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New insights into the pathogenesis and detection of *Mycoplasma pneumoniae* infections

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Abstract

Mycoplasma pneumoniae is a common cause of upper and lower respiratory tract infections in persons of all ages and may be responsible for up to 40% of community-acquired pneumonias. A wide array of extrapulmonary events may accompany the infections caused by this organism, related to autoimmunity or direct spread. This review includes a discussion of the latest knowledge concerning the molecular pathological basis of mycoplasmal respiratory disease, how the organism interacts with the host immune system and its association with the development of chronic conditions such as asthma, recent emergence of macrolide resistance and the status of laboratory diagnostic methods.

Keywords

antimicrobial resistance; asthma; community-acquired pneumonia; cytoadherence; enzyme-linked immunoassay; *Mycoplasma pneumoniae*; PCR

Although more than 200 species in the genus *Mycoplasma* are now recognized, relatively few are pathogenic in humans. The best known and most intensely studied of these species is *Mycoplasma pneumoniae*. The initial descriptions of *M. pneumoniae* as a human pathogen, realization that it was not a virus, characterization of clinical manifestations of mycoplasmal respiratory disease, mode and extent of transmission, and development of serological assays began more than 40 years ago. However, very little was known at that time about how this mycoplasma interacts with and damages host cells, affects the immune system, and the extent to which it may mediate illness outside of the respiratory tract.

Progress in understanding the biological properties of *M. pneumoniae* and its true role as a human pathogen have been hindered significantly over the years by its very slow replication rate (~6 h), fastidious demands for successful laboratory cultivation and the relatively low sensitivity and specificity of the earliest complement fixation serological tests, which were much better suited for less antigenically complex viral pathogens. Until recent years, as more sophisticated laboratory techniques have become available, dependence on nonstandardized sero-logical tests performed in reference laboratories requiring measurement of antibodies in

acute and convalescent sera meant that laboratory confirmation of mycoplasmal infection was seldom sought. Physicians could not easily distinguish mycoplasmal respiratory infection from clinically similar illnesses caused by several other bacteria including *Chlamydomphila pneumoniae* and various respiratory viruses, and therefore did not appreciate how often it occurred in their patient populations. A frequent, but incorrect assumption was that mycoplasmal respiratory infection was uncommon, rarely significant from a clinical standpoint and limited to select age groups. Primary-care physicians seldom considered a mycoplasmal etiology when patients presented with a more severe respiratory infection, or extrapulmonary manifestations, or when an elderly person, very young child or infant was involved. Moreover, the benefit of antimicrobial therapy was not always appreciated, allowing untreated persons to continue to spread the infection within their families, schools and communities. Spread among susceptible populations is also facilitated by the fact that many infectious persons are asymptomatic or very mildly ill and may not take precautions to limit exposure to others.

Knowledge gained over the past several years has proven that *M. pneumoniae* is a significant respiratory pathogen in persons of all ages, sometimes causing severe respiratory disease, and it may induce clinically significant manifestations in extrapulmonary sites by direct invasion and/or immunologic effects. Although most cases can be managed on an outpatient basis, *M. pneumoniae* is estimated to cause more than 100,000 adults hospitalizations each year in the USA [1]. Cytoadherence and subsequent close association of the organism on the respiratory tract mucosa lead to a variety of effects that induce local inflammation and stimulate the host immune system to produce additional manifestations. The ability to detect acute *M. pneumoniae* infection has improved substantially owing to the development and commercialization of improved serological immunoassays, some of which are now point-of-care tests, and the introduction of molecular-based nucleic acid-amplification assays available in some clinical reference laboratories. Despite these significant advances, much remains to be learned about how this organism invades the body, interacts with the host immune system and produces disease. The biological properties of *M. pneumoniae* and typical clinical manifestations of infection were comprehensively reviewed in 2004 [1] and are not revisited here, since these aspects have not changed dramatically since then. The present article focuses on newer knowledge gained about how this organism produces disease, multisystem extrapulmonary manifestations, how infections can be detected using currently available technology and a discussion of future perspectives and unmet needs.

Cellular & molecular basis of pathogenesis

Respiratory disease caused by *M. pneumoniae* stems from the close association between the organism and the mucosal epithelium that occurs as a result of cytoadherence, which is considered to be the major virulence factor. A substantial amount of research has been carried out in recent years to improve understanding of what happens at the subcellular level to enable *M. pneumoniae* to adhere to the host's respiratory mucosa and produce local effects that translate into clinical manifestations of disease.

The interaction between *M. pneumoniae* and host cells is mediated through a polarized attachment organelle comprised of a 170-kDa protein, designated P1, and a number of other proteins, including HMW1, -2 and -3, and P90, -40 and -30, as illustrated in Figure 1. HMW1, -2 and -3 are involved in formation and stabilization of the attachment organelle, including localization of other adhesin proteins. Once this polar structure is established, an independently assembled complex of proteins B, C and P1 is drawn to the structure to complete formation of the functional attachment organelle [2]. *M. pneumoniae* and its subcellular components interact with several types of receptors on the host cell such as sialoglycoconjugates, a sialic acid-free glycoprotein and sulphated glycolipids.

