REVIEW

Laboratory diagnosis of *Mycoplasma pneumoniae* infection

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Diagnosis of *Mycoplasma pneumoniae* infection is challenging due to the fastidious nature of the pathogen, the considerable seroprevalence, and the possibility of transient asymptomatic carriage. During recent years, various new techniques have been adapted for the diagnosis of *M. pneumoniae* infection, notably in the field of molecular biology. Standard polymerase chain reaction (PCR) is currently the method of choice for direct pathogen detection, but several PCR-related methods provide enhanced sensitivity or more convenient handling procedures, and have been successfully applied for research purposes. Among these techniques are real-time PCR, nested PCR, reverse transcriptase PCR (RT-PCR) and multiplex PCR. Generally, amplification-based methods have replaced hybridization assays and direct antigen detection. Serology, which is the basic strategy for mycoplasma diagnosis in routine clinical practice, has been improved by the widespread availability of sensitive assays for separate detection of different antibody classes. For the diagnosis of mycoplasma pneumonia, serology and direct pathogen detection should be combined. Extrapulmonary diseases may be diagnosed by direct pathogen detection alone, but the value of this diagnostic approach is limited by the probably immunologically mediated pathogenesis of some manifestations. This review summarizes the current state of *Mycoplasma pneumoniae* diagnosis, with special reference to molecular techniques. The value of different methods for routine diagnosis and research purposes is discussed.

Keywords *Mycoplasma pneumoniae*, laboratory diagnosis, serology, mycoplasma isolation, molecular methods, extrapulmonary manifestations

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INTRODUCTION

In most studies, 10–30% of cases of community-acquired pneumonia are due to *Mycoplasma pneumoniae* [1,2]. These cases represent less than 10% of *M. pneumoniae* infections. Most patients develop tracheobronchitis or upper respiratory tract disease, and about 15% of infections remain asymptomatic [3].

M. pneumoniae pneumonia is classically referred to as atypical pneumonia, presenting as an illness of gradual onset associated with headache, myalgias, sore throat, and, initially, dry cough [4,5]. The leukocyte count is often normal or only moderately elevated [5,6]. However, although these signs and symptoms are often associated with mycoplasma pneumonia, they are not necessarily of diagnostic value in a given case [5]. Recent work has confirmed that clinical and laboratory findings are insufficient to distinguish between mycoplasma pneumonia and pneumonia caused by other pathogens [1,7,8]. Therefore, correct etiologic diagnosis strongly depends on laboratory diagnosis.

M. pneumoniae pneumonia is most prevalent in children and young adults [3,9], and young children show the highest attack rates [10]. In contrast, the agent is rarely observed in the elderly [11]. Epidemics of *M. pneumoniae* infection occur every 3–7 years [3]. It is noteworthy that several studies

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have found a low incidence of mycoplasma pneumonia in immunocompromised patients [12,13]. However, some reports on cases of severe mycoplasma pneumonia in patients with impaired immune function have been published [14–16]. To date, no individual risk factors for *M. pneumoniae* pneumonia have been found. The usefulness of mycoplasma diagnosis is therefore not restricted to defined groups of patients. However, patients with clinically severe pneumonia, notably after treatment failures with β-lactam antibiotics, are most likely to profit from comprehensive mycoplasma diagnosis and subsequent adequate antimicrobial therapy.

M. pneumoniae pneumonia is usually characterized by a benign outcome, even in cases without adequate antimicrobial therapy. The beneficial effect of adequate treatment in terms of shortening the course of the illness has been demonstrated [7,17], but discordant findings have also been published [18]. It is the current conventional opinion that adequate treatment is indicated in microbiologically proven cases of *M. pneumoniae* pneumonia [19]. Current therapeutic options notably include macrolides, doxycycline and newer quinolones, e.g. levofloxacin and moxifloxacin, whereas the pathogen displays primary resistance to antimicrobial agents acting on the cell wall, e.g. β-lactam antibiotics.

Extrapulmonary manifestations of *M. pneumoniae* infection are less frequent than respiratory tract disease, but often life-threatening. Neurologic and cardiac involvement are observed in 6–7% and up to 5% of hospitalized patients, respectively [20]. Furthermore, joints and red blood cells may be affected [21,22]. This review summarizes the current state of *M. pneumoniae* diagnosis, with an emphasis on molecular approaches. The value of different methods for routine diagnosis and research purposes is discussed.

SEROLOGY

Serology is an important tool for the diagnosis of *M. pneumoniae* infection. The predominant role of serology in routine diagnosis is partly due to the ease of specimen collection and the widespread availability of serologic tests. However, also with regard to the definite proof of a causative role of *M. pneumoniae* in a given episode of pneumonia, serology is far from being replaced by direct pathogen detection.

Before the availability of more advanced serologic techniques, detection of cold agglutinins was considered a valuable tool for *M. pneumoniae* diagnosis. The formation of cold agglutinins is the first humoral response to *M. pneumoniae* [23]. Within 6 weeks after infection, the cold agglutinin titer declines towards the pre-existing level. Determination of these autoantibodies is fast and simple to perform. However, cold agglutinins are not very reliable indicators of *M. pneumoniae* infection, as they are elevated in only 50–60% of patients [24,25]. Furthermore, they are also induced by various other infectious agents, e.g. Epstein–Barr virus, cytomegalovirus and *Klebsiella pneumoniae*, as well as in the course of malignancies of lymphoid cells and autoimmune diseases [26].

Until a decade ago, the complement fixation (CF) test was considered standard in *M. pneumoniae* serology. However, the lack of both sensitivity and specificity of the CF test has been described extensively [27,28]. The antigen used in this test is a chloroform–methanol glycolipid extract of *M. pneumoniae*, which may undergo cross-reactions with human, bacterial and plant epitopes. For example, a significant rise in antibody titer has been observed in patients with bacterial meningitis, while no increase in specific antibodies could be demonstrated by immunoblotting [29]. For metabolic inhibition tests, which have also been replaced by newer techniques, the same glycolipid antigen was used. A more advanced alternative to the CF test is the microparticle agglutination assay (MAG) Serodia Myco II (Fujirebio, Tokyo, Japan) [30]. The principle of this test is hemagglutination by specific antibodies to *M. pneumoniae*. Erythrocytes are replaced by latex particles to avoid nonspecific reactions.

