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Mycoplasma pneumoniae and Its Role as a Human Pathogen

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INTRODUCTION

The first mycoplasma to be isolated in culture was the bovine pleuropneumonia agent now known as *Mycoplasma mycoides* subsp. *mycoides*, which was described initially by Nocard and Roux in 1898 (315). Over the next 50 years, evidence accumulated relating to the importance of the parasitic bacteria known at that time as pleuropneumonia-like organisms (PPLO) in various diseases of animals and their possible involvement in human disease. In the 1930s Klieneberger introduced the concept that mycoplasmas were "L-forms" of bacteria lacking cell walls and living symbiotically with other, walled bacteria (232). This theory started a spirited debate pitting those who believed that mycoplasmas were unique species against those who believed that mycoplasmas were wall-less variants of other known bacterial species and were undeserving of a unique taxonomic representation. This controversy was not completely settled until the 1960s, when guanine-plus-cytosine (G+C) content assays and DNA-DNA hybridization assays showed that mycoplasmas were indeed unique forms of life and lacked the ability to produce cell walls under any circumstances. A summary of the mycoplasma and ureaplasma species known to occur in humans, excluding occasional isolates or mycoplasmas of animal origin that sometimes infect humans, is shown in Table 1.

Dienes and Edsall detected the first mycoplasma isolated from humans in a Bartholin's gland abscess in 1937 (104). This mycoplasma was probably the organism we now know as *Mycoplasma hominis*. Other human mycoplasmas, including *My-*

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TABLE 1. Mycoplasmas isolated from humans[a]

Organism	Primary site of colonization		Role in disease[b]	Reference(s)
	Respiratory tract	Genitourinary tract		
Acholeplasma laidlawii	+	−	No	119
Mycoplasma buccale	+	−	No	154
Mycoplasma faucium	+	−	No	154
Mycoplasma fermentans	+	+	Yes?	118
Mycoplasma hominis	−	+	Yes	118
Mycoplasma genitalium	−	+	Yes	423
Mycoplasma lipophilum	+	−	No	100
Mycoplasma orale	+	−	No	409
Mycoplasma pirum	?	?	No	101
Mycoplasma penetrans	−	+	?	270
Mycoplasma primatum	+	+	No	99
Mycoplasma salivarium	+	−	No	118
Mycoplasma pneumoniae	+	−	Yes	68
Mycoplasma spermatophilum	−	+	No	186
Ureaplasma urealyticum	−	+	Yes	350,376
Ureaplasma parvum	−	+	Yes	350,376

[a] The organisms shown in this table represent those species that have been shown to occur in humans, with humans as their primary host. This listing excludes occasional isolates and those known to be primarily of animal origin that have been recovered from humans in isolated instances, usually in association with immunocompromise.

[b] In immunocompetent persons.

coplasma fermentans, *Mycoplasma salivarium*, and T-strains, later known as ureaplasmas, had been described by the 1950s. The organism eventually known to be *Mycoplasma pneumoniae* was first isolated in tissue culture from the sputum of a patient with primary atypical pneumonia by Eaton et al. in 1944, and thereafter it became known as the Eaton agent (115). Tests on volunteers and field studies conducted in the 1950s and early 1960s provided solid evidence that the Eaton agent caused lower respiratory tract infections in humans (66, 69, 268), but it was considered to be a virus until it became clear that antibiotics could be effective against it. In 1961 Marmion and Goodburn postulated that the Eaton agent was a PPLO and not a virus (281). Chanock et al. succeeded in culturing the Eaton agent on cell-free medium (68) and proposed the taxonomic designation *M. pneumoniae* in 1963 (67).

The numerous commensal mycoplasmal species that commonly inhabit the human oropharynx, especially the most common species, *Mycoplasma orale* and *M. salivarium*, may occasionally cause diagnostic confusion with *M. pneumoniae* if they happen to find their way to the lower respiratory tract or if diagnostic specimens from the lower respiratory tract are contaminated with oropharyngeal secretions. However, with rare exceptions, usually involving immunosuppressed persons, the oropharyngeal commensal mycoplasmal species are not pathogenic.

