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REVIEW

Acute respiratory infection due to *Mycoplasma pneumoniae*: current status of diagnostic methods

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Abstract Because of the absence of well-standardized both in-house and FDA-approved commercially available diagnostic tests, the reliable diagnosis of respiratory infection due to *Mycoplasma pneumoniae* remains difficult. In addition, no formal external quality assessment schemes which would allow to conclude about the performance of *M. pneumoniae* diagnostic tests exist. In this review, the current state of knowledge of *M. pneumoniae*-associated respiratory infections in the context of epidemiological studies published during the past 5 years is discussed, with particular emphasis on the diagnostic strategies used and their impact on results. The role of *M. pneumoniae* as a cause of respiratory tract infections (RTIs) differs from study to study due to geographical and epidemiological differences, as well as to the application of different diagnostic techniques and criteria used.

Introduction

In 2003, we already stated that proper validation and standardization of nucleic acid amplification techniques (NAATs) are often lacking, and that the different methods used must be compared in order to define the most sensitive and specific tests [1]. This is similar for existing serological tests and other new diagnostic tests as well. Studies comparing different methods still remain to be undertaken and will be critically important for the development of a standardized test for clinical laboratories.

Mycoplasma pneumoniae belongs to the class of the Mollicutes and has been associated with a wide variety of acute and chronic diseases. Respiratory tract infections (RTIs) with *M. pneumoniae* occur worldwide and in all age groups.

Serological methods, in particular, such as the complement fixation test (CFT) and enzyme immunoassays (EIAs), are most widely used to diagnose an *M. pneumoniae* infection. The application of polymerase chain reaction (PCR) is more and more often accepted as a rapid diagnostic test, since culture is too slow and too insensitive to be therapeutically relevant. Only a few of the currently available NAATs have been extensively validated against culture, which remains the reference standard, despite its low sensitivity and variable yield, depending on the specimens tested and the isolation protocols used. The sensitivity of NAATs is almost always superior to that of the traditional procedures, and they are more and more often considered as the "new gold standard". However, different studies have used not only different diagnostic tools or combinations thereof, but also different diagnostic criteria for making a diagnosis of an infection, thereby, making comparison between studies difficult. Most importantly, the lack of standardization has resulted in a wide variation of interlaboratory test performance, even when using the same test and criteria [2]. In an effort to standardize diagnostic assays for *C. pneumoniae*, recommendations have been published by the US Centers for Disease Control and Prevention (CDC) and the Canadian Laboratory Centre for Disease Control in 2001 [3]. However, no such recommendations exist for standardizing the diagnostic approach for *M. pneumoniae*. The epidemi-

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ology of *M. pneumoniae*-associated respiratory infections in studies performed all over the world for the purpose of examining the current state of knowledge of *M. pneumoniae* diagnostics since the publication of the 2003 minireview [1] was reviewed.

Epidemiology of respiratory infection due to *M. pneumoniae*

Varying with the population studied and diagnostic methods used, in studies published during the 1990s, in 6 to >30% of lower respiratory tract infections (LRTIs), an association was found with *M. pneumoniae* [4–6]. Over 50 additional studies have been published about *M. pneumoniae*-associated LRTI since 2003. Data from selected studies chosen to represent different populations from around the world are summarized in Tables 1 and 2. As shown in these tables, the proportion of LRTI in children and adults, including community-acquired pneumonia, associated with *M. pneumoniae* infection during the past 5 years has ranged from 0 to 66.7% [7, 8], varying with age and the geographic location of the population examined and the diagnostic methods used. In a lot of studies, the diagnosis of *M. pneumoniae* was based on serology alone [7, 9–11]; some used a PCR assay alone [8, 12, 13] or at least one serological test and a PCR assay [14–20]. Only a limited number of studies used culture in combination with a serological test and/or PCR assay [21–25] and three studies applied two different PCR assays [26–28]. Furthermore, there was a high degree of heterogeneity from study to study in the serological methods and criteria used. These are not necessarily interchangeable. In some studies, no data were presented on the type of assay and the criteria used, thereby, making it difficult, if not impossible, to compare results from one study to another.

One example is a prospective study of the incidence and etiology of community-acquired pneumonia in hospitalized adult patients [29] published in 1999. The researchers used serological methods and PCR for the diagnosis of an *M. pneumoniae* infection. The following serological criteria were used: a 4-fold rise or seroconversion in IgG- and/or an IgM-positive titer for *M. pneumoniae*. Fourteen percent of the patients were thought to have serological evidence of *M. pneumoniae* infection, but no information was provided on the serological method used. Furthermore, the study had no control subjects, and because of their absence, the significance of the reported seroprevalence of the patient group cannot be known. Background rates of seropositivity can be very high in some adult populations, ranging from 36 to 93% for IgG and ranging from 0 to 51% for IgM, depending on the assay used [30].

Another example is the study of Oosterheert et al. [8]. In a randomized controlled trial, nasopharyngeal and oropharyngeal swab specimens from patients admitted for antibiotic treatment of LRTIs were evaluated by means of real-time PCR for respiratory viruses and atypical pathogens, as well as by conventional diagnostic procedures for virus detection. No details on the real-time PCR were given, although no *M. pneumoniae*-positive patient results were identified. No other methods were used in this study to confirm the negative results.