However, neither the CF test nor the MAG enable differentiation between the antibody classes, which may lead to a delay in diagnosis. The level of specific IgG antibodies increases slowly in the course of the illness, reaching peak titers 5 weeks after the onset of clinical symptoms. Usually, no measurable IgG response is observed during the first week of the illness [31]. After infection, these antibodies remain elevated for up to 4 years [3]. Low, but detectable, levels of IgG antibodies may therefore indicate either the early stage of acute infection or a past illness. In case of a low level of specific IgG, a second sample must be examined after an interval of 2 or 3 weeks in order to demonstrate a significant rise of

antibody titer. As the CF test and MAG are subjective assays, at least a four-fold increase of the titer is required to establish a diagnosis. When there is a highly elevated titer as measured by the CF test ($>1:80$) or MAG ($>1:160$), diagnosis can be made on the basis of a single antibody determination.

In order to achieve a rapid diagnosis, separate detection of IgM or IgA antibodies is helpful. IgM antibodies appear during the first week of the illness, and reach peak titers during the third week [31]. They decline towards low levels (below the cut-off value of commercial assays) within a few months. A major disadvantage of IgM-based diagnosis is that these antibodies are not constantly produced in adults, most likely as a result of multiple previous infections [32]. Therefore, a negative IgM result does not rule out acute *M. pneumoniae* infection in the elderly. In contrast, measurement of IgM has been shown to be useful in pediatric patients [33]. Recent studies have suggested that the determination of specific IgA provides superior diagnostic accuracy [34]. These antibodies are also produced early in the course of the disease, but their formation takes place more reliably. Assays for IgA and IgM determination are mostly based on the ELISA principle. A variety of test kits is available from different companies. For IgM determination, a rapid card-based assay has also been developed (ImmunoCard *Mycoplasma*, Meridian, Cincinatti, OH, USA) [35].

A main requirement with regard to the specificity of these ELISA tests is to avoid possible cross-reactions with antibodies to *Mycoplasma genitalium*, the species most closely related to *M. pneumoniae*. The MgPa protein of *M. genitalium* (140 kDa) shows a high degree of similarity with the P1 protein of *M. pneumoniae* (169 kDa) [36]. Both proteins are important for cytoadherence, and the P1 protein is also the most potent immunogenic determinant of *M. pneumoniae* [37]. Elevated levels of antibody to *M. genitalium* mostly result from urogenital infection, whereas the role of this pathogen in respiratory tract diseases is not entirely elucidated [38]. To overcome non-specific reactions, short synthetic peptides may be used instead of whole protein preparations [23]. A change in antigenic pattern, e.g. antigenic variation (sequential expression of different epitopes during the same infectious episode to evade the host's immune response), which has been shown for several *Mycoplasma* species, does not hinder the serologic diagnosis of *M. pneumoniae* infection [37].

A Western immunoblot technique for *M. pneumoniae* has been recently developed and is commercially available (Virotech, Rüsselsheim, Germany). This method is currently the most specific technique for detection of anti-*M. pneumoniae* antibodies. Moreover, it enables the detection of lower antibody levels than other assays.

The detection of specific antibodies in cerebrospinal fluid (CSF) provides additional information in suspected cases of mycoplasma central nervous system disease. Recent work has shown that there may be intrathecal synthesis of at least IgG and IgM antibodies to *M. pneumoniae* [39]. However, even PCR and culture-proven cases without detection of specific antibodies in CSF have been described [40,41]. These findings are in favor of the hypothesis that high levels of antibodies effectively hinder the growth of the organism.

CULTURE TECHNIQUES

For scientific purposes, *M. pneumoniae* culture is mostly performed with cell-free formulations such as Hayflick's, New York City, Soy Peptone or SP-4 medium [42,43]. The last formulation, which had initially been developed for the isolation of *Spiroplasma* spp., is most effective. In order to avoid overgrowth by other pathogens, a broad-spectrum β-lactam antibiotic and an antifungal agent should be added, e.g. penicillin G (100 000 U/mL) and amphotericin B (0.5 mg/mL). There is also a commercially available system for *M. pneumoniae* cultivation (Pneumofast, International Microbic Signes, France). When isolation is to be performed, suitable transport of the clinical specimens to the laboratory is crucial. Most importantly, mycoplasmas are vulnerable to desiccation. In particular, swabs should be kept in an appropriate transport medium, e.g. trypticase soy broth with 0.5% bovine serum albumin. The transport time should be less than 4 h. However, if transport requires more time, viability of the mycoplasmas can be maintained by keeping the specimens at a temperature of about 4 °C. When samples containing concomitant physiologic flora are sent for mycoplasma culture (e.g. respiratory specimens), a broad-spectrum antibiotic should be added to the transport medium.

Isolation of *M. pneumoniae* is slow and insensitive, and therefore not recommended for routine

diagnosis. The difficulty in culturing *Mycoplasma* is due to the extensive nutritive requirements of the pathogen. Like all mycoplasmas, *M. pneumoniae* contains only a small set of enzymes. Complete sequencing of the *M. pneumoniae* genome has revealed that this pathogen possesses 677 predicted protein-coding sequences, compared to approximately 4000 in *Escherichia coli* [44]. *M. pneumoniae* is not capable of amino acid biosynthesis. Also, most cofactors and fatty acids, as well as nucleic acid precursors, must be acquired from the environment.

The required length of incubation strongly depends on the initial inoculum, with a range from 4 days to several weeks. In broth cultures, the growth of *M. pneumoniae* is indicated by phenol red. Acid production as a consequence of glucose utilization by the bacteria leads to a yellowing of the broth. On agar plates, the small colonies of *M. pneumoniae* show a typical granulated appearance. However, differentiation of *M. pneumoniae* from other mycoplasmas on the basis of colony morphology alone is not possible. The presence of *M. genitalium*, which shows a similar colony morphology, may lead to misleading interpretations [45]. Therefore, the use of thallium acetate, which selectively hinders the growth of *M. genitalium*, has been previously recommended, but this substance should be avoided because of its toxicity. On the other hand, *M. genitalium* and *M. pneumoniae* show the same susceptibility to antimicrobial agents [46]. Mixtures of these two species may be observed in urogenital, respiratory and synovial fluid samples.