The flask-shaped cells typical of *M. pneumoniae*, visualized by electron microscopy in Figure 2, show the prominent attachment organelle, which includes a proteinacious, electron-dense core and a base complex [3-5]. This attachment organelle tip structure is responsible for the intimate association between *M. pneumoniae* and cells of the respiratory epithelium in humans that is necessary for the organism to produce clinical disease. The structure has been the focus of numerous investigations over the last several years examining the roles of its various components in the molecular basis of cytoadherence and pathogenicity. It is now known that the core of the attachment organelle consists of a pair of nonequivalent parallel rods with numerous cross-striations, connected to the tip through a terminal button [6,7]. Several additional proteins form a complex at the base of the structure, which may also contact the cell membrane. These include proteins P41 and -24, which are involved in relations with the cell body, including timing and localization of organelle assembly during cell replication [8-10]. Although no correlation between the morphology of the electron-dense core and motility properties was observed in other mycoplasmas [11], it is possible that the core of the attachment organelle is involved in the gliding process in *M. pneumoniae*. Other adhesin molecules, including P1 [12] and -30 [13], are also believed to play a direct role in cell motility. The extent to which both the core proteins and adhesin molecules contribute to motor activity is still under investigation.

Although *M. pneumoniae* is generally considered to be a rather homogeneous organism overall, naturally occurring variations in the primary structure of the P1 adhesin have facilitated studies of *M. pneumoniae* epidemiology, since differences in the amino acid and nucleotide sequences of P1 and its coding gene provide the basis for designation of subtypes of this mycoplasma [14]. The source of these variant sequences may be degenerate repeats of regions of the P1 coding gene present throughout the *M. pneumoniae* genome, although this has not been proven conclusively. As the two main subtypes appear to alternate in successive epidemics of *M. pneumoniae* infection, the variation in P1 might be associated with short-term immunity to individual subtypes, development of subtype-specific antibodies and frequent re-infections with another subtype. Genomic analysis of a clinical isolate of *M. pneumoniae* has provided evidence that recombination occurs in certain genes of unknown function, but thus far recombination-mediated variation of P1 has not been demonstrated [15]. Methods for molecular subtyping of *M. pneumoniae* isolates in clinical samples have been described [16], but they are not standardized nor required for clinical or diagnostic purposes.

Subsequent to cytoadherence, *M. pneumoniae* is believed to partly cause disease through generation of peroxide and superoxide radicals that act in concert with endogenous toxic oxygen molecules generated by the host to induce oxidative stress. Superoxide anions produced by *M. pneumoniae* act to inhibit catalase in host cells, thereby reducing the enzymatic breakdown of peroxides produced endogenously and by the mycoplasma, rendering the host cell more susceptible to oxidative damage.

HPr kinase/phosphorylase (HPrK), one of the nine known regulatory proteins encoded in the *M. pneumoniae* genome, is a key regulator of carbon metabolism in many Gram-positive bacteria [17]. HPrK phosphorylates/dephosphorylates the HPr protein of the bacterial phosphotransferase system on a serine residue in response to the nutrient status of the cell. HPrK activity at low ATP concentrations suggests that *M. pneumoniae*, and probably other mycoplasmas [18], unlike its counterpart in *Bacillus subtilis*, use HPrK in a manner distinct from that of other organisms. In *M. pneumoniae*, the activation of this enzyme requires glycerol, a readily available molecule derived from the phospholipids of the host-cell membrane. Even though the ultimate targets of this pathway in *M. pneumoniae* remain unclear, it appears that metabolism of glycerol may be linked to peroxide production and virulence.

Finally, recent evidence suggests that hydrogen peroxide activates cells through selective inactivation of protein tyrosine phosphatases, an observation that may explain the diverse immunologic events that occur following *M. pneumoniae* infections [19].

The possibility that *M. pneumoniae* produces toxins involved in the mediation of disease is a very recent concept, first demonstrated in 2005 when a protein with significant sequence homology to the pertussis toxin S1 subunit, which carries out ADP-ribosylating activity crucial to the toxin's function, was identified as specifically binding to surfactant protein A [20]. A recombinantly expressed form of this protein was found to function as a protein:ADP-ribosyltransferase, causing vacuolation and ciliostasis in cultured host cells provided its ADP-ribosyltransferase activity was intact. These findings suggested its potential to act as an exotoxin [21]. Owing to the clinical syndrome commonly caused by *M. pneumoniae*, the protein has been named the community-acquired respiratory distress syndrome toxin (CARDS TX). Owing to its very limited genome size, *M. pneumoniae* is not known to possess homologs of proteins associated with the pertussis toxin S1 subunit, which confer its ability to be translocated from the pathogen to the host-cell cytoplasm. Therefore, it is not known how CARDS TX reaches the host cell. However, the C-terminal moiety of CARDS TX is novel and the protein is immunodominant [21], suggesting that novel features of this protein confer the ability to be translocated. Host-cell targets for CARDS TX remain unidentified at present, as does how the toxin's function might relate to its specific binding to surfactant protein A. *M. pneumoniae* has other proteins that act as adhesins to extracellular host proteins, such as fibronectin and mucin [22].

Mammalian cells parasitized by *M. pneumoniae* can exhibit a number of cytopathic effects and impaired metabolism as a result of the local damage that occurs through mechanisms described above. Cells of the respiratory epithelium typically lose their cilia, appear vacuolated, and show a reduction in oxygen consumption, glucose utilization, amino acid uptake and macromolecular synthesis, ultimately resulting in exfoliation. These subcellular events in the airways of the lung are manifested clinically by the persistent, hacking cough typical of *M. pneumoniae* bronchitis and pneumonia [23]. Damage to cells of the upper respiratory tract and the ensuing local inflammatory response is reflected by pharyngitis, hoarseness, coryza, earache, conjunctivitis and cervical adenopathy.

