were performed for the detection of nine serial dilutions of *C. pneumoniae* by NASBA and PCR (18, 27). Furthermore, each BAL pool was spiked in triplicate with a serial dilution of *M. pneumoniae* and tested by both NAATs. Quality control assurance of the panels was performed by NASBA and the in-house-developed PCR.

Methods used in the participating centers for the extraction of *M. pneumoniae* and *C. pneumoniae* nucleic acids. Each laboratory followed its standard procedures for *M. pneumoniae* and *C. pneumoniae* detection. Thus, the procedures varied from laboratory to laboratory.

The following commercially available extraction kits were used (Tables 1 to 4): Amplicor HCV specimen preparation kit (Roche, Mannheim, Germany), Nucleospin tissue (Clontech, Mount View, CA), NucliSens Basic kit (bioMérieux, Boxtel, The Netherlands), Puregene genomic DNA for gram-positive bacteria (Gen trasystems, Minneapolis, MN), QIAamp DNA minikit (QIAGEN, Hilden, Germany), QIAamp DNA blood kit (QIAGEN), QIAamp stool kit (QIAGEN), QIAamp DNA tissue kit (QIAGEN), Roche Amplicor Sputum Preparation kit (Roche), and the Roche High Pure PCR Template Preparation kit (Roche).

Three participants used in-house nucleic acid extraction methods for the *M. pneumoniae* or *C. pneumoniae* 2002 proficiency panels: participant number 5, 10 min of boiling; participant number 10, 5 cycles of 1 min of freeze boiling; and participant number 14, a proteinase K pretreatment followed by 10 min of boiling.

Methods used in the participating centers for the amplification and detection of *M. pneumoniae* and *C. pneumoniae* nucleic acids. Participant 1. Conventional nucleic acid sequence-based amplification (NASBA) for the detection of *M. pneumoniae* RNA in 2002 was done as described earlier (14, 15). Monoplex real-time NASBA for the detection of *M. pneumoniae* RNA was done as described by Loens et al. (16). Conventional and monoplex real-time NASBA for the detection of *C. pneumoniae* RNA was done as described by Loens et al. (18). Multiplex (MX)-real-time NASBA for the detection of RNA from both organisms was done as described previously (19).

Participant 2. *M. pneumoniae* DNA was detected as described by Hardegger et al. (10). *C. pneumoniae* DNA was amplified by an in-house-developed real-time PCR based on the PstI fragment-updated sequence (7) using primers CPNFW (5′-TGG AGATAAAATGGCTGGACG-3′) and CPNREV (5′-TATGGCATATCCGCT TCGG-3′) and detection probe (5′-6-carboxyfluorescein [FAM]-CACGGAAAT AAAGGTGTTGTTTCCAAAATCG-6-carboxytetramethylrhodamine [TAMR A]-3′) and the same amplification conditions as those for *M. pneumoniae* amplification.

Participant 3. *M. pneumoniae* DNA was amplified by an in-house-developed real-time PCR using primers MYPN3 (5′-AGGCTTCAAGTGGACAAA GTGAC-3′) and MYPN4 (5′-GATTGTYCCTGCTGGYCCAT-3′) and detection probe MYPNF (5′-FAM-ACCACACCAAGTTCACGAGCGCTA CG-TAMRA-3′) with the following amplification conditions: 1 cycle of 2 min at 50°C followed by 1 cycle of 10 min at 95°C and 45 cycles of 15 s at 95°C and 60 s at 60°C.

C. pneumoniae DNA was also amplified by an in-house-developed real-time PCR using primers CHPN1 (5′-GAGATGGAGCAAATCCTAAAAGCTA-3′) and CHPN2 (5′-AAATAGGTTGAGTCAACGACTTAAGGT-3′) and detection probe CHPN-F (5′-FAM-TCAGCCATAACGCCGTGAATACGTTCTC-TAMRA-3′) with the same amplification conditions used for *M. pneumoniae*.

Participant 4. In 2002, *M. pneumoniae* DNA was amplified by using the Minerva kit (Minerva Biolabs, Berlin, Germany) according to the instructions of the manufacturer. In 2004, an in-house-developed assay was applied using primers Mpneu02-FW (5′-GCCGGCAGTGGCAGTC-3′) and Mpneu02-RV (5′-AGC CGCTTCGGTTCGG-3′) and detection probe Mpneu02-MGB (5′-FAM-AAC CACGTATGATCCC-nonfluorescent quencher [NFQ]-3′) with the following amplification conditions: 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, and 45 cycles of 15 s at 95°C and 60 s at 60°C.

For the detection of *C. pneumoniae*, primers CHLpnFW (5′-TGGCTAGGC CATTGAGAGTGA-3′) and CHLpnRV (5′-GTTATGGATGGAGGGACT ACTTTTG-3′) and detection probe CHLpnMGB (5′-FAM-CTCAGCGCTTGC C-NFQ-3′) were used. Amplification conditions were 1 cycle of 1 min at 95°C followed by 45 cycles of 15 s at 95°C and 60 s at 60°C.