Among human mycoplasmas, *M. pneumoniae* is by far the best known and most carefully studied. Much has been learned during the past several years about its cell biology, the host immune response that it elicits, laboratory techniques for detection, disease epidemiology, and its role as a respiratory tract pathogen. These developments are summarized in this review.

MOLLICUTE TAXONOMY AND CLASSIFICATION

The term "mycoplasma" (Greek; "mykes" = fungus and "plasma" = formed) emerged in the 1950s (117) and replaced the older PPLO terminology. The allusion to a fungus-like growth pattern in the name "mycoplasma" happens to describe only the growth of *M. mycoides*, but the term was nevertheless adopted and has persisted to this day. In the 1960s, mycoplasmas were designated members of a class named *Mollicutes*, which derives from Latin words meaning soft ("mollis") and skin ("cutis"). The current taxonomic designations included in class *Mollicutes* comprise 4 orders, 5 families, 8 genera, and about 200 known species that have been detected in humans, vertebrate animals, arthropods, and plants. *M. pneumoniae* is a member of the family *Mycoplasmataceae* and order *Mycoplasmatales* (435).

Members of the class *Mollicutes* are characterized by their small genomes consisting of a single circular chromosome containing 0.58 to 2.2 Mbp, a low G+C content (23 to 40 mol%), and the permanent lack of a cell wall (213). The taxonomy of this class has been extensively revised based on 16S rRNA analysis and is discussed in greater detail elsewhere (213). Studies of 16S rRNA sequences suggest that mycoplasmas are most closely related to the gram-positive eubacterial subgroup that includes the bacilli, streptococci, and lactobacilli. According to Maniloff (278), the *Mollicutes* diverged from the *Streptococcus* branch of gram-positive bacteria with low G+C contents and relatively small bacterial genomes about 605 million years ago. Their small genomes are now believed to be the result of a gradual reduction in genome size from a common ancestor in a process known as degenerative evolution (278). The nature of the selective pressure that led to the evolution of *Mollicutes* is not precisely known. Figure 1 shows the mycoplasma phylogeny reconstructed from 16S rRNA sequence comparisons.

CELL BIOLOGY

Mycoplasmas represent the smallest self-replicating organisms, in both cellular dimensions and genome size, that are

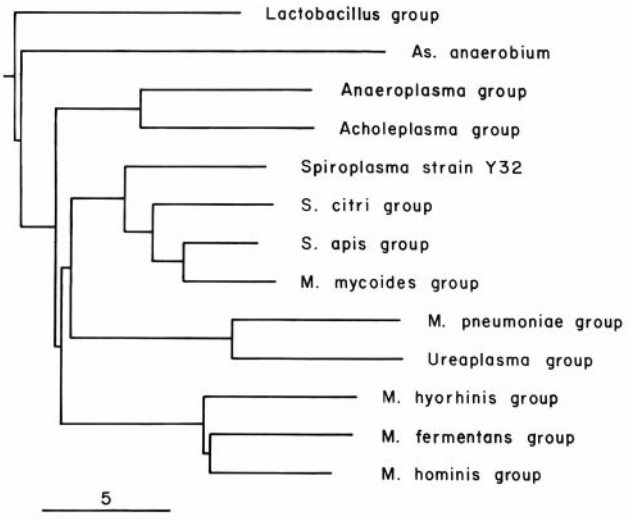

FIG. 1. Phylogeny of mycoplasmas reconstructed from 16S rRNA sequence comparisons. Branch lengths are proportional to evolutionary distance (the number of base changes per 1,000 nucleotides). The scale at the bottom denotes the branch distance corresponding to five base changes per 100 nucleotides. Reprinted from reference 278 with permission.