Mycoplasmal species that infect humans are primarily mucosal pathogens that cause clinical disease by their deleterious effects on the host-cell epithelium, but this concept has been changing recently as evidence accumulates that some species can invade eukaryotic cells. Data obtained from the complete sequencing of the *M. pneumoniae* genome revealed the elimination of a number of genes essential for independent existence, consistent with the life cycle of an intracellular organism [24]. Although intracellular growth and replication has been described for *M. pneumoniae in vitro*, this process has not been proven to occur during natural infections. However, some of the clinical characteristics of *M. pneumoniae* infections are consistent with what may be expected from an intracellular pathogen, including the establishment of latent or chronic infections, limited efficacy of some antimicrobials, necessity for prolonged treatment to eradicate infection in some instances and circumvention of the host immune response [1].

Innate & adaptive immune response

Recent studies have improved our understanding of the role of the host innate and adaptive immune systems in protection from disease, as well as contributing to clinical manifestations of *M. pneumoniae* infection. Mycoplasmas reaching the lower respiratory tract are opsonized by antibody and complement, and then phagocytized by activated macrophages that are drawn to the site of infection by chemotaxis. This process results in an inflammatory exudate that also

includes neutrophils and lymphocytes. Complement-mediated cytolysis may then play a role in limiting the growth of the mycoplasma.

Even though mycoplasmal infection of the respiratory tract typically elicits an immune response in an immunocompetent host, immunity is typically short-lived and re-infections are common. Thus, it is not surprising that the immune response may not eliminate the organisms, allowing long-term carriage in some persons. The importance of humoral immunity is apparent, considering that persons with congenital hypogammaglobulinemia may experience chronic mycoplasmal respiratory infections sometimes accompanied by dissemination of infection to extrapulmonary sites [1].

Two recent studies by Hoek and colleagues demonstrate how *M. pneumoniae* stimulates production of IL-4 by mast cells in co-culture experiments [25,26]. Cytokine production by mast cells is dependent upon the presence of sialic acid residues on the target cell membrane and the P1 adhesin components of the attachment organelle, and requires expression of the heavily sialylated FcεRI α-chain by the mast cell [26]. Mycoplasma adherence to surface sialoglycoproteins results in cellular activation through the normal receptor signaling mechanisms. Hydrogen peroxide produced by *M. pneumoniae* has recently been shown to specifically inhibit protein tyrosine phosphatases and the lipid phosphatase and tensin homolog by reversibly oxidizing an essential cysteine in the catalytic site of mast cells [27,28].

M. pneumoniae also directly activates and induces cytokine production from peripheral blood leukocytes, respiratory epithelial cells and macrophages. TNF-α, IL-8 and IL-1β mRNA are produced by cultured human lung alveolar type II pneumocytes infected with *M. pneumoniae*, supporting the idea that adherence to human airway epithelial cells leads to the production of cytokines and recruitment of lymphocytes and other inflammatory cells, and that these cytokines may subsequently modulate the activity of the inflammatory infiltrates [1]. Activation of phagocytes for TNF-α production does not appear to require cell contact and can be induced with mycoplasma culture supernatants [29]. This process appears to proceed through the activation of Toll-like receptor (TLR)-1 and -2 by mycoplasma-derived lipopeptides [30]. Mycoplasma cell membrane-derived dipalmitoylated lipoprotein subunit b of F₀F₁-type ATPase was also shown to activate nuclear factor-κB through TLR-1, -2 and -6 in a human monocytic cell line, THP-1 [31]. Further studies with F₀F₁-type ATPase in a rodent model suggest this molecule is important in the induction of the inflammatory response through induction of chemokines and inflammatory cytokines followed by neutrophil infiltration [32].

Cytokine production and lymphocyte activation may either minimize disease through the enhancement of host defense mechanisms and subsequent elimination of the infecting organisms, or exacerbate disease through the development of immunologic hypersensitivity, worsening damage to the respiratory epithelium and contributing to the development of the inflammatory response. The more vigorous the cell-mediated immune response and cytokine stimulation, the more severe the clinical illness and pulmonary injury becomes. Persistence of *M. pneumoniae* in the host leads to the provocation of ineffective immune-mediated inflammatory responses, regulated by opposing T-cell activities. Additional information regarding the host immune response and immunomodulatory effects of *M. pneumoniae* has been reviewed by Waites and Talkington [1].

Autoimmunity & extrapulmonary manifestations

Host responses that develop after *M. pneumoniae* infection likely contribute to autoimmunity and a variety of extrapulmonary complications involving the skin and the nervous, cardiovascular, renal, gastrointestinal, musculoskeletal and hematologic systems, which occur in as many as 25% of infected persons, as recently reviewed [1]. Neurologic complications are among the most common extrapulmonary manifestations of *M. pneumoniae* infection. In a