Participant 5. In 2002, *M. pneumoniae* DNA was amplified by an in-house-developed PCR using primers OJPU1 (5′-GCCACCCTCGGGGGCAGTCAG-3′) and OJPU3 (5′-GAGTCGGGATTCCCCGCGGAGG-3′) (12). The following amplification conditions were used: 1 cycle of 3 min at 90°C, followed by 39 cycles of 1 min at 90°C, 2 min at 67°C, and 2 min at 72°C, and 1 final cycle of 10 min at 72°C.

C. pneumoniae DNA was amplified by an in-house-developed real-time PCR using slightly modified major outer membrane protein (MOMP) VD2 primers of Tondella et al. (26), FPCPN (5′-CTCGTTGGTTTATTCGGAGTT-3′) and RPCPN (5′-CCAAGAGAAAGAGGTGTCTGTG-3′), and the slightly modified MOMP VD2 detection probe described by Tondella et al. (26), CPN (5′-FA

TABLE 4. Procedures used for detection of *C. pneumoniae* in 2004[a]

Lab no.	NA extraction method	NAAT	Detection method	Target	Monitoring inhibition	Monitoring of the procedure		dUTP-UDG	Sample volume (μl)		
						Negative contr.	Positive contr.		Input extraction	Elution	Amplification
1	NucliSens Basic kit	In-house NASBA	Real time	16S rRNA	N	Prep + Amp	Prep + Amp	N	100	50	5
1	NucliSens Basic kit	In-house MX NASBA	Real time	16S rRNA	N	Prep + Amp	Prep + Amp	N	100	50	5
2	Roche Amplicor sputum	In-house PCR	Real time	PstI fragment	Y	None	None	Y	300	60	5
3	QIAamp DNA	In-house PCR	Real time	16S rRNA gene	Y	Prep + Amp	Prep + Amp	Y	200	100	10
4	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	PmP4 gene	Y	Prep + Amp	Prep + Amp	Y	400	100	10
5	Pure Gene genomic DNA for gram$^+$ bacteria	In-house PCR	Real time	PstI fragment	Y	Amp	Amp	Y	400	50	10
6	QIAamp DNA blood	In-house PCR	Real time	MOMP gene	Y	Prep + Amp	Amp	N	200	100	4
8	Roche High Pure PCR Template Preparation kit	In-house PCR	Real time	MOMP gene	N	Prep	Amp	N	200	100	5
9	QIAamp DNA	In-house PCR	Real time	MOMP gene	Y	Amp	Amp	N	200	200	5
10	QIAamp DNA stool	In-house PCR	Real time	16S rRNA gene	Y	Prep + Amp	Prep + Amp	Y	200	200	5
11	QIAamp DNA blood	In-house PCR	Real time	16S rRNA gene	Y	Prep + Amp	Prep + Amp	N	200	100	10
13	QIAamp DNA tissue	In-house PCR	Real time	PstI fragment	Y	Prep + Amp	Prep + Amp	Y	200	200	5
14	QIAamp DNA tissue	In-house PCR	Real time	PstI fragment	Y	Prep + Amp	Amp	Y	200	200	5
16	QIAamp DNA	In-house PCR	Real time	PstI fragment	Y	Amp	Prep + Amp	Y	200	100	5
17	QIAamp DNA blood	In-house PCR	Real time	MOMP gene	Y	Prep + Amp	Amp	Y	200	100	5
18	Amplicor HCV	In-house PCR	EIA	MOMP gene	Y	Prep + Amp	Prep + Amp	N	100	30	4.5
18	Amplicor HCV	In-house MX PCR	EIA	MOMP gene	Y	Prep + Amp	Prep + Amp	N	100	30	4.5
19	Nucleospin tissue	Com. MX-PCR	EIA	NS	Y	Prep + Amp	Prep + Amp	N	400	100	10

[a] Amp, amplification; com., commercial assay (Pneumoplex from Prodesse); dUTP-UDG, deoxyuridine triphosphate–uracyl-*N*-glycosylase (dUTP replaces dTTP in PCR reactions, and the enzyme excises uracyl from any contaminating PCR product to prevent false positives); EIA, enzyme immunoassay; MX, multiplex; N, no; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; NS, not specified; prep, preparation; Y, yes; contr., control.

M-AAGGTACTACTGTAAATGCAAATGAACTACCAAACGTTTCTTTAA GTAACGGAG-TAMRA-3′).

In 2004, *M. pneumoniae* DNA was amplified by an own in-house-developed real-time PCR using primers M823F (5′-AGCGAACCGAGAGTGGTCAA-3′) and M973R (5′-GATTGGCCAGATCCAGATGTG-3′) and the detection probe (5′-FAM-CTCCAGGGCGCTGAGGCCACT-TAMRA-3′). Amplification conditions were 1 cycle of 2 min at 50°C and 1 cycle of 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C each.