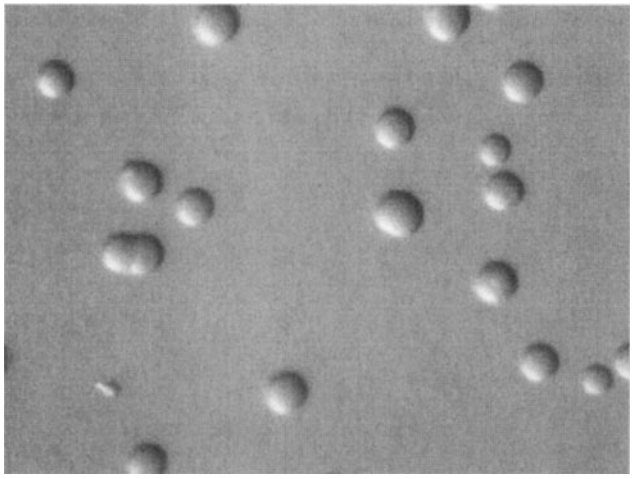

FIG. 2. Spherical colonies of *M. pneumoniae* growing on SP4 agar. Magnification, ×95.

capable of cell-free existence (444). Individual spindle-shaped cells of *M. pneumoniae* are 1 to 2 μm long and 0.1 to 0.2 μm wide, compared with a typical bacillus of 1 to 4 μm in length and 0.5 to 1.0 μm in width. Accordingly, the *M. pneumoniae* cell volume is less than 5% of that of a typical bacillus. The small size and volume of mycoplasmal cells allow them to pass through 0.45-μm-pore-size filters that are commonly used to filter sterilize media. The small cellular mass also means that mycoplasmas cannot be detected by light microscopy, and they do not produce visible turbidity in liquid growth media. Typical colonies of *M. pneumoniae*, shown in Fig. 2, rarely exceed 100 μm in diameter when cultivated on enriched medium such as SP4 agar and require examination under a stereomicroscope to visualize their morphological features.

The genome of *M. pneumoniae* was completely sequenced in 1996 and shown to consist of 816,394 bp with 687 genes (187). In contrast, the *Escherichia coli* genome comprises 4,600,000 bp and about 4,300 genes, making it more than five times larger than that of *M. pneumoniae* (356).

The small genome of *M. pneumoniae* and its limited biosynthetic capabilities are responsible for many of the biological characteristics and requirements for complex medium supplementation in order for the organism to be cultivated in vitro. Mollicutes have no ability to synthesize peptidoglycan cell walls, since the genes responsible for these processes are not present in the genome. The lack of a rigid cell wall confers pleomorphism on the cells and makes them unable to be classified as cocci or bacilli in the manner of conventional eubacteria. Mollicutes have never been found as freely living organisms in nature, since they depend on a host cell to supply them with the things they need for their parasitic existence. Another characteristic of most mollicutes and all members of the genus *Mycoplasma* is the requirement for sterols in artificial growth media, supplied by the addition of serum. Sterols are necessary components of the triple-layered mycoplasmal cell membrane that provide some structural support to the osmotically fragile mycoplasma.

Maintenance of osmotic stability is especially important in mollicutes due to the lack of a rigid cell wall. Although these organisms can flourish within an osmotically stable environment in their chosen eukaryotic host, they are extremely susceptible to desiccation, a fact that has a great impact on the need for proper handling of clinical specimens from which cultural isolation is to be attempted and the need for close contact for transmission of infection from person to person by airborne droplets. Another structural component of the *M. pneumoniae* cell that is important for extracellular survival is a protein network that provides a cytoskeleton to support the cell membrane.

One aspect of *M. pneumoniae* cell biology that is not widely appreciated is the fact that this organism, along with several other mollicute species, may elaborate capsular material external to the cell membrane. The first report indicating the presence of capsular material in *M. pneumoniae* appeared in 1976 in a review of its ultrastructure as determined by electron microscopy (444). It was suggested that this capsular material may have a role in adherence, but this has not been conclusively proven (444).