recent study of 1988 children with encephalitis, *M. pneumoniae* was the most common infectious agent identified, mainly by serological means as PCR assays on cerebrospinal fluid were rarely positive [33]. Occasionally, clusters of encephalitis may also occur during epidemics of *M. pneumoniae* respiratory illness with the neurological manifestations sometimes overshadowing the respiratory problems. Immune-mediated pathological mechanisms have been suspected to be the main cause of neurological manifestations associated with *M. pneumoniae* infections owing to the presence of cross-reactive antibodies to the brain and other neurologic structures that may develop [34]. Neurologic manifestations following *M. pneumoniae* infection can occur as a result of molecular mimicry with carbohydrate moieties of the abundant glycolipids in the *M. pneumoniae* membrane and lipoglycan capsule [35]. Anti-GM1 and galactocerebroside antibodies have been the primary autoantibodies implicated in the ascending paralysis of Guillain-Barré syndrome and in encephalitis associated with *M. pneumoniae* [33,36]. Cytokines including IL-6 and -8 can be elevated in the cerebrospinal fluid of individuals with *M. pneumoniae* encephalitis [37], and appear to be important mediators of inflammation. In recent years, the additional or alternative role of invasion of the CNS by the organism itself has gained renewed interest. *M. pneumoniae* RNA can be detected in brain tissue by nucleic acid hybridization, and the presence of the organism has been demonstrated in cerebrospinal fluid by PCR and culture, and in a recent case of fatal encephalitis, *M. pneumoniae* antigens were immunohistochemically detected in histopathologically involved areas of a brain biopsy and at autopsy [38].

Autoimmune hematologic phenomena frequently occur following *M. pneumoniae* infection, including the well-known cold agglutinins, formerly a primary diagnostic tool, which can occasionally induce a transient brisk hemolytic anemia, most often in children, termed paroxysmal cold hemoglobinuria. These antibodies are primarily of the IgM isotype, and the antigen is primarily of the Ii blood group, a branched (I) or linear (i) polymer of *N*-acetylgalactosamine found on surface glycoproteins or glycolipids of erythrocytes and other cells. Although the antigen is not found in the *M. pneumoniae* membrane, it acts as a surface receptor for the organism and is thereby apparently rendered immunogenic.

The PCR assay has greatly enhanced understanding of how *M. pneumoniae* can disseminate throughout the body. The presence of *M. pneumoniae* in blood, synovial fluid, cerebrospinal fluid, pericardial fluid and skin lesions has also been documented by PCR and/or culture. Thus, direct invasion must also be considered in addition to an autoimmune process in the pathogenesis of extrapulmonary manifestations involving any organ. The frequency of direct invasion of these sites is unknown because the organism is rarely sought, and most extrapulmonary syndromes reported have been attributed to *M. pneumoniae* infection based on serology.

Role of *M. pneumoniae* in asthma & chronic lung disease

Mycoplasmal species that infect animals and humans are particularly well known for their ability to induce chronic disease states in which clearance of the organism is extremely difficult. Intracellular localization, immunomodulatory effects and surface-antigen variations may all contribute to this process. Infection by *M. pneumoniae* is suspected to play a role in some chronic human diseases, including adult rheumatoid arthritis, juvenile idiopathic arthritis, Crohn's disease and asthma [39-43]. A role for *M. pneumoniae* in the pathogenesis of asthma was suggested more than 30 years ago, and the support for this theory is now strong, implicating this organism both in pathogenesis as well as in exacerbation of acute attacks [43]. Evidence implicating *M. pneumoniae* in asthma, summarized by Waites and Talkington [1], includes the following:

The organism can be isolated in higher prevalence from asthmatics than from healthy persons;

Administration of macrolide antibiotics can result in improvement in pulmonary function in asthma patients who are known to have mycoplasmal infection;

Follow-up studies in children have demonstrated prolonged airway dysfunction consistent with a persistent infection;

M. pneumoniae induces a number of inflammatory mediators such as IgE, substance P and neurokinin 1, and IL-5, implicated in the pathogenesis of asthma that may play a role in exacerbations, which often include wheezing;

Models of chronic respiratory infection in mice have demonstrated that the organisms can produce pneumonia, and stimulate cytokine production, airway hyper-responsiveness resembling chronic asthma and a Th2-dominant airway inflammatory process that potentiates organism survival in the lungs.

A diagram indicating how various aspects of the host immune system and inflammatory mediators may interact with *M. pneumoniae* to cause airway hyper-reactivity is provided in Figure 3.

The possibility of mycoplasmal association in the pathogenesis of asthma has stimulated experimental work using rodent models of chronic respiratory infection. Prior allergic sensitization of mice to hen egg ovalbumin has been shown to be associated with downregulation of TLR-2 expression and decreased clearance of *M. pneumoniae* in mouse lungs [44,45]. A Th17-dominant inflammatory response appears to be important in organism clearance, however, mycoplasma infection of mouse lungs can be prolonged when alveolar macrophages are depleted and IL-23-mediated IL-17F production is neutralized [46,47]. Another effect of allergic pulmonary inflammation in this model is to decrease the humoral immune response to the pathogen. Interestingly, in a study of 55 adult, chronic stable asthmatics, Martin *et al.* found a 43% prevalence of PCR positivity for *M. pneumoniae* in bronchioalveolar lavage fluid and bronchial biopsies, but none of the subjects had elevated levels of antibody [48]. In a 5-year prospective study of 82 allergic children, significantly fewer were found to be positive for IgG antibody compared with controls, although there was no difference in the detection of IgM or genomic DNA [49]. Thus, humans with allergic sensitization of the lungs may be at greater risk of developing chronic airway colonization by *M. pneumoniae*, with resultant augmentation of airway hyper-reactivity, even in the absence of a measurable humoral immune response.