To assess the sensitivity of isolation or direct pathogen detection, the number of mycoplasmas is expressed in terms of colony-forming units (CFUs), color-changing units (CCUs), or copy numbers. One CFU is estimated to contain 160 bacterial cells according to Harris et al., and 10–1000 cells according to Razin [47,48]. One CCU is estimated to contain 10–100 organisms. Between 10^2 and 10^4 CCU/mL are present in respiratory secretions during acute infection [49].

Approximately 10^5 CFU/mL can be detected by culture methods. This is roughly three log dilutions less sensitive than PCR. When resolved by PCR, the sensitivity of *M. pneumoniae* isolation from respiratory specimens has turned out to be as low as 61% [50]. The sensitivity, and also the speed of mycoplasma growth, are enhanced by cell-sheet culture systems, e.g. using HeLa 229 cells [51]. If this method is applied, the growth of *M. pneumoniae* can be determined after 5 days of incubation, by immunofluorescence (cells), PCR or antigen detection (fluid phase) [52]. The sensitivity of isolation on agar plates can be increased by using diphasic media. Culture-enhanced PCR is useful to remove inhibitors of the DNA polymerase, thus avoiding a significant loss in sensitivity [52]. For this purpose, clinical samples, e.g. throat swabs, are incubated in broth culture media overnight before DNA extraction.

Despite the low sensitivity of *M. pneumoniae* culture, the isolation of the pathogen has led to some insights into the pathogenesis of extrapulmonary manifestations, because successful isolation provides evidence of direct invasion by viable mycoplasmas. A similar conclusion cannot necessarily be drawn from positive PCR results, because target DNA may persist for some time after the death of the bacteria. Furthermore, it has been assumed that *Mycoplasma* DNA may be carried to extrapulmonary sites by antigen-presenting cells [53]. Considerable interest has been focused on the possible invasion of the central nervous system by *M. pneumoniae*, which had initially been demonstrated by culture techniques [41,54]. Other non-respiratory sites where *M. pneumoniae* has been detected are summarized in Table 1.

DIRECT ANTIGEN DETECTION AND NUCLEIC ACID HYBRIDIZATION

Assays for direct antigen detection have been largely replaced by amplification-based techniques. These assays provide a sensitivity of approximately 10^4 CFU/mL [51]. ELISA kits for direct antigen detection are commercially available (Virion, Rüschlikon, Switzerland). This diagnostic approach, although not recommended for diagnosis of extrapulmonary manifestations, is a convenient alternative for detection of *M. pneumoniae* in respiratory secretions if no PCR equipment is available. Monoclonal antibodies for direct staining of the pathogen have been elaborated, but this method has poor sensitivity [55]. However, by immune peroxidase staining, the presence of mycoplasma antigen in renal tissue has been demonstrated in an isolated case [56].

Hybridization with specific probes was the first molecular application in *M. pneumoniae* diagnosis. Hybridization assays provide a similar sensitivity to ELISA tests for antigen detection. The GenProbe

Table 1 Detection of *M. pneumoniae* in clinical samples outside the respiratory tract

Type of specimen	Isolation	PCR	Other method	Reference
Cerebrospinal fluid	Yes	Yes	–	[52,54]
Brain parenchyma	No	Not done	DNA/RNA hybridization	[58]
Pericardial fluid	Yes	Not done	–	[76]
Whole blood	Yes	Yes	–	[81,82]
Peripheral blood mononuclear cells	Not done	No	–	[83]
Coronary atherectomy specimens	Not done	Yes	–	[84]
Renal tissue	Not done	Not done	Immunoperoxidase staining	[56]
Urogenital specimens	Yes	Yes	–	[85,86]
Synovial fluid	Yes	No	–	[21]
Bone (osteomyelitis)	Not done	Not done	PCR (eubacterial 16S rDNA) + sequencing	[73]
Skin (Stevens–Johnson syndrome)	Yes	Not done	–	[87]

Rapid System (GenProbe, San Diego, CA, USA), which involves a ^{125}I-labeled DNA probe to an rRNA sequence specific for *M. pneumoniae*, was widely used before the widespread availability of PCR. Evaluation of this assay showed high degrees of sensitivity (0.95) and specificity (0.85) for mycoplasma detection in sputum samples when resolved by culture and serology. However, this assay is of limited value for mycoplasma detection in throat swabs [57]. With the General-Probe Rapid System, *M. pneumoniae* was detected for the first time in the brain parenchyma, namely in a child with lethal mycoplasma encephalitis [58]. Hybridization assays have also been largely replaced by PCR and related methods. This is due to the higher sensitivity of amplification-based assays, and also to inconvenient handling procedures if radioactively labeled probes are involved.

NUCLEIC ACID AMPLIFICATION

PCR was first applied for *M. pneumoniae* diagnosis in 1989 by Bernet et al. [59]. The first set of primers was chosen from the ATPase operon gene, and enabled the amplification of a 144-bp fragment. Several additional primer sequences targeting the genes coding for the P1 protein [52,60,61] and the 16S rRNA [62] have now been constructed (Table 2). Although the latter gene is highly conserved, regions V2 and V3 of the 16S rDNA display sufficient interspecies differences for specific detection of *M. pneumoniae* [63]. Cross-reactions are most likely to occur with the *M. genitalium* genome. The 16S rDNA also enables PCR at the genus level, e.g. detection of most *Mycoplasma*, *Ureaplasma*, *Spiroplasma* and *Acholeplasma* species, by a single assay [63]. This application is useful for screening cell cultures for mycoplasma contamination. However, *M. pneumoniae* is rarely observed as a cell-culture contaminant.