M. pneumoniae possesses very limited metabolic and biosynthetic activities for proteins, carbohydrates, and lipids in comparison to conventional bacteria. Like other mollicutes, it scavenges for nucleic acid precursors and apparently does not synthesize purines or pyrimidines de novo (91). For many years it has been known that fermentation of glucose to lactic acid by means of substrate phosphorylation effected by phosphoglyceric acid kinase and pyruvate kinase is a means by which *M. pneumoniae* generates ATP. Beyond that, there has been considerable speculation about which enzyme systems were actually present. Much of what is now known about the metabolic properties of *M. pneumoniae* has been made possible through annotation of its genome and direct identification of the genes encoding enzyme systems responsible for various metabolic pathways (91, 187). However, some interesting and perhaps unexpected findings have occurred since the sequence and annotation of the genome have been published. For example, there is genomic evidence for enzymes such as arginine deiminase, even though biochemical activity evidenced by ammonia production has not been directly observed in *M. pneumoniae* (328). Pollack et al. (327) have recently reviewed the central carbohydrate pathways of mollicutes and reported that *M. pneumoniae* possesses all 10 reactions of glycolysis but that the tricarboxylic acid cycle and a complete electron transport chain containing cytochromes are absent. Thus, the lactic acid end product of fermentation is still relatively reduced with electrons whose energy could be trapped if the pyruvate precursor could be diverted to the tricarboxylic acid cycle.

M. pneumoniae reduces tetrazolium either aerobically or anaerobically, and this has been one of several characteristics that have been used historically to identify the species and distinguish it from commensal mycoplasmas of the oropharynx. The availability of nucleic acid amplification techniques such as the PCR assay has made older methods of identification such as tetrazolium reduction and hemadsorption with guinea pig erythrocytes less important than they were previously. A pathway listing all of the relevant enzymes encoded in the *M. pneumoniae* genome is available at www.bork.embl-heidelberg.de/Annot/MP/ (91). Another unique property of *Mycoplasma* spp. is the use of the universal stop codon UGA as a codon for tryptophan (200).

M. pneumoniae, like other mollicutes, has developed special-

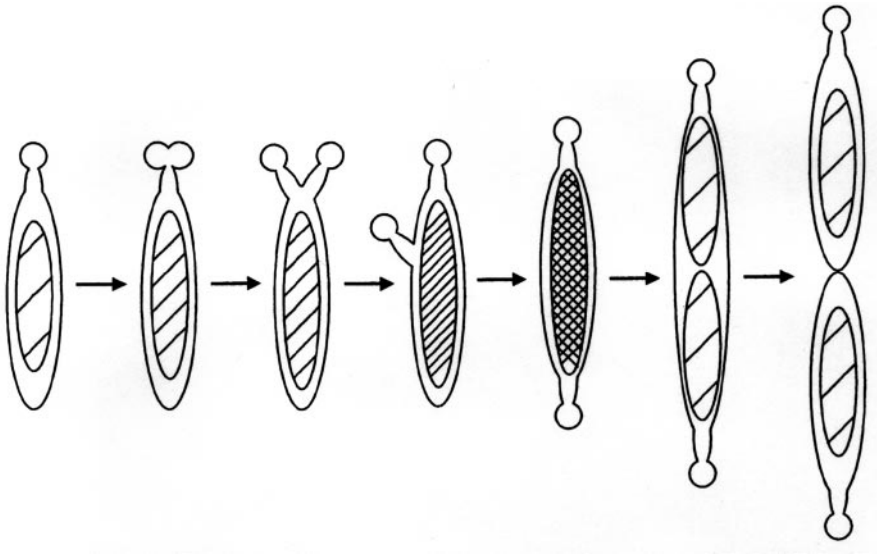

FIG. 3. Proposed scheme for cell division and duplication of the terminal attachment structure in *M. pneumoniae.* Reprinted from reference 375 with permission.