Laboratory detection

Since the original descriptions of *M. pneumoniae* as an agent of human disease more than 40 years ago, serology has been the most widely used means for laboratory confirmation of mycoplasmal respiratory infections, since there were no other practical alternatives. Although serology is a useful epidemiologic tool to investigate and characterize outbreaks in circumstances where the likelihood of mycoplasmal disease is high, it is less suited for assessment of individual patients. The fastidious growth requirements and length of time necessary to culture *M. pneumoniae* (as much as 6 weeks) make growing the organism impractical for patient management. Therefore, few clinical laboratories offer culture as a means to diagnose *M. pneumoniae* infections.

M. pneumoniae has both lipid and protein antigens, which elicit an antibody response that can be detected after approximately 1 week of illness, with a peak at 3–6 weeks followed by a gradual decline. In most clinical laboratories the original serological test used for detection of *M. pneumoniae* infection, complement fixation, has been replaced by newer methods that have greater sensitivity and specificity, many of which have been developed and sold as commercial kits. Serological assays used to detect acute *M. pneumoniae* infection include immuno-

fluorescent antibody assays, direct and indirect hemagglutination using IgM capture, particle agglutination antibody assays and enzyme immunoassays (EIAs). The most widely used commercial serological assays are the EIAs, which allow the antibody class to be measured and can be comparable in sensitivity to the PCR assay, providing a sufficient time has elapsed since infection for antibodies to develop and for the patient to have a functional immune system. EIAs may be qualitative or quantitative, may or may not require specialized equipment for performing the assay and reading the results and can be performed with very small volumes of serum.

The need for acute and convalescent sera is a significant limitation for prompt point-of-care diagnosis. However, qualitative point-of-care EIA-based serologic assays that detect both IgM and IgG or IgM alone in a single test format have been developed. IgA is typically produced early in the course of *M. pneumoniae* infection, but very few commercial assays currently include reagents for its detection.

There are concerns about the use of single qualitative tests to identify acute *M. pneumoniae* infections in adults, since many persons may not mount an IgM response, presumably because of re-infection, and when it is produced IgM may persist for long periods. The percentage of individuals with acute infection who demonstrated a positive IgG response in the acute phase was less than 50% in a recent study [50]. When convalescent sera were tested, the number of IgG-positive specimens rose to 82%. This same study found that only 14 out of 27 (52%) acute-phase sera tested positive by various IgM assays, but this number rose to 39 (88%) when convalescent sera were tested. Another recent study found that a single assay using the Meridian IgM ImmunoCard (Meridian Biosciences, OH, USA) had a sensitivity of only 31.8% for detection of acute *M. pneumoniae* infection in seropositive children with pneumonia, but this increased to 88.6% when paired sera were analyzed [51]. The findings that a substantial proportion of healthy blood donors have measurable IgG, IgA and/or IgM anti bodies against *M. pneumoniae* suggests cross-reactivity with antigen preparations used in some of the commercial EIAs and the likelihood that these tests result in overdiagnosis of these infections [52]. The variability of results from comparative studies underscores the need for improved sensitivities and specificities among serological reagents for detecting acute *M. pneumoniae* infection [50,53].

Other limitations of serology for diagnosis of acute *M. pneumoniae* infection include the possibilities that antibody production can be delayed in some infections, or even absent if the patient has an impaired humoral immune response from immunosuppression. False-negative tests can also occur if serum is collected after antibiotics are administered.

PCR assays were developed in the late 1980s and in the two decades since that time there have been more than 200 publications describing its utility for detection of *M. pneumoniae* in human infections. Gene targets used in various types of PCR assays for *M. pneumoniae* include 16S rRNA, P1 adhesin, ATPase operon gene and *tuf* gene, and repetitive element *repMp1*, among others. The sensitivity of PCR is very high, corresponding to the presence of a single organism when purified DNA is used. Other advantages include the ability to complete the procedure in 1 day, the possibility of obtaining a positive result more quickly after onset of illness than when using serology, the need for only one specimen containing organisms that do not have to be viable and the ability to detect nucleic acid in preserved tissues [1]. The PCR assay is also valuable in identifying a mycoplasmal etiology in persons with a variety of extrapulmonary syndromes in which an obvious contribution of respiratory infection may not be readily apparent. Detection of the organism by PCR is possible in blood and cerebrospinal fluid, where culture has rarely been successful.

Real-time PCR assays have significant advantages over traditional PCR, including more rapid turnaround time and less handling of PCR products by electrophoretic analysis [54]. The advantage of real-time PCR over traditional PCR in detection of systemic spread of *M. pneumoniae* was demonstrated in a recent study, which found that 15 out of 29 (52%) patients with serologically proven *M. pneumoniae* infection had a positive PCR on their sera, while conventional PCR was uniformly negative [55].

Molecular-based assays typically demonstrate superior sensitivity for detection of acute mycoplasmal infection compared with serology and culture, but this is not always the case as some studies have shown [56,57]. Culture-negative persons who are PCR positive and do not have clinical evidence of respiratory disease suggest that assay specificity is inadequate, persistence of the organism after infection or asymptomatic carriage, perhaps in an intracellular compartment that does not yield culturable organisms. Positive PCR results in serologically-negative persons may be due to an inadequate immune response, early successful antibiotic treatment or collection of specimens before specific antibody synthesis could occur. Negative PCR results in culture or serologically proven infections suggests that inhibitors may be present, or other technical problems with the assay and its gene target exist. The use of a second PCR assay with a different gene target may help interpret results and resolve such discrepancies. This is particularly important in the setting where a PCR assay based on a single gene target is positive and the corresponding culture and/or serology does not confirm the presence of infection. The common occurrence of several nonpathogenic commensal mycoplasmal species in the respiratory tract mandate rigorous specificity testing of any PCR assay for *M. pneumoniae* to ensure that it does not amplify any of these other mycoplasmal species.