In general, in more recent studies using PCR assays, lower rates of *M. pneumoniae*-associated LRTI have been reported than in studies using serological testing (Tables 1 and 2). During a community outbreak of *M. pneumoniae*, Nilsson et al. [28] compared semi-nested and real-time PCR of oropharyngeal swabs with serology for the diagnosis of *M. pneumoniae* infections at different time points after the onset of disease. *M. pneumoniae* was diagnosed in 48/164 patients with a respiratory tract infection. Forty-five (29%) were PCR-positive, whereas a significant rise in IgG titer or IgM antibodies was detected in 44/154 (27%) subjects. Although the authors found that persistence of *M. pneumoniae* DNA in the throat was common and could be present for up to 7 weeks after the onset of disease, they concluded that PCR was superior to serology for the diagnosis of an *M. pneumoniae* infection during the early phase of infection. When examining 73 children with RTIs for *M. pneumoniae* by real-time PCR and two serological assays (a passive agglutination test and the ImmunoCard assay), Otomo et al. [31] confirmed the results of Nilsson et al. [28]. They found sensitivities of 100% and 33.3% and specificities of 100% and 82.1% for PCR and the ImmunoCard assay, respectively. According to the authors, real-time PCR or a related molecular assay is suitable for rapid diagnosis as a first screening test. These data confirmed the lack of correlation of serological methods with culture and/or PCR assays reported in earlier studies [1].

The epidemiological data emerging from pediatric studies have revealed similar inconsistencies of the methods and criteria used to make a diagnosis of an acute *M. pneumoniae* infection (Table 2). Examples include the use of single IgG titers by some studies (Table 2). A more specific example is a small uncontrolled pediatric study in Turkey [17] collecting nasopharyngeal samples for the PCR detection of *M. pneumoniae* DNA and blood for serology on the first admission to the hospital. However, they did not specify the PCR test applied, and although blood was only collected once (on hospital admission), serological diagnosis was made according to the materials and methods section by demonstrating an increment of IgM greater than 1/10 and a 4-fold increase in IgG with enzyme-linked immunosorbent assay (ELISA). No positive case definition

Table 1 Summary of studies of respiratory infections due to *Mycoplasma pneumoniae* in adults published since 2003

Reference	Location	Subject age (years)	No. of subjects tested	Diagnostic method(s)	No. (%) of subjects infected with *M. pneumoniae*	Comments	Methodological problem(s)[a]
[16] 2004	The Netherlands	1–88	159	P1 gene-based PCR, particle agglutination and ELISA	19 (11.9)	7 PCR-positive, all also positive in at least one serological test, 2-fold titer increase in IgG was defined as positive	D
[7] 2004	Trinidad and Tobago	>2	132	IgM and IgG EIA on acute phase serum	88 (66.7)	36/88 IgM-positive	B
[14] 2005	France	≥18	3,198	P1-based PCR, Ag detection by EIA	109 (3.6)	114 PCR-positive, Ag detection test had very low sensitivity and the results were not mentioned as such	E
[27] 2005	Chile	60–96	84	P1 gene-based PCR, 16S rRNA gene-based PCR, IgM and IgG indirect IF	11 (13.1)	8 positive by IFI (of which 4 by IFI alone), 7 PCR-positive (of which 3 only by PCR)	...
[8] 2005	The Netherlands	≥18	107	PCR	0	PCR not described	A
[26] 2006	UK	≥18	80 patients 49 controls	P1 gene-based PCR, 16S rRNA-based PCR	1 (1.3)	1 PCR-positive patient	...
[18] 2006	Denmark	18–96	235 patients 113 controls	P1 gene-based PCR and CFT	14 (5.5)	13 positive patients, 1 positive control Proportion of positive results by each test not specified	...
[25] 2007	India	≥18	100	Culture, IgM ELISA, cold agglutination test on acute-phase sera	31 (31)	31 positive by culture, 21 positive by IgM ELISA, 34 positive by cold agglutination test	...
[22] 2008	Belgium	≥18	147	Mono and MX real-time NASBA, real-time PCR, culture, IgM and IgG EIA	19 (12.9)	8 positive by culture, 15 positive by PCR, 19 positive by real-time MX NASBA, 23 by mono real-time NASBA	...
[19] 2008	The Netherlands	≥18	201	PCR, CFT	8 (4.0)	7 positive by PCR, 8 positive by serology PCR not specified	A
[23] 2008	Japan	16–>80	NS	Culture, IgM, and IgG serology	210	210 IgG *M. pneumoniae*-positive, 38 culture-positive	...

[a] A: no information provided about the PCR and/or serological assay used; B: single IgG titer used as part of the criteria used to define an acute infection; C: EIA used as the only serodiagnostic tool; D: serological titers vary from those recommended for the diagnosis of acute infections; E: one PCR assay used as the only diagnostic tool; F: (single) IgM titer used as the only serodiagnostic tool in some or all patients

Ag: antigen; CFT: complement fixation test; IFI: indirect immunofluorescence; IgA/G/M: immunoglobulin A/G/M; MX: multiplex; NASBA: nucleic acid sequence-based amplification; …: no major methodological problems identified

Table 2 Summary of studies of respiratory infections due to *M. pneumoniae* in pediatric patients published since 2003