ized reproductive cycles as a result of its adaptation to existence with a limited genome and a parasitic life style that requires attachment to host cells (91). It reproduces by binary fission, temporally linked with duplication of its attachment organelle, which migrates to the opposite pole of the cell during replication and before nucleoid separation (15). A graphic model proposed for *M. pneumoniae* cell division, illustrating the formation and migration of the attachment organelles, is shown in Fig. 3. *M. pneumoniae* has been shown to bind and glide on glass and other solid surfaces, with the organism moving with the attachment organelle at the leading end. Neither genomic analysis nor electron microscopy of *M. pneumoniae* has demonstrated the presence of structures such as flagella or pili, suggesting that gliding motility occurs by an unknown mechanism involving the attachment organelle (290).

PATHOGENESIS OF DISEASE

Mycoplasmas are primarily mucosal pathogens, living a parasitic existence in close association with epithelial cells of their host, usually in the respiratory or urogenital tracts. *M. pneumoniae* exclusively parasitizes humans, whereas some of the other human mycoplasmas have also been recovered from nonhuman primates. A comprehensive review of the molecular biology and pathogenicity of mycoplasmas that includes considerable information devoted to *M. pneumoniae* was published in 1998 by Razin et al. (345), and the reader is referred to that excellent summary for details at the cellular and subcellular levels of how mycoplasmas cause disease in their eukaryotic hosts.

Cytadherence

Evidence accumulated since the 1960s through animal models as well as in vitro cell and organ culture systems indicates that attachment of *M. pneumoniae* to the respiratory epithelium is a prerequisite for later events that culminate in production of disease (403). This close interaction between the mycoplasma and host cells protects it from removal by the host's mucociliary clearance mechanism and allows it to produce a variety of local cytotoxic effects.

Because *M. pneumoniae* is primarily an extracellular pathogen that depends on close association with host cells to survive, it has evolved a complex and specialized attachment organelle to facilitate its parasitic existence, as shown by electron microscopy (Fig. 4 and 5). This attachment organelle consists of a specialized tip structure with a central core of a dense rodlike central filament surrounded by a lucent space that is enveloped by an extension of the organism's cell membrane. The tip structure is actually a network of adhesins, interactive proteins, and adherence accessory proteins that cooperate structurally and functionally to mobilize and concentrate adhesins

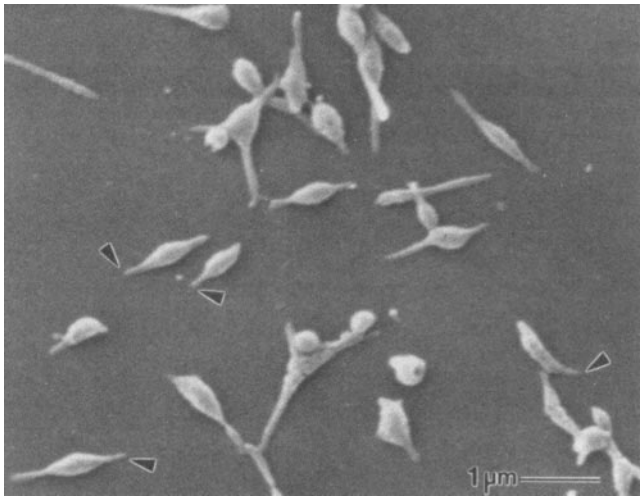

FIG. 4. Scanning electron micrograph of *M. pneumoniae* cells. Whole mycoplasmas are shown, with terminal attachment structures indicated by arrowheads. Reprinted from reference 245 with permission.