Use of the PCR assay combined with serology in symptomatic persons may be the optimum approach for diagnosis of *M. pneumoniae* respiratory infection, but it may be less useful in adults who do not mount an IgM response and would add to the complexity and expense of laboratory testing. Combining serology with PCR may also provide some interpretive guidance in distinguishing colonization from active disease. Thus far, there are no commercial PCR kits for detection of *M. pneumoniae* sold in the USA, but many reference laboratories offer assays that they have validated themselves using various gene targets. A variety of commercial PCR kits to detect *M. pneumoniae* are available in Europe.

Clinical samples suitable for *M. pneumoniae* PCR include nasopharyngeal and oropharyngeal secretions, sputa, bronchoalveolar lavage and lung tissue obtained by biopsy. Michelow and colleagues reported that nasopharyngeal and oropharyngeal specimens are equally effective for detection of *M. pneumoniae* by PCR, but combining results from both sites provided the greatest diagnostic yield [56]. By contrast, Raty and colleagues found sputa to be superior to nasopharyngeal aspirates and throat swabs in young adults with serologically proven *M. pneumoniae* infection [58]. These conclusions regarding the optimum specimen type for PCR assays have not been corroborated by other studies [1]. From a practical standpoint, many children and adults with *M. pneumoniae* respiratory infections may not produce sputum and are rarely ill enough to warrant invasive procedures to sample the lower respiratory tract. In these settings, it is more expedient to sample the throat or nasopharynx if a microbiological diagnosis is needed for optimum patient management.

M. pneumoniae is one of a group of fastidious, slow-growing pathogenic microbes that also includes *C. pneumoniae* and *Legionella pneumophila* that can cause clinically similar respiratory tract infections. Owing to the inherent difficulties in laboratory diagnosis of these pathogens, efforts in recent years have focused on development of multiplex PCR assays for their simultaneous detection. Commercial availability of a sensitive and specific multiplex PCR assay for these common respiratory pathogens that are not amenable to easy detection in clinical

laboratories would be very beneficial from a patient care standpoint and would likely be widely used if the technical aspects are not too complex and if the assay is not too costly.

Macrolide resistance & susceptibility testing

Historically, *M. pneumoniae* has been considered to be susceptible to macrolides, tetracyclines and fluoroquinolones, and each of these drug classes may have a role in treating the infections it causes in various circumstances. As *M. pneumoniae* is only very rarely isolated from clinical specimens, and performance of *in vitro* susceptibility tests is almost never done, there are no data to document whether naturally occurring resistance to antimicrobial agents exists in most countries. No naturally occurring resistance to tetracyclines and fluoroquinolones has been reported to our knowledge, but mutations in the quinolone resistance-determining regions, generated by *in vitro* selection, resulted in MICs of up to 32 µg/ml for ciprofloxacin [59].

It has been known for many years that persons with mycoplasmal respiratory infections may continue to shed the organisms following clinical resolution of the illness. Naturally occurring macrolide-resistant strains have been documented since the 1970s. Macrolide-resistant *M. pneumoniae* selected *in vitro* contain point mutations in the peptidyl transferase loop of domain V of 23S rRNA, which reduces affinity of these agents for ribosomes [1]. The likelihood that these mutations can develop under natural conditions is quite plausible, since there is only a single rRNA operon in the genome. *M. pneumoniae* has never been shown to harbor plasmids or *erm* genes that mediate ribosomal modification, or any enzymes that break down macrolides.

Reports published over the past 5 years have documented the occurrence of macrolide-resistant *M. pneumoniae* possessing a 23S rRNA mutation in Japanese children with pneumonia and bronchitis. Characterization of strains isolated in Japan prior to 1999 did not detect macrolide resistance, indicating it is of recent development [60,61]. Among 76 *M. pneumoniae* strains isolated in Japan between 2000 and 2003, 13 (17%) were resistant to erythromycin, 12 of which had MICs of more than 256 µg/ml [60]. The mechanisms of macrolide resistance in these bacteria was proven by nucleotide sequencing of 23S rRNA domains II and V, and ribosomal proteins L4 and -22. This analysis revealed ten strains had an A–G transition at position 2063, one strain had an A–G transition at position 2064, and one strain had an A–G transition at position 2617 within domain V. Domain II and ribosomal proteins L4 and -22 were not involved with this resistance. In addition, using PCR these investigators detected A–G mutations in 23 out of 94 (24%) PCR-positive oral samples taken from children with respiratory infections. Moruzumi identified 12 out of 183 (6.6%) *M. pneumoniae* isolates from Japanese children with respiratory tract infections collected between 2002 and 2004 that were resistant to erythromycin with MICs of 32–64 µg/ml [62]. They found an A2063G transition in nine strains and A2064G transition in two strains. All 12 strains had a C785T transition in domain II. One strain did not have a transition in the 23S rRNA gene. Pulsed-field gel electrophoresis classified seven strains as group I and five as group IIb. Additional reports from Japan found macrolide resistance in 10–33% of *M. pneumoniae* strains obtained between 2001 and 2006, all of which had mutations in domain V of 23S rRNA [61,63]. It seems likely the increase in macrolide resistance since 2000 in Japan is due to antibiotic pressure owing to widespread usage of macrolides during recent years [62]. Another report has confirmed the presence of macrolide-resistant *M. pneumoniae* in France with the domain V 23S rRNA mutations [14]. The CDC described three out of 11 cases (27%) of *M. pneumoniae* infections from an outbreak that occurred in 2006/2007 in northeastern USA that were macrolide-resistant and had the A2064G mutation [64]. The same study also reported a new real-time PCR assay that simultaneously detected the organism and its macrolide resistance.