Reference	Location	Subject age (years)	No. of subjects tested	Diagnostic method(s)	No. (%) of subjects infected with *M. pneumoniae*	Comments	Methodological problem(s)[a]
[21] 2003	Chile	0–14	106	16S rRNA gene-based PCR, IgM serology (two tests) on acute serum, culture	31 (29.2)	31 positive by IgM, in 28/31 cases, serology was confirmed by PCR, 19 positive by culture	...
[12] 2004	The Netherlands	0–16	168	P1 gene-based PCR	4 (2.4)		E
[15] 2004	Greece	0.5–14	65	P1 gene-based PCR and IgM serology on acute-phase serum	18 (27.5)	18 positive by IgM, 9 PCR-positive	F
[70 2004	Japan	0–14	369	16S rRNA gene-based PCR, CFT, culture	69 (18.7)	68 positive by PCR, 53 culture-positive, 76 serology-positive	...
[9] 2005	Finland	0.3–16	101	CFT, IgM, and IgA serology	27 (27)	27 patients positive with two or more tests, 12 patients positive with only one test	C
[13] 2005	China	0–5	85 patients 185 controls	ATPase-based PCR	6 (7.1)	All positive results were found inpatients	E
[11] 2005	India		93	IgM ELISA on acute-phase serum	22 (24)	All positive by single IgM determination	F
[10] 2006	Finland	0–16	220	2 IgM EIAs	11 (5)	Not clear whether positive in acute, convalescent, or both sera	F
[20] 2006	Japan	0–6	339	P1 gene-based PCR, passive agglutination, IgM, IgG, and IgA ELISA	81 (23.9)	66 PCR-positive, 106 PA titers above 1:40, among PCR-positive patients, 30/36 had a ≥4-fold increase in PA titer, 36/81 positive for IgG, 16/81 positive for IgA, and 54/81 positive for IgM	...
[24] 2007	Japan	0–15	194	Culture, CFT, rapidtest	45 (23.2)	14 culture-positive, ImmunoCard-positive in 39 paired sera and in 14 acute sera, CFT results not mentioned	...
[17] 2007	Turkey	5–15	284	PCR, IgM ELISA onacute-phase serum	NS	33/203 PCR-positive, 86/284 IgM patients, only in 13 cases positive by both PCR and IgM	...
[31] 2008	Japan	0–15	73	16S rRNA gene-based PCR, IgM and IgG serology	6 (8.2)	6/6 PCR-positive, 5/6 IgG seroconversion/ significant rise, one additional positive by IgG serology, 2/6 ImmunoCard assay-positive, 12 additional patients positive by ImmunoCard assay	...

[a] A: no information provided about the PCR and/or serological assay used; B: single IgG titer used as part of the criteria used to define an acute infection; C: EIA used as the only serodiagnostic tool; D: serological titers vary from those recommended for the diagnosis of acute infections; E: one PCR assay used as the only diagnostic tool; F: (single) IgM titer used as the only serodiagnostic tool in some or all patients

Ag: antigen; CFT: complement fixation test; IFI: indirect immunofluorescence; IgA/G/M: Immunoglobulin A/G/M; MX: multiplex; NASBA: nucleic acid sequence-based amplification; NS: not specified; …: no major methodological problems identified

was mentioned either. The authors concluded that serological tests were more sensitive and specific than PCR, since the false-positive ratio for PCR was 16.2%. Another small uncontrolled pediatric study in Dallas, Texas [32], identified *M. pneumoniae* as the cause of infection in 14% of patients with pneumonia. The center applied an ELISA for serological testing and used a 4-fold increase of IgG or single IgM titers of ≥1:10 as evidence of acute infection. However, they did not specify the proportion of positive results by the different antibody classes and no information on the ELISA was presented.

The true role of *M. pneumoniae* in RTIs remains a challenge given the wide variations of data from studies with equally wide variation of and lack of standardized diagnostic methods.

Serology

The serologic measurement of specific antibody responses has limited application for an etiologic diagnosis of an *M. pneumoniae* infection because diagnostic results are only available retrospectively.

A great number of antigen preparations have been proposed: whole organisms, protein fractions, glycoprotein fractions, recombinant antigens. Some commercialized assays lack both sensitivity and specificity, emphasizing the need for more validation and quality control [30, 33–35].

The sensitivity of the serological assays depends on whether the first serum sample is collected early or late after the onset of disease and on the availability of paired sera, since for an accurate diagnosis to be made, paired serum samples are required, with a 4-fold rise in titer appearing after three to four weeks after the onset of disease [36]. In practice, however, often, only one serum sample, from the acute-phase of the illness, is available or the two samples are collected within a too short time interval to detect a titer rise. Solitary high IgG titers have no diagnostic meaning for an acute infection, since the moment of the seroconversion is unknown and necessarily took place some time before the illness under observation started. Single high titers, for which a cut-off value has to be determined by a local evaluation, are useful only in prevalence studies among population groups.

Since IgM antibodies appear earlier than IgG antibodies, the detection of IgM in serum is a widely used approach for the early serologic diagnosis of an *M. pneumoniae* infection, especially in children. It should be realized that IgM antibodies are often not produced in children under 6 months of age, in a proportion of primary infections and during reinfections. A single IgM measurement may detect an acute infection with higher sensitivity if the test is performed after at least 7 days following the onset of disease [37]. In some patients, IgM antibodies appear even later [38]. Ozaki et al. [24] found that a single assay using the IgM ImmunoCard (Meridian Biosciences) had a sensitivity of 31.8% for the detection of an acute *M. pneumoniae* infection, which increased to 88.6% when paired sera were analyzed from seropositive children with pneumonia. Furthermore, an elevated IgM may persist for months after the acute infection [39]. IgM tests are usually less sensitive and specific than 4-fold changes in antibody titers between paired specimens separated by several weeks [40].