C. pneumoniae DNA was amplified as described by Welti et al. (32).

Participant 6. In 2002 and 2004, *M. pneumoniae* DNA was amplified as described previously by Ieven et al. (12) and Ursi et al. (30), respectively. For *C. pneumoniae* DNA amplification, PCR was applied in 2002 and 2004 as described by Ursi et al. (29) and Hoymans et al. (11), respectively, using for the latter the MOMP VD4 primers and one detection probe described by Tondella et al. (26). Amplification conditions were the same as those for *M. pneumoniae*.

Participant 7. *M. pneumoniae* DNA was amplified by an in-house-developed real-time PCR using reverse primer 5′-CCAGGGCACATAATCCAACAC-3′ and forward primer 5′-AAGGAACAAACTGATCCCACTTCT-3′ and detection probe 5′-FAM-TCTCCACCGGGTTCAACCTTGTGG-NFQ-3′, with the following amplification conditions 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C.

Participant 8. *M. pneumoniae* DNA was amplified by an in-house-developed real-time PCR using primers MPF (5′-CCAACCTCCATGTAGCTGATAG CT-3′) and MPR (5′-TATCGCCAGGTAAAAACTCCTTCT-3′) and the detection probe (5′-FAM-ATCCTTGTTGTAAGGCTTGTAATCG-TAMRA-3′). Amplification conditions were 1 cycle of 2 min at 50°C and 1 cycle of 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C.

C. pneumoniae DNA was amplified by an in-house-developed real-time PCR using primers CPF (5′-AAGGGCTATAAAGGCGTTGCT-3′) and CPR (5′-T GGTCGCAGACTTTGTTCCA-3′) and detection probe (5′-FAM-TCCCCTTG CCAACAGACGCTGG-TAMRA-3′). The amplification conditions used were the same as those for *M. pneumoniae*.

Participant 9. *M. pneumoniae* DNA was amplified by an in-house-developed real-time PCR using primers FPMPN (5′-TCTGGCGTGGATCTCTCCC-3′) and RPMPN (5′-GACACTCGTGCTTGGTAACTGC-3′). Detection was done by using SYBR green. Amplification conditions were 1 cycle at 95°C for 10 min followed by 50 cycles each of 10 s at 95°C and 20 s at 65°C. In 2004, the same assay conditions were used, but real-time detection was done using probe MPN (5′-FAM-GAAGG AATGATAAGGCTTCAAGTGGACAAAGTG-TAMRA-3′).

C. pneumoniae DNA was amplified by an in-house-developed real-time PCR using the slightly modified MOMP VD2 primers of Tondella et al. (26), FPCPN (5′-CTCGTTGGTTTATTCGGAGTT-3′) and RPCPN (5′-CCAAGAGAAAGA GGTGTCTGTG-3′). Detection was done by using SYBR green. Amplification conditions were the same as those for *M. pneumoniae*. In 2004, the slightly modified MOMP VD2 detection probe described by Tondella et al. (26), CPN (5′-FAM-A AGGTACTACTGTAAATGCAAATGAAC-TAMRA-3′), was used.

Participant 10. *M. pneumoniae* DNA was amplified by an in-house-developed real-time PCR using primers MNP1FW (5′-CCAACCAAACAACAACGTTC A-3′) and MNP1REV (5′-CCTTGACTGGAGGCCGTTAA-3′) and detection probe MPN (5′-FAM-TCAATCCGAATAACGGTGACTTCTTACCACTG-T AMRA-3′) in 2004. Amplification conditions were 1 cycle at 50°C for 2 min followed by 1 cycle at 95°C for 10 min and 40 cycles each 15 s at 95°C and 60 s at 60°C.

C. pneumoniae DNA was amplified by an in-house-developed real-time PCR using primers CPF1 (5′-GGACCTTACCTGGACTTGACATGT-3′) and CPR1 (5′-CCATGCAGCACCTGTGTATCTG-3′) and detection probe (5′-FAM-TG ACCACTGTAGAAATACAGCTTTCCGCAAGG-TAMRA-3′). The amplification conditions used were the same as those for *M. pneumoniae*.

Participant 11. The *M. pneumoniae* and *C. pneumoniae* monoplex real-time PCR assays were done as described previously by Templeton et al. (24, 25). For the real-time multiplex PCR, primers, probes, and amplification conditions similar to those in the monoplex assays were used.

Participant 12. *M. pneumoniae* and *C. pneumoniae* DNA was detected as described by Abele-Horn et al. and Tong and Sillis (1, 27), respectively.

Participant 13. *C. pneumoniae* DNA was amplified by an in-house real-time PCR developed by participant number 14 using primers chlapneu 171F and chlapneu 250R and detection probe chlapneu 200T. Amplification conditions were 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 45 cycles each of 15 s at 95°C and 60 s at 60°C.