Some evidence exists to support the idea that macrolide resistance in *M. pneumoniae* has some clinical implications. Suzuki *et al.* reported that patients with macrolide-resistant *M.*

pneumoniae who received macrolide treatment experienced more febrile days than patients with macrolide-susceptible isolates, but there were no apparent treatment failures or serious illnesses reported [61]. Another study reported that children with macrolide-resistant *M. pneumoniae* required therapeutic changes with substitution of minocycline or levofloxacin because of persistent fever, cough, no resolution or worsening of chest radiographs [63]. However, it should be mentioned that use of fluoroquinolones and tetracyclines in children is generally discouraged because of safety concerns, except in unusual or life-threatening situations.

In vitro susceptibility testing of *M. pneumoniae* is neither practical nor necessary for routine direction or monitoring of treatment for individual patients. However, accurate and reproducible methods to quantify antimicrobial susceptibilities of *M. pneumoniae* to currently available agents is needed in some settings. An example is an immunocompromised person with a culture-proven systemic infection caused by *M. pneumoniae* that has not responded to treatment. Accurate and reproducible susceptibility testing techniques are necessary to recognize the occurrence and magnitude of emerging macrolide resistance, as is now the case in Japan and possibly other countries. Finally, there is a continuing need to evaluate investigational antimicrobial agents to determine their spectrum of activities. It is important to understand that the slow growth and fastidious cultivation requirements of *M. pneumoniae* dictate that the usual methods for broth microdilution and agar dilution have to be modified to determine MICs for conventional bacteria. Since the first attempts to perform *in vitro* susceptibility testing on human mycoplasmas in the 1960s, many studies have been published, but methods were never standardized and results have been quite variable. A Subcommittee on Mycoplasma Antimicrobial Susceptibility Testing was established in 2001 by the Clinical and Laboratory Standards Institute (CLSI) to develop and validate, in multilaboratory studies, protocols for standardized methods for performance, interpretation and quality control of *in vitro* antimicrobial susceptibility tests. A major component of this project has been to select reference strains with defined MIC ranges that can be used for quality control purposes with individual MIC assays. Preliminary results summarize the recommended test methods for agar and broth dilution techniques and establish quality control guidelines [65,66]. A CLSI guideline for susceptibility testing of human mycoplasmas and ureaplasmas is expected to be produced.

Conclusion

M. pneumoniae is a common cause of upper and lower respiratory tract infections in children and adults. Serious infections requiring hospitalization, while rare, occur in persons of all age groups, and may affect multiple organ systems. Clinical manifestations of respiratory tract disease occur as a result of cytoadherence of the organism on the host's respiratory epithelium followed by the production of a variety of substances that induce local damage and stimulate release of inflammatory mediators by the host. Severity of disease appears to be related to the degree to which the host immune response reacts to the infection. Extrapulmonary complications involving all of the major organ systems can occur in association with *M. pneumoniae* infection as a result of direct invasion and/or autoimmune response. Evidence is accumulating regarding this organism's contributory role in chronic lung conditions such as asthma. Qualitative and quantitative serological assays of many types are sold commercially, and this technique is still widely used for diagnostic purposes. Owing to inherent limitations of serological testing, PCR assays were developed for *M. pneumoniae* nearly 20 years ago, and this technology is becoming more widely accepted as a means for rapid diagnosis, even though it has very limited availability and is expensive. Effective management of *M. pneumoniae* infections can usually be achieved with macrolides, tetracyclines or fluoroquinolones, but the recent emergence of macrolide resistance in Japan is of concern. Development of standardized methods with appropriate quality control measures to perform and interpret antimicrobial susceptibility tests for *M. pneumoniae* is now being achieved and this is very important to

monitor the development and spread of drug resistance and to evaluate new pharmacologic agents.

Future perspective

Within the last 10 years, molecular-based techniques such as the PCR assay, along with older technology such as serology and culture, supplemented by knowledge obtained from the publication of the complete genome sequence of *M. pneumoniae* in 1996, have been applied to epidemiologic investigations, animal models of disease, evaluation of diagnostic tests and clinical trials of antimicrobials. As a consequence, our understanding of the cell biology, molecular basis of pathogenesis, role in chronic disease processes, detection of antimicrobial resistance and assessment of efficacy of antimicrobial treatment have improved. Earlier studies were greatly hampered by the difficulty in laboratory detection of *M. pneumoniae* infection. Despite these many advances, much is still unknown about this microbe. Most mycoplasmal respiratory infections never have a microbiological diagnosis because rapid, sensitive, specific and reasonably priced methods for its direct detection are not readily available in physician offices or hospital laboratories.