It has been reported that the detection of IgA-specific antibody seems to be a good indicator of a recent *M. pneumoniae* infection in both children and adults [41–43]. On the other hand, when evaluating the Medac IgM, IgG, and IgA assay on 159 serum samples from 113 patients with acute RTIs, Narita [44] did not find a significant advantage of detecting IgA in children.

Talkington et al. [35] compared eight commercial EIAs (two single-use EIAs and six plate-type EIAs) for the detection of specific IgM/IgG antibodies, using paired serum samples from 51 patients with a confirmed *M. pneumoniae* infection and a positive complement fixation test (CFT). The results from acute-phase sera ranged from 14% ImmunoWELL IgM-positive to 45% positive by Zeus IgG EIA. When both the acute-phase and convalescent phase serum samples were analyzed, positive results ranged from 39% by the ImmunoWELL IgM assay to 88% positive by the Remel IgG-IgM EIA. In their study, the single-use EIAs proved to be more reliable than the plate-type EIAs. Beersma et al. [30] evaluated the sensitivity and specificity of 12 assays for the detection of *M. pneumoniae* IgM and IgG, as well as the CFT. Some of the assays had a low sensitivity (Novum and ImmunoCard IgM), while the best performances in terms of sensitivity and specificity were recorded for ANILab systems (77 and 92%, respectively) and the CFT (65 and 97%, respectively). Petitjean et al. [34] found similar IgM sensitivities with four *M. pneumoniae* tests in children: between 89 and 92%, but wide variations in adults: Platelia and BMD 16%, Biotest 50% and Sorin 58%. The specificities of the tests were 100, 90, 65, and 25% respectively. The latter two IgM tests cannot, thus, be used for diagnosis. The sensitivities of the IgG tests in children varied between 52 and 78%. The sensitivities for the IgG tests in adults were comparable: between 89 and 92%. When comparing four IgM-, IgG-, and IgA-specific EIAs in sera from 504 blood donors and 102 patients with infections not caused by *M. pneumoniae*, Csángó et al. [45] reported the detection frequencies of IgM in blood donors varying between 2.8 and 16% and in patients between 9.8 and 42.2%. IgA was detected in 22.8 to 68.5% of blood donor sera and in 53.8 to 100% of

patients, illustrating again that the use of some serological kits may lead to a serious overdiagnosis of *M. pneumoniae* infections. Finally, Nir-Paz et al. [33] compared eight commercially available tests for *M. pneumoniae* using 204 single sera from healthy individuals. The study showed that age was associated with test positivity in healthy individuals, with the IgM peaking at primary/secondary school age, and declining thereafter, while IgG rose progressively into adulthood. The high IgM positivity in these age groups casts doubt on the suggestion that combining IgM tests with amplification-based tests in the pediatric population might be of benefit [6, 46, 47]. Inter-assay agreement was poor. The study confirmed that single serum serology is unsuitable for the diagnosis of *M. pneumoniae* infection, and that commercially available tests need further improvement.

In conclusion, serologic tests can never offer an early diagnosis and are, therefore, an epidemiological than a diagnostic tool. The clinical significance of a serologic test, for both IgM and IgG, should be defined by studies of patients with a documented infection and for whom detailed information concerning the time lapses between the onset of disease and the collection of the serum specimens are known.

Culture

M. pneumoniae was first recovered on a medium devised by Hayflick in which PPLO agar [48] was supplemented with a fresh yeast extract preparation of Edward [49] and 20% horse serum. However, *M. pneumoniae* grows slowly, so cultures may require up to 6 weeks to become positive. Although the culture of *M. pneumoniae* is still considered to be the gold standard, it is seldomly performed as a diagnostic test.

Culture is, and will remain, essential for further biological and molecular characterization of clinical isolates (including antibiotic resistance studies); however, its use as a routine diagnostic tool is suboptimal.

PCR

Over the last 20 years, NAATs have become a major tool for the detection of micro-organisms, for diagnostic testing, and for research purposes in the field of infectious diseases. NAATs offer significant sensitivity and speed compared to culture and do not require the presence of viable organisms. Diagnostic testing for micro-organisms based on NAATs has become increasingly complex and the field is changing and expanding rapidly. Thus, an NAAT established 10 years ago and designed with the best information and knowledge available at that time may not necessarily be state-of-the-art today.

Validated commercially available FDA-cleared assays exist only for a limited number of organisms and not for the detection of *M. pneumoniae* in respiratory or other specimens. There are also a number of so-called analyte-specific reagents commercially available. Besides these standardized kits, the use of NAATs for research purposes kits and in-house developed NAATs has expanded tremendously. The assays range from those that are well validated to those that are not. Carefully reading many of these publications reveals that, often, little or no information is provided on the validation of the NAATs applied. Yet, these assays are frequently used and cited in the literature.