The main advantage of PCR in comparison to methods that are not based on amplification is the superior sensitivity. Single-step PCR assays usually have a detection level of 10^2–10^3 genome copies per milliliter of sample [49,64]. The sensitivity can be increased by nested PCR, which involves the reamplification of a PCR product with a second primer set. DNA dilution experiments have revealed that this method enables the detection of 30–100 fg of DNA, roughly corresponding to 10–100 organisms [52]. Hence, application of nested PCR may lead to a 10^2-fold increase in sensitivity. However, when clinical samples are examined, the tendency of *M. pneumoniae* cells to form conglomerates is a limitation for ultrasensitive detection. For this reason, it has been assumed that a detection level lower than 250 genome copies/mL may not be achieved [65]. Carryover contamination is a major problem with PCR diagnosis. This is especially true for nested PCR, because aerosol-forming PCR products have to be handled when the second run is prepared. Shortly after the development of PCR, guidelines for avoiding false-positive results were developed [66].

Table 2 Detection of *M. pneumoniae* in respiratory specimens by PCR set-up

Type of specimen	Gene	Amplicon	Reference
Sputum	16S rDNA	277 bp	[62]
Throat swabs	ATPase operon gene	144 bp (104 bp)[a]	[52]
	16S rDNA	76 bp[b]	[72]
	P1 adhesin gene	375 bp	[88]
Nasopharyngeal aspirates	ATPase operon gene	144 bp	[33]
	ATPase operon gene	144 bp (104 bp)[a]	[52]
	16S rDNA	277 bp	[50]
	16S rDNA	76 bp[b]	[72]
	P1 adhesin gene	209 bp	[50]
Tracheal aspirates	ATPase operon gene	144 bp (104 bp)[a]	[52]
Bronchoalveolar lavage (BAL)	ATPase operon gene	144 bp	[49]
	16S rDNA	427 bp	[61]
Transthoracic needle aspirate	ATPase operon gene	144 bp	[89]
Fixed lung tissue[c]	P1 adhesin gene	102 bp	[90]
Open lung biopsy specimen[c]	P1 adhesin gene	285 bp (170 bp)[a]	[91]

[a]Nested PCR.
[b]Real-time PCR.
[c]Data available only from isolated cases.

By the application of such recommendations, contamination may be reduced to less than 0.5% even for nested PCR, [52]. Kits for *M. pneumoniae* PCR diagnosis are commercially available (Roche, Basel, Switzerland; Minerva Biolabs, Berlin, Germany).

Hybridization of PCR products is generally thought to increase the sensitivity of PCR compared to analysis by gel electrophoresis and ethidium bromide staining, but discordant findings have also been published [65]. If the detection level of the applied PCR has been determined, a semiquantitative assessment of the mycoplasma load in the sample can be achieved by testing dilutions of extracted nucleic acids [67]. Such a rough quantification may be useful, as recent work has shown that patients with acute *M. pneumoniae* infection harbor greater quantities of the pathogen in respiratory secretions than do convalescent patients [67]. Table 2 summarizes the different types of respiratory specimen where *M. pneumoniae* has been detected by PCR. The specificity of PCR depends on the choice of primers and the stringency of the reaction. It has been shown that, under optimized conditions, detection of *M. pneumoniae* by PCR provides a specificity of 100% [50].

Despite advanced in vitro technology, PCR alone is not always sufficient for the diagnosis of respiratory infections by *M. pneumoniae*. Several studies have revealed a poor correlation between antibody response and positive PCR results in patients with mycoplasma pneumonia. Tjhie et al. have reported concordant results of PCR and MAG in 88.1% of patients [65]. A correlation of 72.4% was reported by Skakni et al. [49]. Waris et al., who determined both the IgM and IgG responses to *M. pneumoniae* by ELISA, achieved a positive PCR result in only 50% of pediatric cases of mycoplasma pneumonia [33]. Comparable observations have been made when culture techniques have been applied. Kenny et al. found sensitivities of 53% and 64% of serology and culture, respectively, when the other approach was regarded as the reference [42]. Similarly, it has been demonstrated that only 58% of patients with positive mycoplasma culture show a four-fold or greater increase in CF antibody titer [68].

There are several explanations for the detection of *M. pneumoniae* in respiratory secretions of patients without antibody response. Most importantly, the presence of the pathogen in the respiratory tract is not necessarily associated with clinical symptoms. Gnarpe et al. found that, depending on the current epidemiologic situation, 5.1–13.5% of healthy adults harbor the organism in the throat [69]. These data were obtained by isolation of the pathogen from throat swabs. Transient asymptomatic carriage of *M. pneumoniae* results from the persistence of the pathogen after disease, and from infections during the incubation period. In this

context, it is important to note that the incubation time is approximately 3 weeks in *M. pneumoniae* respiratory tract diseases [20]. However, there is no evidence that the onset of symptoms is causally related to the appearance of diagnostic antibody levels. Also, during the early stages of disease, there is no diagnostic antibody response, while *M. pneumoniae* is detectable in respiratory secretions. In immunocompromised patients, no diagnostic antibody response may be observed. It is currently unknown whether a diagnostic rise in antibody titer regularly occurs in asymptomatic infections. As a consequence of these considerations, detection of *M. pneumoniae* in respiratory specimens is not necessarily indicative of a causative role of the pathogen in a given episode of pneumonia. However, the constellation of clinical evidence of pneumonia and a positive PCR result points to a mycoplasmal etiology of the disease, and represents an indication for antimycoplasma therapy.

Although *M. pneumoniae* tends to persist in the respiratory tract for some months after infection in some patients, the pathogen is detected less frequently during the later stages of the disease [33,42]. The early disappearance of *M. pneumoniae* could be due to antimicrobial therapy or the immune response of the host. This may explain negative PCR results in patients with otherwise serologically confirmed mycoplasma respiratory tract disease. In contrast to a positive PCR result, a significant rise in specific antibodies always provides evidence of current *M. pneumoniae* infection.

In addition to PCR and nested PCR, several new amplification-based techniques have been adapted for *M. pneumoniae* diagnosis. The simultaneous detection of *M. pneumoniae* and other respiratory pathogens can be performed by multiplex PCR. Multiplex PCR assays for the detection of *M. pneumoniae* and *Chlamydia pneumoniae*, and *M. pneumoniae*, *C. pneumoniae* and *Chlamydia psittaci*, have now been developed [70,71]. A real-time PCR assay for the detection of *M. pneumoniae* in clinical samples has been evaluated, and was found to be equal to conventional nested PCR with regard to sensitivity [72]. This method allows the quantification of the amplified product during PCR, combined with a significant reduction in hands-on time. The amplification of eubacterial DNA by primers targeting a highly conserved genome region (16S rDNA) and subsequent sequencing of the amplicon enabled the diagnosis of *M. pneumoniae* in a case of osteomyelitis, in which pus cultures had been repeatedly negative [73,74].