Performance of nonamplified antigen-based detection methods have been disappointing owing to low sensitivity for detection, and very little work is being conducted in this area. Emphasis has shifted primarily towards improvement in rapid, point-of-care serology tests and PCR assays, particularly real-time PCR. Owing to the complex problems and inherent limitations of serology, and the uncertainty of the magnitude and duration of IgA and IgM responses in adults versus children, it is unlikely that a completely reliable single test format serological assay suitable for point-of-care use will be developed in the near future. However, research into the improvement of serological assays should be continued, even if paired sera must be analyzed for greatest accuracy.

Diagnostic companies are now introducing automated real-time PCR instruments that readily fit into the workflow of clinical micro-biology laboratories and can provide results in approximately 1 h for detection of organisms such as methicillin-resistant *Staphylococcus aureus*. It is expected that assays for rapid detection of many other microorganisms of clinical importance will soon follow. Thus far, there is no commercially available automated PCR assay for *M. pneumoniae* or other common agents of community-acquired respiratory infections such as *C. pneumoniae* and *L. pneumophila* in the USA, and to our knowledge none of the companies at the forefront for development of these innovative diagnostic modalities is planning to offer an assay that includes detection of *M. pneumoniae* in the near future. Commercial PCR kits sold in Europe have not been rigorously tested on a large scale, nor extensively compared with one another. A real-time, automated, multiplex PCR assay for detection of the atypical respiratory bacterial pathogens, including *M. pneumoniae*, that is suitable for use on an instrument that can also be employed to detect other microorganisms of interest for hospital epidemiological purposes would be cost-effective in many clinical laboratories and may also encourage more physicians to seek a microbiological diagnosis for community-acquired respiratory infections rather than rely entirely on empiric management. Development of a safe vaccine that offers protective immunity would be a major step in reducing the extent of *M. pneumoniae* infections, especially in high-risk populations such as patients with chronic respiratory disease and the military, as well as in schools, hospitals and other institutions where large numbers of people dwell in close proximity; however, the generally disappointing results from vaccine trials performed during the 1960s through to the 1990s suggest that such a development may be some time away. No new strategies appear to be on the horizon, although the recent discovery of the CARDS TX of *M. pneumoniae* suggests a possible new vaccine target that has yet to be explored.

Since the worldwide adoption of macrolides, especially azithromycin, it is prudent to perform global surveillance for the occurrence of macrolide-resistant *M. pneumoniae* to identify whether macrolide resistance is emerging elsewhere and whether adults are affected. The impact of macrolide resistance on the outcomes

Executive summary

Mycoplasma pneumoniae is a common cause of upper and lower respiratory tract infections in children and adults of all ages, with re-infections commonly occurring over time.

Most *M. pneumoniae* respiratory infections can be managed in an ambulatory setting, but serious infections requiring hospitalization sometimes occur, affecting multiple organ systems as a result of direct invasion and/or autoimmune response.

Clinical manifestations of *M. pneumoniae* respiratory tract disease occur as a result of cytoadherence of the organism on the host's respiratory epithelium followed by production of a variety of substances that induce local damage and stimulate release of inflammatory mediators by the host.

Severity of *M. pneumoniae* respiratory disease appears to be related to the degree to which the host immune response reacts to the infection.

Evidence is accumulating for a contributory role of *M. pneumoniae* in chronic lung conditions such as asthma.

Qualitative and quantitative serological assays to detect the host immune response to *M. pneumoniae* infection are widely used, although there are some significant limitations of existing products.

PCR assays are gaining popularity as a means for rapid diagnosis of *M. pneumoniae* respiratory infections, even though there is currently a very limited availability of the technology and it is expensive.

Effective management of *M. pneumoniae* infections can usually be achieved with macrolides, tetracyclines or fluoroquinolones, but the recent emergence of macrolide resistance in Asia as a result of antibiotic pressure is of concern since there may be an impact on patient outcome when macrolides are used.

Development of standardized methods with appropriate quality control measures to perform and interpret antimicrobial susceptibility tests for *M. pneumoniae* is now being achieved and this is very important to monitor development and spread of drug resistance and to evaluate new pharmacologic agents.

of respiratory infections is not clear, since the number of cases described thus far is very small and the limited data available is largely descriptive and difficult to quantify. Further studies in this area such as those recently reported from Japan are needed, but they are likely to be hampered to a considerable extent because the time and expertise needed to detect *M. pneumoniae* and to perform susceptibility tests are not widely available. This means that PCR-based clinical studies to evaluate clinical specimens for the presence of *M. pneumoniae* containing specific ribosomal mutations should be performed to monitor the spread of macrolide resistance since clinical isolates are so difficult to obtain.

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Figure 1.
Terminal attachment organelle of *Mycoplasma pneumoniae*.

Figure 2. Scanning electron micrograph of *Mycoplasma pneumoniae* cells grown on glass coverslips
 Electron micrograph is provided courtesy of Jennifer Hatchel and the Miami University
 Electron Microscopy Facility. Bar length = 1 μ .

Figure 3. Role of *Mycoplasma pneumoniae* in chronic asthma

Initial infection in a subject with established allergic asthma and Th2-biased airway inflammation followed by chronic phase with multiple virulence factors (CARDS TX, H₂O₂, lipopeptides and direct adherence to surface immunoreceptors) contributing to maintenance of chronic airway inflammation and hyper-reactivity. CARDS TX: Community-acquired respiratory distress syndrome toxin.