At the time of the 2003 minireview [1], there were 34 published in-house NAATs for the detection of *M. pneumoniae* DNA or RNA. However, validation was primarily analytical; none of these assays were extensively evaluated using clinical specimens from well-defined patient populations from a wide geographic area. Since then, an additional 27 assays have been described (Tables 3 and 4). There is a great variation of the methods used from study to study, including variability of target (P1 gene, 16S rRNA, ATPase gene, *parE* gene, *tuf* gene; monoplex versus multiplex targets) and of NAAT (conventional, nested, and real-time; RNA vs. DNA targets; and PCR and nucleic acid sequence-based amplification technologies) and detection formats (agarose gel electrophoresis, SYBR green, TaqMan probe, hybridization probes, molecular beacons, and microchip electrophoresis). Furthermore, there is no consensus on the optimal respiratory specimen to be used for *M. pneumoniae* detection by nucleic acid amplification tests and culture. Different specimens have been used, such as sputum, nasopharyngeal, or oropharyngeal swabs or washes; bronchoalveolar lavage; or pleural fluid. In a review on optimal sampling for the detection of respiratory pathogens, Loens et al. concluded that, if sputum is available, it might be the best specimen for *M. pneumoniae* detection by culture and NAATs. A nasopharyngeal swab, nasopharyngeal aspirate, or oropharyngeal swab might be the second best option for analysis by NAATs [50].

Interstudy variation is related to the reference diagnostic assay with which the new assay is compared (a serological test, culture, or a pre-existing PCR assay). Due to the lack of conformity between different studies, it is very difficult to compare the data from study to study.

Winchell et al. [51] evaluated three real-time PCR assays targeting the ATPase gene and newly described CARDS toxin genes during an *M. pneumoniae* outbreak. A total of 54 respiratory specimens from patients (n=35) and controls (n=19) were tested in triplicate with each PCR assay. The assay targeting the CARDS toxin gene proved to be the most sensitive (lower ct-values) in identifying positive

Table 3 Summary of recent mono polymerase chain reaction (PCR) assays for the detection of *M. pneumoniae* published since 2003 and previously validated assays used as comparators

Reference	Assay type	Detection format	Target gene (bp)	PCR assay used as a comparator for the new assay	Non-PCR comparator test	Specimens tested for the validation of sensitivity and/or specificity
2003 [71]	PCR	Molecular beacons	P1 gene (151)	[72, 73]	Serology	Various bacterial species, DNA dilutions, clinical specimens
2004 [70]	PCR	Agarose gel electrophoresis	16S rRNA gene (225)	ND	Culture, serology	*M. pneumoniae* dilution series
2004 [74]	Broad-range PCR	Microarray	*parE* gene (±300)	ND	ND	Various bacterial species, DNA dilutions, clinical specimens
2004 [60]	Nested PCR	Reverse line blot hybridization	16S-23S rRNA spacer (94)	ND	ND	21 Mollicute reference strains, 92 contaminated cell cultures, 80 Mollicute isolates, 14 *M. pneumoniae*-positive NPAs, 6 *M. pneumoniae*-negative specimens Pathogens targeted: *M. arginini*, *M. fermentans*, *M. hyorhinis*, *M. orale*, *Acholeplasma laidlawii*, *M. pneumoniae*, *M. hominis*, *M. genitalium*, *Ureaplasma parvum*, *U. urealyticum*
2005 [75]	PCR	Real-time	16S RNA gene (NS)	ND	Culture	Spiked sputa and BALs, clinical specimens with known *M. pneumoniae* status by culture
2005 [76]	LAMP	Turbidimeter	P1 gene (NS)	[77]	ND	Various bacterial species, DNA dilutions, clinical specimens
2006 [78]	PCR	Molecular beacon	16S rRNA gene (225)	ND	Culture, serology	Various bacterial species, bacterial dilution series, clinical specimens
2006 [79]	PCR	Real-time	P1 gene (141)	ND	Culture, serology	Various bacterial strains, dilutions of cloned DNA, clinical specimens with known *M. pneumoniae* status by culture and serology
2007 [80]	PCR	Scorpion probe	P1 gene (72)	[81, 82]	Serology	Various bacterial strains, dilutions of *M. pneumoniae* DNA, clinical specimens with unknown status
2007 [83]	PCR	Real-time	repMp1 in P1 (184)	P1 gene-based PCR (177 bp)	ND	Various bacterial species, dilutions of plasmids containing target sequence, clinical specimens with known *M. pneumoniae* status by PCR
2008 [51]	PCR	Real-time	CARDS toxingene (73) ATPase gene(68) ATPase gene(106)	ND	ND	Various bacterial species, bacterial dilution series, clinical specimens from an outbreak
2009 [59]	Broad-range PCR	Real-time	*tuf* gene (160)	VenorGeM-DI Mycoplasma detection kit, MycoSensor QPCR Assay kit	ND	32 Mollicute species, various other bacterial species, cell culture supernatants, clinical specimens (80sputa, 5 throat swabs)

A-test: passive agglutination-test; LAMP: loop-mediated isothermal amplification; ND: not done; MycoSensor QPCR Assay (Stratagene, LaJolla, CA, USA); VenorGeM-DI Mycoplasma detection kit (Minerva Biolabs GmbH, Berlin, Germany)

Table 4 Summary of recent multiplex PCR assays for the detection of *M. pneumoniae* published since 2003 and previously validated assays used as comparators