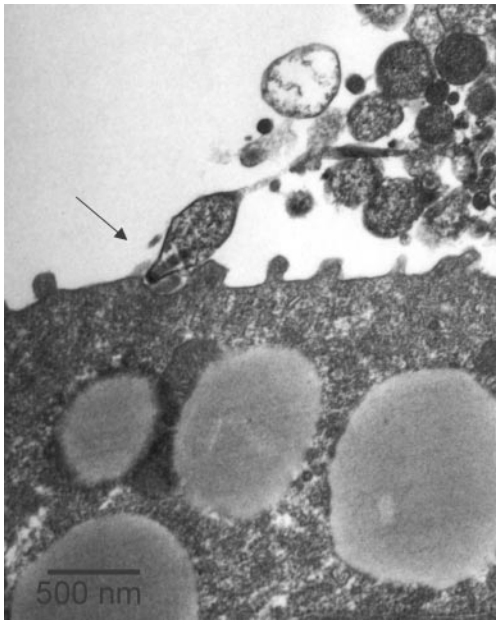

FIG. 5. Transmission electron micrograph of *M. pneumoniae*-infected hamster tracheal ring, demonstrating the close association of the attachment structure to the epithelium (arrow). (Copyright J. L. Jordan and D. C. Krause.)

at the tip of the organism. The host cell ligand for mycoplasmal adhesins has not been characterized conclusively, although sialoglycoconjugates and sulfated glycolipids have been implicated (247, 347). Recent data (89) have shown that two proteins expressed on the *M. pneumoniae* cell surface, elongation factor TU and pyruvate dehydrogenase E1 β, are also involved in binding *M. pneumoniae* to fibronectin, a very common component of eukaryotic cell surfaces, basement membranes, and the extracellular matrix.

The P1 adhesin is a 170-kDa protein concentrated in the attachment tip that is now known to be the major structure responsible for interaction of *M. pneumoniae* with host cells (27, 30, 31, 82, 88, 90, 196, 240, 241). In addition to its presence in the attachment organelle, lower concentrations of P1 are also widespread on the cell's surface (15, 375). Loss of P1 activity through spontaneous mutation or by trypsin treatment results in avirluence by reduced adherence of mycoplasmas to eukaryotic cells (26). Spontaneous reversion to the cytadhering phenotype is accompanied by the reappearance of the implicated proteins, restoration of structurally and functionally intact tips, and return of full infectivity. Further proof for the functional role of P1 as an adhesin comes from evidence that monoclonal antibodies against P1 block adherence in a hamster model of mycoplasma respiratory disease, whereas antibodies produced against some other *M. pneumoniae* proteins have no effect on attachment (242).

Jacobs (202) postulated that the immunodominant epitopes of the *M. pneumoniae* adhesins differ from the highly conserved adherence-mediating domains, thereby suggesting that one reason for the lack of protective immunity against reinfection even in the midst of a serological response could lie in the fact that antibodies directed against the variable domains are unlikely to generate effective cytadherence-blocking antibodies. Studies by Baseman and coworkers suggest that the P1 protein expression alone is not sufficient to mediate adherence of *M. pneumoniae* to certain host cells and cause disease and that the cooperative activity of other proteins is needed (29, 243, 244). P30 is one of several additional proteins that have been implicated in the adherence process, based on the knowledge that antibodies developed against P30 can block *M. pneumoniae* hemadsorption (29, 88, 298). Balish and Krause (15) recently suggested that P30 may be involved in gliding motility as well as coordination of cell division with biogenesis of the attachment organelle.

Other structures produced by *M. pneumoniae* that have been studied as mediators in cytadherence in *M. pneumoniae* include proteins HMW1, HMW2, HMW3, HMW4, HMW5, P90, and P65, which, in addition to P30, are believed to participate in the establishment of the polar structure. 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 terminal attachment organelle (15). A more in-depth discussion of mycoplasma interactions with host cells and the process of cytadherence at the subcellular level was published recently by Rottem (354).

Intracellular Localization

Mycoplasmas are known primarily as mucosal pathogens that reside extracellularly on epithelial surfaces. However, during the past few years, the potential for several mycoplasmal species to fuse with and enter host cells that are not normally phagocytic has been demonstrated (355). Such an occurrence should not be unexpected for microorganisms lacking a rigid cell wall that are typically closely associated with host cell surfaces. Rottem (355) has summarized current knowledge concerning the features that enable *M. penetrans*, a mycoplasma of uncertain pathological significance that has been isolated from urine specimens from human immunodeficiency virus-infected persons, and *M. fermentans* to invade host cells, some of which may be relevant to enhance understanding of similar events that may occur with *M. pneumoniae*. Dallo and Baseman (87) recently described the ability of *M. pneumoniae* to survive, synthesize DNA, and undergo cell replication in artificial cell culture systems over a 6-month period.