Participant 14. *M. pneumoniae* DNA was amplified by an in-house-developed real-time PCR using primers MP-T1 (5′-ACACCAAGTT CGCGAGTGCTA-3′) and MP-T2 (5′-CCGTCCTGCGTGGTTAAACTAT-3′) and detection probe

TABLE 5. Results of *M. pneumoniae* detection on individual samples in 2002

Sample no. and test description	Input (CCU/100 μl)	Laboratory no. and test result[e]												% Positive[b]
		6	11	1[a]	9	4	12	2	3	5	8	7	10	
B-001	5,000	+	+	+	+	+	+	+	+	+	+	+	−	100
B-005	5,000	+	+	+	+	+	+	+	+	+	+	+	−	100
B-010	5,000	+	+	+	+	+	+	+	+	+	+	+	−	100
B-002	500	+	+	+	+	+	+	+	+	+	+	+	−	100
A-004	500	+	+	+	+	−	+	−	+	+	−	−	−	66.7
A-007	500	+	+	+	+	+	+	−	−	+	−	−	−	66.7
A-010	500	+	+	+	+	−	−	+	−	+	−	−	−	58.3
B-003	500	+	+	+	−	+	−	+	−	−	−	−	−	50.0
B-008	500	+	+	−	−	+	−	+	+	−	inh	−	−	45.5
A-001	50	+	+	+	+	−	−	−	−	+	−	−	−	50.0
A-009	50	+	+	−	−	inh	−	+	−	−	−	−	−	27.3
A-002	50	−	−	−	−	−	+	+	−	−	−	−	−	16.7
A-003	0	−	−	−	−	−	−	+	−	+	−	−	−	16.7
A-008	0	−	−	−	−	inh	−	+	−	−	−	−	−	9.1
A-006	0	−	−	−	−	−	−	+	−	−	−	−	−	8.3
B-004	0	−	−	−	−	−	−	−	−	+	−	−	−	8.3
A-005	0	−	−	−	−	−	−	−	−	−	−	−	−	0
B-006	0	−	−	−	−	−	−	−	−	−	−	−	−	0
B-007	0	−	−	−	−	−	−	−	−	−	−	−	−	0
B-009	0	−	−	−	−	−	−	−	−	−	−	−	−	0
% Correct results		95.0	95.0	85.0	80.0	77.8	75.0	70.0	70.0	70.0	63.2	60.0	40.0	
Routine-application NAAT[c]		Y	Y	Y	N	Y	Y	N	Y	Y	NS	N	Y	
No. of samples tested monthly[d]		2	1	3	0	0	3	2	2	1	1	0	1	

[a] Identical results were obtained by detection by real-time mono-NASBA and by electrochemiluminescence.
[b] No. 10 was excluded from analysis.
[c] All operations were performed in separate rooms, except for participant 7, who used a single room.
[d] 0 = 0; 1 = 1 to 10 samples monthly; 2 = 11 to 45; 3 is more than 50.
[e] inh, inhibition.

MP-S (5′-FAM-ATCCCGACTCGTTAAAGCAGGATAAGATT-TAMRA-3′). Amplification conditions were 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 45 cycles each of 15 s at 95°C and 60 s at 60°C.

C. pneumoniae DNA was amplified by an in-house-developed real-time PCR using primers chlapneu 171F (5′-CGGCTAGAAATCAATTATAAGACTGAAG-3′) and chlapneu 250R (5′-TGGCGAATGACACCATGATC-3′) and detection probe chlapneu 200T (5′-FAM-AAATCTGCATCTCCCTCACGAATATGCTCA-TAMRA-3′). The amplification conditions used were the same as those for *M. pneumoniae*.

Participant 15. Conventional and monoplex real-time NASBA for the detection of *C. pneumoniae* RNA was done as described previously (23). MX-real-time NASBA was done as described previously (19).

Participant 16. *M. pneumoniae* and *C. pneumoniae* DNA was detected as described by Hardegger et al. and Welti et al. (10, 32), respectively.

Participant 17. *M. pneumoniae* and *C. pneumoniae* MOMP VD4 DNA was detected as described by Hardegger et al. and Tondella et al. (10, 26), respectively.

Participant 18. *M. pneumoniae* and *C. pneumoniae* MOMP VD2 DNA was detected by monoplex PCR using primers and probes described by Grondahl et al. and Tondella et al. (9, 26), respectively. Amplification conditions were 1 cycle of 10 min at 94°C followed by 40 cycles of 30 s at 94°C, 50°C, and 72°C, with a final cycle of 5 min at 72°C. The same primers, probes, and amplification conditions were used in the multiplex PCR.

Participant 19. The commercially available Pneumoplex assay (Prodesse, Waukesha, Wis.) was used for the detection of both *M. pneumoniae* and *C. pneumoniae* according to the instructions of the manufacturer.

Participant 20. *M. pneumoniae* DNA was amplified for participant 20 as described by Ursi et al. (30).