Reference	Assay type	Detection format	Target gene (bp)	PCR assay used as a comparator for the new assay	Non-PCR comparator test	Specimens tested for the validation of sensitivity and/or specificity
2004 [84]	MX-PCR	Microchip electrophoresis	16S rRNA gene(88)	Mono-assay	Serology	*M. pneumoniae* dilution series, various bacterial species, clinical specimens Pathogens targeted: *M. pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila*
2005 [85]	Mass Tag MX-PCR	Masscode Tag	NS	ND	ND	DNA dilutions, *M. pneumoniae*-negative clinical specimens Pathogens targeted: influenza A and B, RSV A and B, metapneumovirus, SARS, coronavirus OC43 and 229E, parainfluenza 1-3, *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila*, enterovirus, adenovirus
2005 [57]	MX-PCR Chlamylege	Hybridization	P1 gene (298)	[86]	Serology	Various bacterial species, bacterial dilutions, clinical specimens with known status, clinical specimens with unknown status Pathogens targeted: *C. pneumoniae*, *M. pneumoniae*, *Legionella* spp.
2005 [56]	MX-PCR Pneumoplex	Real-time	16S rRNA gene	NS	ND	Various bacterial species, dilutions of recombinant DNA, dilutions of organisms, spiked BALs Pathogens targeted: *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *L. micdadei*, *Bordetella pertussis*
2005 [87]	MX-PCR	Agarose gel electrophoresis	P1 gene (360)	[72]	ND	Various bacterial specimens, dilutions of organisms, clinical specimens with known *M. pneumoniae* status by PCR Pathogens targeted: *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*
2005 [88]	MX-PCR	Agarose gelelectrophoresis	P1 gene (483)	ND	ND	Various bacterial species, serial dilutions of DNA, clinical respiratory specimens Pathogens targeted: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *M. pneumoniae*, *C. pneumoniae*
2007 [89]	MX-PCR	Agarose gelelectrophoresis	P1 gene (225)	*M.pneumoniae* OligoDetect PCR kit, P1-based in-house PCR	ND	Extracts from samples known to be positive for some common respiratory bacterial pathogens, clinical specimens from children with RTIs Pathogens targeted: *M. pneumoniae*, *C. pneumoniae*, *B. pertussis*, *B. parapertussis*
2007 [90]	Nested MX-PCR	Agarose gel electrophoresis	P1 gene (343, 160)	ND	ND	DNA dilutions, clinical specimens Pathogens targeted: *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, adenovirus
2007 [55]	MX-PCR	Resequencing microarray	NS	ND	ND	Various bacterial species, *M. pneumoniae* cells, DNA dilutions, archived throat swabs Pathogens targeted: adenovirus, *Bacillus anthracis*, *C. pneumoniae*, influenza virus A and B, *Francisella tularensis*, coronavirus 229 E and OC43, rhinovirus, Lassa virus, *M. pneumoniae*, parainfluenza virus 1, 3, RSV A and B, *S. pneumoniae*, *S. pyogenes*, vaccinia virus, *Yersinia pestis*, Ebola virus, Variola major virus

2007 [91]	MX-PCR	Microarray with electrochemical detection	*dnaK* gene (654), *pdhA* gene (284), *tuf* gene (604)	ND	ND	Various bacterial and viral species, dilution series The *pdhA* primers and probes were found to be the most sensitive combination Pathogens targeted: *B. pertussis*, *S. pyogenes*, *C. pneumoniae*, *M. pneumoniae*, adenovirus, coronavirus OC43, 229E, and HK, influenza A and B, parainfluenza types 1, 2, and 3, RSV
2008 [58]	MX-PCR ResPlex	Luminex technology	ATPase (NS)	NS	ND	*M. pneumoniae* type I and II , >110 strains of other bacterial species found in the respiratory tract or related spp., 10-fold serial DNA dilutions, 49 NPS with known *M. pneumoniae* status by PCR Pathogens targeted: *S. pneumoniae*, *Neisseria meningitidis*, *H. influenzae*, *L. pneumophila*, *M. pneumoniae*, *C. pneumoniae*
2008 [92]	MX-PCR	Molecular beacons	P1 gene (158)	[93]	ND	Reference strains of common bacterial respiratory pathogens or related species, serial dilutions of DNA, spiked samples, samples with known status by PCR Pathogens targeted: *M. pneumoniae* and *C. pneumoniae*
2008 [94]	MX NASBA	Molecular beacons	16S rRNA	[81, 73]	Culture, serology	Various bacterial species, bacterial dilutions, dilutions of wild-type *M. pneumoniae* 16S rRNA generated in vitro, spiked specimens, specimens with known status by PCR Pathogens targeted: *M. pneumoniae*, *C. pneumoniae*, *Legionella* spp.
2008 [95]	MX-PCR	Reverse line blot hybridization	16S-23S rRNA spacer (93)	[60]	ND	12 reference strains and 63 clinical isolates of common bacterial respiratory pathogens, 10-fold serial DNA dilutions, 100 NPAs from children with CAP Pathogens targeted: *Staphylococcus aureus*, *S. pneumoniae*, *S. pyogenes*, *Moraxella catarrhalis*, *H. influenzae*, *B. pertussis*, *Klebsiella pneumoniae*, *L. pneumophila*, *Mycobacterium tuberculosis*, *C. pneumoniae*, *M. pneumoniae*
2008 [96]	MX-PCR	Enzyme hybridization or electronic microarray detection	P1 gene (299)	ND	ND	Various bacterial and viral species, bacterial dilutions, spiked clinical specimens, clinical specimens from carriage study, re-analysis of samples with discrepant results Pathogens targeted: influenza virus A, influenza virus B, RSV A and B, *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *L. micdadei*, *B. pertussis*, *S. aureus*, *S. pneumoniae*