Suspected extrapulmonary manifestations of *M. pneumoniae* infection represent an important application of molecular methods. In cases of rarely encountered manifestations, direct pathogen detection in the affected site provides most diagnostic accuracy. Otherwise, it remains mostly uncertain whether the positive serologic results are related to the disease under investigation. On the other hand, the negative predictive value of molecular pathogen detection is obviously low in some extrapulmonary manifestations that are potentially immunologically mediated and hence not associated with direct invasion by the pathogen. Most importantly, the pathogenesis of neurologic manifestations is currently unknown. It is assumed that there exist different pathogenetic mechanisms of central nervous system involvement in *M. pneumoniae* infection, each involving either direct pathogen invasion or an aberrant immune response [75]. Invasion of cardiac structures by *M. pneumoniae* in cases of pericarditis or perimyocarditis seems to be a more constant event [76]. However, it has to be noted that large pericardial effusions, which required puncture or even pericardiectomy, were mostly investigated, while mycoplasma pericarditis primarily occurs with small pericardial effusions [77].

Most extrapulmonary sites do not provide a favorable environment for mycoplasma growth. As the pathogen is probably present in small quantities, the application of molecular methods of enhanced sensitivity, e.g. nested PCR, may be necessary [40]. However, if other potential etiologies are carefully ruled out, the diagnosis can be made by comprehensive serologic examinations in the majority of cases. There exists only one report of a severe extrapulmonary manifestation (encephalitis) with detection of the pathogen from the affected site (CSF) in the absence of a generalized serologic response [40].

RNA amplification techniques are also promising with regard to *M. pneumoniae* diagnosis. The advantage of RNA detection is the high sensitivity, which is due to the large number of rRNA copies ($>10^3$) per bacterial cell. As RNA is degraded rapidly in vivo by RNases, the detection of this nucleic acid is more indicative of viable mycoplasmas in a clinical sample than is DNA detection. RT-PCR assays targeting sequences of the 16S

rRNA have been developed, for both the genus and species levels [63]. A single-tube multiplex RT-PCR for the detection of *M. pneumoniae* and eight additional respiratory pathogens has been developed recently [78]. There are also RNA amplification techniques which have not yet been introduced into mycoplasma diagnosis, although they have been shown to be useful for the detection of other infectious agents. Transcription-mediated amplification (TMA) assays, which are commercially available for the detection of *Chlamydia trachomatis* [79] and *Mycobacterium tuberculosis* [80] (GenProbe), may improve mycoplasma diagnosis.

CONCLUSION

For serologic diagnosis of *M. pneumoniae* pneumonia, an assay for the separate detection of specific IgM or IgA antibodies should be applied. Elevated IgM antibodies represent a reliable indicator of mycoplasma infection in children, but IgA-based diagnosis provides higher sensitivity in older patients. In order to rule out the possibility that the elevated antibodies are due to past infection, serology has to be combined with direct pathogen detection. However, it has to be noted that *M. pneumoniae* may be detected in the respiratory tract of asymptomatic persons. In suspected extrapulmonary manifestations, detection of *M. pneumoniae* from the affected, primary sterile site is diagnostic. Some manifestations, notably involving the central nervous system, are assumed to have an immunologically mediated pathogenesis that does not involve direct pathogen invasion. These diseases must be diagnosed by serologic examination.

Standard PCR is currently the method of choice for direct detection of *M. pneumoniae*. PCR has replaced hybridization and direct antigen detection because of its higher sensitivity. During recent years, several PCR-related methods have been applied for research purposes. Nested PCR is of high sensitivity, which may be required for the detection of *M. pneumoniae* from extrapulmonary sites, where the pathogen is usually present in low copy numbers. Multiplex PCR assays were designed for the simultaneous detection of *M. pneumoniae* and other respiratory pathogens. Real-time PCR enables the monitoring of amplification during PCR and provides comfortable handling procedures. Among the new techniques in the field of molecular mycoplasma diagnosis, the latter method is most likely to become widely used for routine diagnosis.

Methods involving amplification of specific RNA sequences have been exclusively applied for research purposes. RNA-based diagnosis is of high sensitivity, due to the presence of approximately 10^3 copies of 16S rRNA per mycoplasma cell. Detection of RNA provides further evidence of viable mycoplasmas in the specimen, because RNA is destroyed more rapidly than DNA after the death of the mycoplasma cell. Whereas RT-PCR assays for the detection of *M. pneumoniae* have been developed, the TMA technology has not yet been introduced into mycoplasma diagnosis. TMA assays have been shown to be useful for routine diagnosis of other infections, and may also facilitate the diagnosis of *M. pneumoniae* infection.

REFERENCES

1. Lieberman D, Schlaeffer F, Lieberman D, Horowitz S, Porath A. *Mycoplasma pneumoniae* community-acquired pneumonia: a review of 101 hospitalized adult patients. *Respiration* 1996; 63: 261–6.
2. Gomez J, Banos V, Ruiz-Gomez J *et al*. Prospective study of epidemiology and prognostic factors in community-acquired pneumonia. *Eur J Clin Microbiol Infect Dis* 1996; 15: 556–60.
3. Foy HM. Infections caused by *Mycoplasma pneumoniae* and possible carrier state in different populations of patients. *Clin Infect Dis* 1993; 17(suppl 1): S37–46.
4. Clyde WA Jr. Clinical overview of typical *Mycoplasma pneumoniae* infections. *Clin Infect Dis* 1993; 17(suppl 1): S32–6.
5. Denny FW, Clyde WA Jr, Glezen WP. *Mycoplasma pneumoniae* disease: clinical spectrum, pathophysiology, epidemiology, and control. *J Infect Dis* 1971; 123: 74–92.
6. Bochud P-Y, Moser F, Erard P *et al*. Community-acquired pneumonia. A prospective outpatient study. *Medicine* 2001; 80: 75–87.
7. Principi N, Esposito S, Blasi F, Allegra L, the Mowgli Study Group. Role of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in children with community-acquired lower respiratory tract infections. *Clin Infect Dis* 2001; 32: 1281–9.
8. Marrie TJ, Peeling RW, Fine MJ, Singer DE, Coley CM, Kapoor WN. Ambulatory patients with community-acquired pneumonia: the frequency of atypical agents and clinical course. *Am J Med* 1996; 101: 508–15.
9. Noah ND. *Mycoplasma pneumoniae* infection in the United Kingdom 1967–73. *BM J* 1974; 2: 544–6.
10. Foy HM, Kenny GE, McMahan R, Mansy AM, Grayston JT. *Mycoplasma pneumoniae* pneumonia in an urban area. *JAMA* 1970; 214: 1666–72.