Internal controls. Most participants used a generic internal control such as the ones described by Hoymans et al., Ieven et al., and Tong and Sillis (11, 12, 27) or phocine herpes virus (25), except for participant 1, who measured for the presence of U1A mRNA in 2002, participant 2, who spiked another aliquot of the same sample with the respective organism, and the commercially available assays, which had their own internal controls.

Statistical analysis. The Fisher exact test or the chi-squared test was applied for the calculation of the significance between results during the preparation of the QC panels and for the performance of the different assays.

RESULTS

Preparation of the QC panels. To monitor the stability of the samples in the panels, aliquots kept at −80°C were examined by PCR and NASBA after one and two freezing-thawing cycles. All samples tested positive in 9 to 15 different runs for *C. pneumoniae* and *M. pneumoniae*, respectively, except for the samples with the lowest concentrations of *M. pneumoniae*, which produced positive results in 2 out of 15 PCR runs and 6 out of 15 NASBA runs ($P = 0.10$) in 2002.

When spiked BAL pools were tested by NASBA, an input of 50 CCU/100 μl, 500 CCU/100 μl, and 5000 CCU/100 μl of *M. pneumoniae* yielded 9 out of 9 positive results each time. When spiked BAL pools were tested by NASBA, an input of 4.9 IFU/100 μl, 49 IFU/100, and 490 IFU/100 μl of *C. pneumoniae* yielded 9 out of 9 positive results each time.

Performance of the laboratories. Some laboratories provided two amplification results obtained by two different procedures, such as mono- and multiplex reactions. Primers and probes used, as well as amplification conditions applied, were highly diverse.

Tables 1 to 4 show the different extraction, amplification, and detection procedures applied, as well as the target molecules used.

TABLE 6. Results of *M. pneumoniae* detection in 2004

Sample no. and test description	Input (CCU/ 100 μl), pool no.[a]	Laboratory no. and result																		% Positive
		1	1[b]	2	3	4	5	6	8	9	11	11[c]	14	16	17	20	18	18[c]	19[c]	
4	5,000, p2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
6	5,000, p1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	94.4
1	500, p1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	94.4
7	500, p2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	88.9
8	5,000, p3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	88.9
9	500, p3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	88.9
2	0, p3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
3	0, p1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
5	0, p2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
10	0, p1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
% Correct results		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	90	70	60	
Routine application NAAT[d]		Y	Y	N	Y	N	Y	Y	Y	N	Y	Y	N	Y	N	Y	Y	?	N	
No. of samples tested monthly[e]		3	3	0	1	0	1	2	2	0	3	?	0	2	0	1	1	?	0	

[a] p1, clear BAL pool; p2, more viscous BAL pool; p3, BAL pool blood stained.
[b] Results of multiplex NASBA.
[c] Results of multiplex PCR.
[d] All operations were performed in separate rooms.
[e] 0 = 0; 1 = 1 to 10 samples monthly, 2 = 11 to 45; 3 means more than 50.

The 2002 *M. pneumoniae* QC. For *M. pneumoniae*, 13 datasets were obtained (Table 5). All procedures were in-house developed, except one participant, no. 4, who used the commercial kit from Minerva Biolabs GmbH (Berlin, Germany).

Eleven out of 13 assays applied one PCR and two NASBA; 9 were based on real-time detection, 3 used agarose gel electrophoresis, and 1 used electrochemiluminescent detection. The target most often used was the P1 gene in 9 out of 13 (69.2%) assays.

One data set, from participant 10, was excluded from the global analysis because of the uniformly negative results. All samples containing 5,000 CCU/100 μl were found positive. The samples containing 500 CCU/100 μl were scored positive in 20 out of 36 (55.5%) of the cases when suspended in saline and in 21 out of 36 (58.3%) of the cases when suspended in BAL fluid ($P = 0.8$). The lowest concentrations tested, 50 CCU/100 μl, scored positive in 10 out of 36 (27.8%) of the cases, a score which is not different from that obtained during the assessment of the panels before their distribution ($P = 0.3$).

Participant 2 did not include any negative controls and scored 3 out of 8 (37.5%) false-positive results. False-positive results were reported by a second laboratory (no. 5), and inhibition of the reaction in three instances was reported by two laboratories. In 7 out of 13 tests (53.9%), an internal control was used to monitor inhibition of the reaction in each sample. Eight (66.7%) participants reported the use of dUTP–uracyl-*N*-glycosylase (dUTP-UDG) to avoid false-positive results due to carryover, but nevertheless participants 2 and 5 using dUTP-UDG produced false-positive results.

The 2004 *M. pneumoniae* QC. The 2004 *M. pneumoniae* QC resulted in 18 datasets; 11 sets were obtained from 10 laboratories that participated in the 2002 QC (Table 6).

All participants used a commercially available nucleic acid extraction kit. Agarose gel electrophoresis was no longer used by any of the participants. In 16 out of 18 assays PCR was used, NASBA was used twice. Real-time detection was applied in 15 out of 18 tests, and an enzyme immunoassay (EIA) was applied in 3 out of 18 tests. The target most often used was the P1 gene (66.7%).