A-test: passive agglutination-test; MX-PCR: multiplex PCR; NASBA: nucleic acid sequence-based amplification; NPA: nasopharyngeal aspirate; NS: not specified; Pneumoplex (GenProbe Prodesse Inc., Waukesha, WI, USA); ResPlex (Qiagen GmbH, Valencia,CA, USA); *M. pneumoniae* OligoDetect PCR kit (Millipore/Chemicon, Eugene, OR, USA)

specimens. The analytical sensitivity of this assay was between 1–5 CFU, whereas it was between 5–50 CFU for the other two assays. However, the authors concluded that the inclusion of a second PCR assay may provide an increased level of confidence for the reporting of results. Dumke and Jacobs [52] compared under standardized conditions the performance of two commercial PCR assays (Artus RepMp1 [QIAGEN GmbH, Hilden, Germany] and the Venor Mp-QP *M. pneumoniae* kit [Minerva Biolabs GmbH, Berlin, Germany] and three in-house PCR assays for the detection of *M. pneumoniae* on the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). All five procedures were able to demonstrate *M. pneumoniae* DNA in a concentration comparable to 1 CFU/µl, but the differences in the mean crossing points between the tested procedures (up to 4.6) caused differences of the calculated mean concentration of the genome equivalents by a factor of up to 20.

Multicenter studies that use a large and geographically diverse repertoire of clinical specimens and compare data from >2 centers independently are likely to provide important insights into the performance of new assays. To date, only two such studies describing multicenter comparisons of the performance of various NAATs for the detection of *M. pneumoniae* in respiratory specimens have been published, and both studies revealed significant variations of test performance from laboratory to laboratory [2, 53]. Ursi et al. collected a panel of 78 respiratory samples from 43 patients which were analyzed in three different centers for the presence of *M. pneumoniae* DNA by different PCR assays [53]. Nucleic acids were extracted at one site and subsequently amplified in three centers. Loens et al. [2] used spiked respiratory specimens to compare the performance of several NAATs being used by 18 laboratories, each with their own extraction and amplification protocols. Both of these studies revealed significant intercenter discordance of detection rates, using different or even the same tests, despite the fact that the laboratories participating were very experienced with the use of PCR assays. In the spring of 2008, a pilot panel for the molecular diagnosis of *M. pneumoniae* was produced by Quality Control for Molecular Diagnostics (QCMD). An external quality assessment (EQA) panel consisting of a total of 13 samples in bronchoalveolar lavage (BAL) or transport medium were prepared to assess the proficiency of laboratories in the correct detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* by NAATs (six samples containing various concentrations [4.9–490 inclusion forming units (IFU)/ml] of *C. pneumoniae*, five samples containing various concentrations [20–5,000 color changing units (CCU)/ml] of *M. pneumoniae*, and two samples negative for both) [54]. Seventy-nine laboratories from 18 countries participated in this EQA study. Sixty-seven datasets were obtained for *M. pneumoniae* (n=5 conventional commercial, n=10 conventional in-house, n=4 real-time commercial, n=46 real-time in-house, n=2 strand displacement amplification [SDA]). For the total panel, correct results per sample varied between 53.7 and 95.5% for *M. pneumoniae*.

Respiratory viruses and other so-called "atypical bacteria" are all responsible for RTIs that may produce clinically similar manifestations. In order to reduce costs and hands-on time, multiplex nucleic acid amplification techniques (MX-NAATs) have been developed (Table 4). Originally only two or three organisms were targeted in one assay. Currently, some assays detect up to 22 targets [55]. However, comparison between monoplex and multiplex assays has been rarely performed. Findings and conclusions result frequently in contradictory and conflicting data concerning the sensitivity and specificity of the MX-NAATs compared to the monoplex NAAT. Owing to the complexity of the variables in a multiplex PCR, including different combinations of primer concentrations, magnesium ion concentrations, and annealing temperatures, this is not unexpected. The results of MX-NAATs on proficiency panels [2] seem to confirm that multiplex assays are somewhat less sensitive than monoplex assays, but until the number of organisms present in the clinical specimens of diseased individuals is known, it is impossible to state whether the degree of sensitivity attained is clinically acceptable.

It has been proposed that industry-produced assays in kit form may enable standardization. The Pneumoplex assay (Prodesse Inc.) (Table 4) was reported to have 100% sensitivity (when the sample contained 5 CFU/ml of *M. pneumoniae*) and 96% specificity for the detection of *M. pneumoniae* in spiked specimens [56]. The sensitivity of the Chlamylege assay (Argene Inc.) (Table 4) was 5.10^{-2} CCU per reaction tube for *M. pneumoniae* [57]. A cohort of 154 clinical samples from patients with documented respiratory infections was analyzed by the same kit, including two samples from patients with *C. pneumoniae* infection, nine samples from patients with *M. pneumoniae* infection, 19 samples from patients with *Legionella* species infection, and 114 samples that tested negative for the three pathogens. All of the positive specimens were correctly detected and identified by the Chlamylege kit, and no false-positive result was observed with the negative samples. The kit was then evaluated in a pediatric prospective study that included 220 endotracheal aspirates, and the results were compared with those obtained by three monoplex in-house PCR assays. Six specimens were found to be positive for *M. pneumoniae* by using both strategies. The Chlamylege kit detected two additional samples positive for *M. pneumoniae*. A comparative analysis of the limits of detection of the ResPlex I assay (Table 4) and real-time single PCR assays

demonstrated that the ResPlex I assay is 10-fold less sensitive in detecting *M. pneumoniae* [58]. Furthermore, the ResPlex I assay was performed on 49 nasopharyngeal swab specimens known to be positive by real-time PCR for three pathogens (*C. pneumoniae*, *M. pneumoniae*, and *S. pneumoniae*) and detected 50, 59, and 81% of the *C. pneumoniae*-, *M. pneumoniae*-, and *S. pneumoniae*-positive samples, respectively. However, since the calculation of the sensitivities of the industry-produced multiplex assays was mainly dependent on the DNA copy number, further evaluation and standardization using an extended number of clinical specimens that may have a low load of the organism are needed.