11. Marrie TJ. *Mycoplasma pneumoniae* pneumonia requiring hospitalization, with emphasis on infection in the elderly. *Arch Intern Med* 1993; 153: 488–94.
12. Mansel JK, Rosenow EC, Smith TF, Martin JW. Mycoplasma pneumoniae pneumonia. *Chest* 1989; 95: 639–46.
13. Tarp B, Jensen JS, Østergaard L, Andersen PL. Search for agents causing atypical pneumonia in HIV-positive patients by inhibitor-controlled PCR assays. *Eur Respir J* 1999; 13: 175–9.
14. Perez CR, Leigh MW. *Mycoplasma pneumoniae* as the causative agent for pneumonia in the immunocompromised host. *Chest* 1991; 100: 860–1.
15. Foy HM, Ochs H, Davis SD, Kenny GE, Luce RR. *Mycoplasma pneumoniae* infections in patients with immunodeficiency syndromes: report of four cases. *J Infect Dis* 1973; 127: 388–93.
16. Jensen JS, Heilmann C, Valerius NH. *Mycoplasma pneumoniae* infection in a child with AIDS. *Clin Infect Dis* 1994; 19: 207.
17. Kingston JR, Chanock RM, Mufson MA *et al.* Eaton agent pneumonia. *JAMA* 1961; 176: 118–23.
18. Macfarlane J, Holmes W, Gard P *et al.* Prospective study of the incidence, aetiology and outcome of adult lower respiratory tract illness in the community. *Thorax* 2001; 56: 109–14.
19. Taylor-Robinson D, Bébéar C. Antibiotic susceptibilities of mycoplasmas and treatment of mycoplasmal infections. *J Antimicrob Chemother* 1997; 40: 622–30.
20. Taylor-Robinson D. Infections due to species of Mycoplasma and Ureaplasma: an update. *Clin Infect Dis* 1996; 23: 671–84.
21. Davis CP, Cochran S, Lisse J *et al.* Isolation of *Mycoplasma pneumoniae* from synovial fluid samples in a patient with pneumonia and polyarthritis. *Arch Intern Med* 1988; 148: 969–70.
22. Daxboeck F, Zedtwitz-Liebenstein K, Burgmann H, Graninger W. Severe hemolytic anemia and excessive leucocytosis masking mycoplasma pneumonia. *Ann Hematol* 2001; 80: 180–2.
23. Jacobs E. Serological diagnosis of *Mycoplasma pneumoniae* infections: a critical review of current procedures. *Clin Infect Dis* 1993; 17(suppl 1): S79–82.
24. Ali NJ, Sillis M, Andrews BE, Jenkins PF, Harrison BDW. The clinical spectrum and diagnosis of *Mycoplasma pneumoniae* infection. *Q J Med* 1986; 58: 241–51.
25. Hosker HSR, Tam JS, Chan CHS, Lai CKW. *Mycoplasma pneumoniae* infection in Hong Kong—clinical features during an epidemic. *Respiration* 1993; 60: 237–40.
26. Geißler RG, Köbberling J. Kälteagglutininkrankheit. *Klin Wochenschr* 1988; 66: 277–83.
27. Ruuskanen O, Nohynek H, Ziegler T *et al.* Pneumonia in childhood: etiology and response to antimicrobial therapy. *Eur J Clin Microbiol Infect Dis* 1992; 11: 217–23.
28. Pönka A, Pönka T, Sarna S, Penttinen K. Questionable specificity of lipid antigen in the *Mycoplasma pneumoniae* complement-fixation test. *J Infect* 1981; 3: 332–8.
29. Kleemola M, Kayhty H. Increase in titers of antibodies in patients with purulent meningitis. *J Infect Dis* 1983; 146: 284–8.
30. Barker CE, Sillis F, Wreghitt TG. Evaluation of Serodia Myco II particle agglutination test for detection of *Mycoplasma pneumoniae* antibody: comparison with μ-capture ELISA and indirect immunofluorescence. *J Clin Pathol* 1991; 43: 163–5.
31. Jacobs E, Bennewitz A, Bredt W. Reaction pattern of human anti-*Mycoplasma pneumoniae* antibodies in enzyme-linked immunosorbent assays and immunoblotting. *J Clin Microbiol* 1986; 23: 517–22.
32. Sillis M. The limitations of IgM assays in the serological diagnosis of *Mycoplasma pneumoniae* infections. *J Med Microbiol* 1990; 33: 253–8.
33. Waris ME, Toikka P, Saarinen T *et al.* Diagnosis of *Mycoplasma pneumoniae* pneumonia in children. *J Clin Microbiol* 1998; 36: 3155–9.
34. Watkins-Riedel T, Stanek G, Daxboeck F. Comparison of SeroMP IgA with four other commercial assays for serodiagnosis of *Mycoplasma pneumoniae* pneumonia. *Diagn Microbiol Infect Dis* 2001; 40: 21–5.
35. Matas L, Dominguez J, De Ory F *et al.* Evaluation of Meridian ImmunoCard *Mycoplasma* test for the detection of *Mycoplasma pneumoniae*-specific IgM in paediatric patients. *Scand J Infect Dis* 1998; 30: 289–93.
36. Morrison-Plummer J, Lazell JA, Baseman JB. Shared epitopes between *Mycoplasma pneumoniae* major adhesin protein P1 and a 140-kilodalton protein of *Mycoplasma genitalium*. *Infect Immun* 1987; 55: 49–56.
37. Vu AC, Foy HM, Cartwright FD, Kenny GE. The principal antigens of isolates of *Mycoplasma pneumoniae* as measured by levels of immunoglobulin G in human serum are stable in strains collected over a 10-year period. *Infect Immun* 1987; 55: 1830–6.
38. Taylor-Robinson D, Gilroy CB, Hay PE. Occurrence of *Mycoplasma genitalium* in different populations and its clinical significance. *Clin Infect Dis* 1993; 17(suppl 1): S66–8.
39. Benèina D, Dovè P, Mueller-Premru M *et al.* Intrathecal synthesis of specific antibodies in patients with invasion of the central nervous system by *Mycoplasma pneumoniae*. *Eur J Clin Microbiol Infect Dis* 2000; 19: 521–30.
40. Narita M, Matsuzono Y, Togashi T, Kaji N. DNA diagnosis of central nervous system infection by *Mycoplasma pneumoniae*. *Pediatrics* 1992; 90: 250–3.
41. Abramovitz P, Schvartzman P, Harel D, Lis I, Naot Y. Direct invasion of the central nervous system by