The samples containing 5,000 CCU/100 μl scored positive in 51 out of 54 (94.1%) of the cases, and those containing 500 CCU/100 μl were scored positive in 49 out of 54 (90.2%) of the cases. Four participants applied a multiplex assay. In three instances, participants 1, 11, and 18, an in-house-developed multiplex assay was performed in parallel with a monoplex real-time reaction; in one of these the multiplex reaction was less sensitive. The commercially available multiplex assay, the Pneumoplex assay from Prodesse (Waukesha, Wis.) (used by participant 19), was also less sensitive.

An internal control to monitor inhibition of the reaction in each sample was used in 15 out of 18 (83.3%) of the tests. Nine (60.0%) participants reported the use of dUTP-UDG to avoid false-positive results; none were recorded. One participant did not include negative controls.

The 2002 *C. pneumoniae* QC. The *C. pneumoniae* panels resulted in 16 datasets, of which 10 were delivered within the requested period of 4 weeks (Table 7). Twelve out of 16 amplification assays applied a PCR, 4 used a NASBA, 13 datasets used real-time detection, 2 participants used agarose gel electrophoresis, and 1 used electrochemiluminescent detection. Targets most often used were the 16S rRNA (NASBA), a cloned PstI fragment, the MOMP gene, or a 16S rRNA gene fragment in four (25%), four (25%), three (18.8%), and three (18.8%) of the assays, respectively. The other targets were only used once and were the PmP4 gene and a cytadhesin gene.

No false-positive results were recorded among the 124 samples. Samples containing 490 IFU/100 μl scored positive in 44 out of 45 (97.8%) instances, samples containing 49 IFU/100 μl suspended in saline scored positive in 46 out of 48 (95.8%) cases, and samples suspended in BAL fluid scored positive in 39 out of 45 (86.7%) cases ($P = 0.1$). The lowest concentration

TABLE 7. Results of *C. pneumoniae* detection on individual samples in 2002

Sample no. and test description	Input (IFU/100 μl)	Laboratory no. and result															% Positive
		1[a]	2	6	11	15	3	8	14	4	9	13	15[b]	5	12	10	
B-004	4.9×10^2	+	+	+	+	+	+	+	+	+	NA	+	+	+	+	+	100
B-008	4.9×10^2	+	+	+	+	+	+	+	+	+	NA	+	+	+	+	+	100
A-008	4.9×10^1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
B-005	4.9×10^2	+	+	+	+	+	+	+	+	+	NA	+	+	+	−	+	93.3
A-002	4.9×10^1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	93.8
A-006	4.9×10^1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	93.8
B-002	4.9×10^1	+	+	+	+	+	+	+	+	+	NA	+	−	+	+	−	86.7
B-003	4.9×10^1	+	+	+	+	+	+	+	+	+	NA	+	−	+	+	−	86.7
B-009	4.9×10^1	+	+	+	+	+	+	+	+	+	NA	+	+	+	−	−	86.7
A-003	4.9	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−	75.0
A-009	4.9	+	+	+	+	+	−	+	+	−	−	−	+	−	+	−	62.5
A-010	4.9	+	+	+	+	+	+	+	−	−	+	−	+	−	−	−	62.5
A-001	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
A-004	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
A-005	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
A-007	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
B-001	0	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	0
B-006	0	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	0
B-007	0	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	0
B-010	0	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	0
% Correct results		100	100	100	100	100	95.0	95.0	95.0	90.0	90.0	90.0	90.0	85.0	80.0	60.0	
Routine application NAAT[c]		Y	Y	Y	N	Y	Y	Y	N	N	N	Y	Y	Y	Y	?	
No. of samples tested monthly[d]		3	2	2	0	3	2	1	0	0	0	1	3	1	3	1	

[a] Both conventional and real-time NASBA gave the same results.
[b] Results of multiplex NASBA.
[c] All operations were performed in separate rooms.
[d] 0 = 0; 1 = 1 to 10 samples monthly; 2 = 11 to 45; 3 = more than 50. NA, technical error.

tested, 4.9 IFU/100 μl, scored positive in 32 out of 48 (66.7%) of the cases.

The multiplex NASBA, participant 15[b], targeting the *M. pneumoniae*, *C. pneumoniae*, and *Legionella pneumophila* 16S rRNA, was significantly less sensitive than the monoplex NASBA of participant 15; both were in-house developed in the same laboratory.

Six out of 16 tests (37.5%) used an internal control to monitor inhibition of the reaction in each sample. Nine (64.3%) participants reported the use of dUTP-UDG.

The 2004 *C. pneumoniae* QC. The *C. pneumoniae* panels resulted in 18 datasets (Table 8). Thirteen sets were obtained from 12 laboratories that participated in the 2002 QC.