There is also more and more a trend of adapting commercially available (e.g., MicroSeq Mycoplasma, Applied Biosystems) or in-house PCR assays for screening cell cultures for the presence of Mollicutes to respiratory specimens for the specific or generic detection of *M. pneumoniae* [59, 60]. These assays need to be extensively validated in terms of sensitivity and specificity as well before they can be applied to clinical respiratory specimens.

Ideally, a newly proposed NAAT assay should be validated by comparison with a sensitive culture system and at least one validated PCR or another NAAT assay that targets a different gene or a different sequence of the same gene.

Conventional manual nucleic acid extraction for the isolation of pathogen DNA or RNA from clinical samples is the most labor-intensive and critical part in current nucleic acid diagnostic assays. Automated nucleic acid extraction systems with high flexibilities in the type and numbers of samples to be handled, and with a wide range of sample input and elution volumes and short turnaround time will improve the application of NAATs to clinical services. Data from the literature indicate that the sensitivity of an NAAT after nucleic acid extraction with an automated system is similar to or better than the sensitivity after manual nucleic acid extraction. When the easyMAG nucleic acid extractor (bioMérieux) was applied retrospectively to clinical specimens, better amplification results were obtained for *M. pneumoniae* and *C. pneumoniae* detection compared with manual methods such as the Qiagen blood mini kit and the NucliSens miniMAG platform [61, 62]. This and other automated nucleic acid extraction instruments need to be further evaluated.

Antibiotic resistance

Since in 2001 a report was published describing the first macrolide-resistant *M. pneumoniae* strain possessing a 23S rRNA gene mutation [63], other reports followed [64–67]. Although most macrolide-resistant strains were detected in Japan so far, the first macrolide-resistant *M. pneumoniae* strains in Europe were reported recently in France [68]. Since the impact of macrolide resistance on the outcome of the infections is not clear so far, measures need to be taken to identify these strains and PCR assays have been developed to detect some of these mutations [64, 69]. Both assays target the V-domain of the 23S rRNA gene; the presence of point mutations in the amplicons is detected by using restriction fragment length polymorphism (RFLP) [64] or high-resolution melt curve analysis [69].

Conclusions and future directions

During recent years, significant progress has been made in the microbiological diagnosis of *Mycoplasma pneumoniae* respiratory tract infections (RTIs). Despite these many efforts, much is still unknown about the role of *M. pneumoniae* in respiratory and other infections. Most *Mycoplasma* infections never have a microbiological diagnosis because rapid, sensitive, and specific methods for its direct detection are not readily available in physician offices or hospital laboratories.

Significant limitations continue to surround the accurate and reliable serological diagnosis of *M. pneumoniae* infection. These include the wide variation of the methods and diagnostic criteria used across studies, which results in the subsequent emergence of data that remain incomparable and often controversial because of discordant and, at times, incorrect methodologies used.

Numerous in-house PCR assays to detect *M. pneumoniae* have been developed. Proper validation and standardization are still often lacking, and quality control studies have revealed frequent deficiencies, resulting in both false-negative and false-positive results [2, 54]. Consequently, all newly developed tests must be submitted to extensive validation before their introduction in the molecular diagnostic laboratory. Validation must be performed at several levels, including sample preparation, amplification, and detection. Since respiratory samples often contain substances inhibiting amplification, special attention should be paid to the efficiency of the reaction with these samples. Once a test is validated, it should be further evaluated in proficiency testing programs. Whereas quality control is an essential part of quality assurance in molecular diagnostics, proficiency panels for the detection of *M. pneumoniae* are not readily available. They are urgently needed to allow meaningful comparisons between the results obtained in different laboratories.

In conclusion, the following standards should be followed by all laboratories when validating new tests for the detection of *M. pneumoniae*: (i) having the ability to apply a second PCR at another target to at least some cases in epidemic situations; (ii) performing proficiency testing regularly in a blind fashion to ensure proper test and

personnel performance; and (iii) incorporating hierarchical acceptance criteria monitoring for test failures, including positive and negative controls, re-evaluating multiple consecutive positive specimens, and confirming that positive specimens were collected from persons meeting the clinical case definition. Furthermore, multicenter studies that use a large and geographically diverse repertoire of clinical specimens and compare data from >2 centers independently are likely to provide important insights into the performance of new assays.

Given the high sensitivity and specificity of nucleic acidamplification techniques (NAATs), NAATs are the preferred diagnostic procedures for the diagnosis of *M. pneumoniae* infections, provided that the quality of the procedures is controlled. Additional prospective multicenter studies on large numbers of patients with respiratory signs and symptoms, including hospitalized and non-hospitalized patients, are necessary to extend our knowledge on the epidemiology of *M. pneumoniae*.

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