Mycoplasma pneumoniae: a report of two cases. *J Infect Dis* 1987; 155: 482–7.
42. Kenny GE, Kaiser GG, Cooney MK, Foy HM. Diagnosis of *Mycoplasma pneumoniae* pneumonia: sensitivities and specificities of serology with lipid antigen and isolation of the organism on soy peptone medium for identification of infections. *J Clin Microbiol* 1990; 28: 2087–93.
43. Granato PA, Poe L, Weiner LB. Use of modified New York City medium for growth of *Mycoplasma pneumoniae*. *Am J Clin Pathol* 1980; 73: 702–5.
44. Razin S. Comparative genomics of mycoplasmas. *Wien Klin Wochenschr* 1997; 109: 551–6.
45. Tully JG, Rose DL, Baseman JB, Dallo SF, Lazzell AL, Davis CP. *Mycoplasma pneumoniae* and *Mycoplasma genitalium* mixture in synovial fluid isolate. *J Clin Microbiol* 1995; 33: 1851–5.
46. McCormack GE. Susceptibility of mycoplasmas to antimicrobial agents: clinical implications. *Clin Infect Dis* 1993; 17(suppl 1): 200–1.
47. Harris R, Marmion BP, Varkansis G, Kok PW, Lunn B, Martin. J. Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 2. Comparison of methods for direct detection of specific antigen or nucleic acid sequences in respiratory exudates. *Epidemiol Infect* 1988; 101: 685–94.
48. Razin S. DNA probes and PCR in diagnosis of mycoplasma infections. *Mol Cell Probes* 1994; 8: 497–511.
49. Skakni L, Sardet A, Just J *et al.* Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. *J Clin Microbiol* 1992; 30: 2638–43.
50. Ieven M, Goossens H. Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. *Clin Microbiol Rev* 1997; 10: 242–56.
51. Marmion BP, Williamson J, Worswick DA, Kok T-W, Harris RJ. Experience with newer techniques for the laboratory detection of *Mycoplasma pneumoniae* infection: Adelaide, 1978–1992. *Clin Infect Dis* 1993; 17(suppl 1): S90–9.
52. Abele-Horn M, Busch U, Nitschko H *et al*. Molecular approaches to diagnosis of pulmonary disease due to *Mycoplasma pneumoniae*. *J Clin Microbiol* 1998; 36: 548–51.
53. Abele-Horn M, Franck W, Busch U, Nitschko H, Roos R, Heesemann. J. Transverse myelitis associated with *Mycoplasma pneumoniae* infection. *Clin Infect Dis* 1998; 26: 909–12.
54. Fleischhauer P, Hüben U, Mertens H, Sethi KK, Thürmann D. Nachweis von *Mycoplasma pneumoniae* im Liquor bei akuter Polyneuritis. *Dtsch Med Wschr* 1972; 97: 678–82.
55. Cimolai N, Schryvers A, Bryan LE, Woods DE. Culture-amplified immunological detection of *Mycoplasma pneumoniae* in clinical specimens. *Diagn Microbiol Infect Dis* 1988; 9: 207–12.
56. Andrews PA, Lloyd CM, Webb MC, Sacks SH. Acute interstitial nephritis associated with *Mycoplasma pneumoniae* infection. *Nephrol Dial Transplant* 1994; 9: 564–6.
57. Kleemola SRM, Karjalainen JE, Räty RKH. Rapid diagnosis of *Mycoplasma pneumoniae* infection: clinical evaluation of a commercial probe test. *J Infect Dis* 1990; 162: 70–5.
58. Launes J, Paetau A, Linnavouri K, Iivanaineu M. Direct invasion of the brain parenchyma by *Mycoplasma pneumoniae*. *Acta Neurol Scand* 1997; 95: 374.
59. Bernet C, Garret M, De Barbeyrac B, Bebear C, Bonnet J. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. *J Clin Microbiol* 1989; 27: 2492–6.
60. Ieven M, Ursi D, Van Bever H, Quint W, Niesters HGM, Goossens H. Detection of *Mycoplasma pneumoniae* by two polymerase chain reactions and role of *M. pneumoniae* in acute respiratory tract infections in pediatric patients. *J Infect Dis* 1996; 173: 1445–52.
61. Buck GE, O'Hara LC, Summersgill JT. Rapid, sensitive detection of *Mycoplasma pneumoniae* in simulated clinical specimens by DNA amplification. *J Clin Microbiol* 1992; 30: 3280–3.
62. Van Kuppeveld FJ, Johansson K-E, Galama JM *et al*. 16S rRNA based polymerase chain reaction compared with culture and serological methods for diagnosis of *Mycoplasma pneumoniae* infection. *Eur J Clin Microbiol Infect Dis* 1994; 13: 401–5.
63. Van Kuppefeld FJM, van der Logt JTM, Angulo AF *et al*. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl Environ Microbiol* 1992; 58: 2606–15.
64. Kessler HH, Dodge DE, Pierer K *et al*. Rapid detection of *Mycoplasma pneumoniae* by an assay based on PCR and probe hybridization in a nonradioactive microwell plate format. *J Clin Microbiol* 1997; 35: 1592–4.
65. Tjhie JHT, van Kuppefeld FJM, Roosendaal R *et al*. Direct PCR enables detection of *Mycoplasma pneumoniae* in patients with respiratory tract infections. *J Clin Microbiol* 1994; 32: 11–16.
66. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989; 339: 237–8.
67. Dorigo-Zetsma JW, Zaat SAJ, Vriesema JM, Dankert J. Demonstration by nested PCR for *Mycoplasma pneumoniae* that *M. pneumoniae* load in the throat is higher in patients hospitalized for *M. pneumoniae* infection than in non-hospitalized subjects. *J Med Microbiol* 1999; 48: 1115–22.
68. Grayston JT, Foy HM, Kenny C. The epidemiology of mycoplasma infections of the respiratory tract. In: Hayflick L, ed. *The mycoplasmatales and L phase of bacteria*. Amsterdam: North Holland, 1969: 651–82.