Sixteen assays applied a PCR, and 2 applied a NASBA; 15 datasets used real-time detection, and in 3 assays an EIA was used. Targets most often used were the MOMP gene, the cloned PstI fragment, and the 16S rRNA gene in 6 out of 18 (33.3%), 5 out of 18 (27.8%), and in 3 out of 18 (16.7%) assays, respectively.

Among 72 *C. pneumoniae*-negative samples, 4 were reported positive by one participant who did not use dUTP-UDG. Samples containing 490 IFU/100 μl were scored positive for 17 out of 18 (94.4%) of the cases, samples containing 49 IFU/100 μl scored positive in 14 out of 18 (83.3%) cases, and those containing 4.9 IFU/100 μl scored positive in 15 out of 18 (83.3%) of the cases. The lowest concentration tested, 0.49 IFU/100 μl, scored positive in 18 out of 54 (33.3%) of the cases.

Three participants submitted results of a multiplex NAAT: a real-time in-house developed NASBA (participant 1), an in-house-developed PCR (participant 18), and the commercially available Pneumoplex from Prodesse. The multiplex PCR was less sensitive than the monoplex counterpart. Laboratory 19, applying the Pneumoplex, did not detect a single *C. pneumoniae*-positive sample.

In 15 out of 18 tests (83.3%), an internal control was used to monitor inhibition of the reaction in each sample. Nine (56.3%) participants reported the use of dUTP-UDG to avoid false-positive results. One participant did not include negative controls.

Samples prepared with BAL pool 1 (clear), pool 2 (more viscous), and pool 3 (blood stained) spiked with 0.49 IFU/100 μl scored positive in 4 out of 17, 8 out of 17, and 4 out of 17 cases, respectively. The results of participant 8 were not included in this calculation due to the high number of false-positive results.

Generally, participants targeting the MOMP gene (participants 6, 8, 9, 17, and 18) found fewer samples to be *C. pneumoniae* positive than participants targeting the 16S rRNA, 16S rRNA gene, or PstI fragment. Participants 13 and 14 applied the same in-house real-time assay and obtained identical results.

DISCUSSION

QC for the NAATs for *M. pneumoniae* and *C. pneumoniae* are particularly important, because in the clinical laboratory there is no practical alternative for the detection of both agents. The two QCs reported here illustrate the technical evolution between 2002 and 2004: disappearance of gel electrophoresis for the detection

TABLE 8. Results of *C. pneumoniae* detection in 2004[a]

Sample no. and test description	Input (IFU/100 μl), pool no.	Laboratory no. and result																		% Positive
		4	1	2	3	11	16	5	9	1[b]	6	13	14	17	18	10	18[c]	19[c]	8	
9	490, p1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NI	+	94.4
7	4.9, p3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	83.3
6	49, p2	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	77.8
3	0.49, p2	+	+	−	+	+	+	+	+	−	−	−	−	−	+	−	−	−	+	50.0
5	0.49, p1	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	27.8
10	0.49, p3	+	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	22.2
1	0, p1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	5.6
2	0, p2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	5.6
4	0, p3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	5.6
8	0, p1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	5.6
% Correct results		100	90	90	90	90	90	80	80	70	70	70	70	70	70	60	60	40	30	
Routine application NAAT[d]		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	N	Y	
No. of samples tested monthly[e]		1	3	2	2	1	2	1	1	1	1	1	0	2	3	1	3	0	1	

[a] NI, not interpretable; p1, clear BAL pool; p2, more viscous BAL pool; p3; BAL pool blood stained.
[b] Results of multiplex NASBA.
[c] Results of multiplex PCR.
[d] All operations were performed in separate rooms.
[e] 0 = 0; 1 = 1 to 10; 2 = 11 to 45; 3 = more than 50.

of the amplicons, a substantial increase in monitoring of inhibition of the amplification reaction, and an increased use of positive and negative samples and of multiplex amplification reactions. The use of dUTP-UDG remained constant. Inclusion of positive and negative controls did not differ between the two QCs.

Gradually, laboratories performing NAATs for the detection of both *M. pneumoniae* and *C. pneumoniae* use the same amplification conditions for both assays. The advantage is that in the same run different organisms can be targeted, decreasing the total turnaround time.

In 2002 two participants obtained false-positive results in the *M. pneumoniae* panel (one of these performing routinely up to 50 tests on a monthly basis). In the 2004 *M. pneumoniae* panel, both participants reported all samples correctly. No false-positive *C. pneumoniae* results were recorded in 2002, and 11 out of 15 participants produced correct results. In 2004, one participant produced false-positive results for all four *C. pneumoniae*-negative samples. Other PCR quality assessment studies have also recorded false-positive results (8, 20, 22, 31, 33). Perhaps this problem will not be eliminated until sample processing can be automated.

In this quality control study, the major problem was the occurrence of false negatives, especially when testing the *M. pneumoniae* 2002 proficiency panel. The results obtained by the participants in 2002 for the samples containing 50 CCU/100 μl of *M. pneumoniae* are in line with those of the reference laboratory ($P = 0.13$). This low concentration was not included in 2004. There were no differences between the suspensions prepared in saline (20 positive results) and those prepared in BAL (21 positive results). Participant 10, which failed to detect any *M. pneumoniae*-positive samples, did not participate in the *M. pneumoniae* 2004 panel and also failed to detect small numbers of *C. pneumoniae* in 2002 and 2004.

The lowest concentration of *M. pneumoniae* is clearly at the limit of detectability, therefore only the 5,000 and 500 CCU/100 μl samples were taken into consideration for the global analysis. Thus, 2 out of 12 participants produced correct results, 2 out of 12 produced false-positive results, and 9 participants produced between 0 of 9 and 8 of 9 correct results. There was considerable improvement in 2004, when 15 out of 18 participants produced 100% *M. pneumoniae* correct results without any false positives.

Suspensions of both *M. pneumoniae* and *C. pneumoniae* in saline and in BAL were included in the 2002 panels to reveal possible inhibitors: two samples in saline were found in one laboratory to inhibit the reaction, and one sample in BAL was found in a second laboratory. Both laboratories used an extraction kit from Roche. No inhibitors were recorded in 2004, although one of the BAL pools was blood stained. Both laboratories reporting inhibition in the 2002 panel used the same extraction procedure for the 2004 *M. pneumoniae* panel. Participant 8 did not change the amplification and detection procedures. For the 2004 panel, participant 4 replaced the Minerva Biolabs kit with an in-house-developed real-time PCR.

Two out of three participants (18[b] and 19) obtaining false-negative results in the 2004 *M. pneumoniae* QC applied a multiplex NAAT, one of them being a commercial kit (Pneumoplex; Prodesse). Two other participants using an in-house-developed multiplex NAAT obtained correct results.

Four participants failed to detect *C. pneumoniae* in the sample containing 49 IFU/100 μl in the 2004 QC, two of them applied a multiplex NAAT, with one being a commercial kit (Pneumoplex; Prodesse).

In the 2004 QC tests, multiplex NAAT was applied. PCR was used by participants 11, 18, and 19 (Table 6 and Table 8), and NASBA was used by participants 1 and 15. The multiplex PCRs scored a smaller number of samples positive than most of the monoplex tests. One multiplex PCR was the commercially available Pneumoplex (Prodesse). Although the limit of detection of this assay was reported to be 5 CCU/ml for *M. pneumoniae* and 0.01 50% tissue culture infective dose/ml for *C. pneumoniae* and 10 copies of recombinant DNA for each

organism (13), the test did not perform well in this evaluation. Participant 19 tested a second panel of each organism with similar results. The assay was performed correctly. The manufacturer was contacted and is aware of the sensitivity problems of the Pneumoplex assay. They intend to improve the sensitivity of the test.

Multiplex assays are somewhat less sensitive than monoplex assays, but until the number of organisms present in clinical specimens of diseased individuals is known, it is impossible to state whether the degree of sensitivity attained is acceptable.

From previous investigations, we have learned that the sensitivity of molecular diagnostics using respiratory samples may be compromised by the presence of inhibitory factors in the samples (2, 5, 8, 14, 28, 29). Therefore, three different BAL pools were used in the 2004 QC panels. There was no significant difference in the positivity rates between the three different BAL pools used to prepare the suspensions, although the suspension in the blood-stained BAL had the lowest number of positive results. Unexpectedly, the viscous suspension scored a higher number of positive results than the clear BAL suspension.

There was considerable variation between the different PCR protocols applied by the participants. Evaluation of the various PCR protocols showed no apparent association between their performance and the particular variables of the PCR method used, except for the *C. pneumoniae* MOMP amplification protocols. Participants 6, 17, and 18 used the protocol described by Tondella et al. (26) and obtained lower positivity scores than participants using a different protocol and targeting a different gene for the detection of *C. pneumoniae*. Participant 9 had a slightly better positivity score using slightly modified MOMP VD2 primers and detection probe. However, the results of such a comparison must be interpreted with caution due to the relatively small number of samples, the small number of participating laboratories, and the high diversity of the methods used.

The discrepant results from two successive QC exercises observed among different laboratories, some of which lacked experience in NAAT, illustrate the importance of training of personnel and the use of negative and positive controls in the preparative and amplification phases. This is illustrated by the three laboratories that reported false-positive results; one of them did not use negative controls in the preparation, amplification, and detection procedures.

The different performance characteristics of the amplification-based assays used may explain discrepant findings from published studies that used NAATs to determine *M. pneumoniae* and *C. pneumoniae* prevalence in patient populations. Sensitivity and specificity issues should be addressed before publishing clinical and epidemiological studies of *M. pneumoniae* and *C. pneumoniae* infections based on the detection of bacterial DNA and RNA in clinical specimens by NAAT.

This study also underlines the need for reference reagents and standard operating procedures to enable experienced technicians to perform quality control assessment of nucleic acid amplification methods and thus perform reliable diagnostic molecular amplification techniques on a routine basis.

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