69. Gnarpe J, Lundbäck A, Sundelöf B, Gnarpe H. Prevalence of *Mycoplasma pneumoniae* in subjectively healthy individuals. *Scand J Infect Dis* 1992; 24: 161–4.
70. Corsaro D, Valassina M, Venditti D, Venard V, Le Faou A, Valensin PE. Multiplex PCR for rapid and differential diagnosis of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in respiratory infections. *Diagn Microbiol Infect Dis* 1999; 35: 105–8.
71. Ton CYW, Donnelly C, Harvey G, Sillis M. Multiplex polymerase chain reaction for the simultaneous detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* in respiratory samples. *J Clin Pathol* 1999; 52: 257–63.
72. Hardegger D, Nadal D, Bossart W, Altwegg M, Dutly F. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. *J Microbiol Methods* 2000; 41: 45–51.
73. La Scola B, Michel G, Raoult D. Use of amplification and sequencing of 16S rRNA gene to diagnose *Mycoplasma pneumoniae* osteomyelitis in a patient with hypogammaglobulinemia. *Clin Infect Dis* 1997; 24: 1161–3.
74. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991; 173: 697–703.
75. Pellegrini M, O'Brien TJ, Hoy J, Sedal L. *Mycoplasma pneumoniae* infection associated with an acute brainstem syndrome. *Acte Neurol Scand* 1996; 93: 203–6.
76. Kenney RT, Li JS, Clyde WA Jr *et al.* Mycoplasmal pericarditis: evidence of invasive disease. *Clin Infect Dis* 1993; 17(suppl 1): S58–62.
77. Sands MJ, Satz JE, Turner WE, Soloff LA. Pericarditis and perimyocarditis associated with active *Mycoplasma pneumoniae* infection. *Ann Intern Med* 1977; 86: 544–8.
78. Gröhndahl B, Puppe W, Hoppe A, Kühne I, Weigl JAI, Schmitt H-J. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. *J Clin Microbiol* 1999; 37: 1–7.
79. Pasternack R, Vuorinen P, Miettinen A. Evaluation of the General-Probe *Chlamydia trachomatis* transcription-mediated amplification assay with urine specimens from women. *J Clin Microbiol* 1997; 35: 676–8.
80. Miller N, Hernandez SG, Cleary TJ. Evaluation of General-Probe amplified *Mycobacterium tuberculosis* direct test and PCR for direct detection of Mycobacterium tuberculosis in clinical specimens. *J Clin Microbiol* 1994; 32: 393–7.
81. Naftalin JM, Wellisch G, Kahana Z, Diengott D. *Mycoplasma pneumoniae* septicemia. *JAMA* 1974; 228: 565.
82. Narita M, Matsuzono Y, Itakura O, Togashi T, Kikuta H. Survey of mycoplasmal bacteremia detected in children by polymerase chain reaction. *Clin Infect Dis* 1996; 23: 522–5.
83. Kovacic R, Launay V, Tuppin P *et al.* Search for the presence of six mycoplasma species in peripheral blood mononuclear cells of subjects seropositive and seronegative for human immunodeficiency virus. *J Clin Microbiol* 1996; 34: 1808–10.
84. Maraha B, van der Zee A, Bergmans AMC *et al.* Is *Mycoplasma pneumoniae* associated with vascular disease? *J Clin Microbiol* 2000; 38: 935–6.
85. Goulet M, Dular R, Tully JG, Billowes G, Kasatiya S. Isolation of *Mycoplasma pneumoniae* from the human urogenital tract. *J Clin Microbiol* 1995; 33: 2823–5.
86. Sharma S, Brousseau R, Kasatiya S. Detection and confirmation of *Mycoplasma pneumoniae* in urogenital tract specimens by PCR. *J Clin Microbiol* 1998; 36: 277–80.
87. Stutman HR. Stevens–Johnson syndrome and *Mycoplasma pneumoniae*: evidence for cutaneous infection. *J Pediatr* 1987; 111: 845–7.
88. Ramirez JA, Ahkee S, Tolentino A, Miller RD, Summersgill JT. Diagnosis of *Legionella pneumophila*, *Mycoplasma pneumoniae*, or *Chlamydia pneumoniae* lower respiratory infection using the polymerase chain reaction on a single throat swab specimen. *Diagn Microbiol Infect Dis* 1996; 24: 7–14.
89. Falguera M, Nogues A, Ruiz-Gonzalez A, Garcia M, Puig T. Detection of *Mycoplasma pneumoniae* by polymerase chain reaction in lung aspirates from patients with community-acquired pneumonia. *Chest* 1996; 110: 972–6.
90. Williamson J, Marmion BP, Kok T, Antic R, Harris RJ. Confirmation of fatal *Mycoplasma pneumoniae* infection by polymerase chain reaction. *J Infect Dis* 1994; 170: 1052–3.
91. Talkington DF, Thacker WL, Keller DW, Jensen JS. Diagnosis of *Mycoplasma pneumoniae* infection in autopsy and open-lung biopsy tissues by nested PCR. *J Clin Microbiol* 1988; 36: 1151–3.