An intracellular existence that sequesters *M. pneumoniae* could facilitate the establishment of latent or chronic states, circumvent mycoplasmacidal immune mechanisms, facilitate its ability to cross mucosal barriers and gain access to internal tissues, and impair the efficacy of some drug therapies, accounting for difficulty in eradicating the mycoplasmas under clinical conditions (28, 355, 403, 420). Fusion of the mycoplasmal cell membrane with that of the host may also result in release of various hydrolytic enzymes produced by the mycoplasma as well as insertion of mycoplasmal membrane components into the host cell membrane, a process that could potentially alter receptor recognition sites and affect cytokine induction and expression (355). At present, the extent to which *M. pneumoniae* invades and replicates intracellularly in vivo is not known. Therefore, the clinical significance of these theoretical events associated with cell fusion remains to be proven.

Cytotoxicity and Inflammation

Internalization of a cell-associated mycoplasma into a host cell is not a prerequisite for the initiation of local cytotoxic events and clinical manifestation of disease, although cytadherence in the respiratory tract is the initiating event in disease production by *M. pneumoniae*. It is not known precisely how *M. pneumoniae* injures the respiratory epithelial cell after attachment, but a number of biochemical and immunological properties of the organism that are likely to be involved have been described. Close approximation of the organism to the host cells, facilitated by the adhesin proteins, appears to be important to facilitate localized tissue disruption and cytotoxicity. Unlike many human pathogens, *M. pneumoniae* is not known to produce any exotoxins. Hydrogen peroxide and superoxide radicals synthesized by *M. pneumoniae* act in concert with endogenous toxic oxygen molecules generated by host cells to induce oxidative stress in the respiratory epithelium (420). Consistent with the small genome, *M. pneumoniae* lacks some enzymes that are associated with virulence of other bacteria, such as superoxide dismutase and catalase. Hydrogen peroxide production in *M. pneumoniae* occurs as a result of a flavin-terminated electron transport chain (420). Hydrogen peroxide has been known to be important as a virulence factor in *M. pneumoniae* since Somerson et al. showed it to be the molecule that confers hemolytic activity (385). The ultrastructural effects of peroxide on host cells such as erythrocytes include loss of reduced glutathione, denaturation of hemoglobin, peroxidation of erythrocyte lipids, and eventually lysis of the cells. Almagor et al. (8) suggested that superoxide anion produced by *M. pneumoniae* acts 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. *M. pneumoniae* hemadsorption and lysis of guinea pig erythrocytes, which are low in endogenous catalase, are also mediated by peroxide (420). This property was adapted for use as a diagnostic test to presumptively distinguish *M. pneumoniae* from other commensal mycoplasmas that are commonly found in the human respiratory tract, which do not produce hydrogen peroxide and therefore do not hemadsorb in this manner.

Host cell lactoferrin acquisition by *M. pneumoniae* is yet another possible means by which local injury may occur, through generation of highly reactive hydroxy radicals resulting from the introduction of iron complexes in a microenvironment rendered locally acidic by cellular metabolism that also includes hydrogen peroxide and superoxide anion (419).

Mammalian cells parasitized by *M. pneumoniae* exhibit a number of cytopathic effects that may occur as a result of the local damage mediated biochemically following cytadherence. *M. pneumoniae* infection leads to deterioration of cilia in the respiratory epithelium, both structurally and functionally. Cells may lose their cilia entirely, appear vacuolated, and show a reduction in oxygen consumption, glucose utilization, amino acid uptake, and macromolecular synthesis, ultimately resulting in exfoliation of all or parts of the infected cells (80, 83) These subcellular events can be translated into some of the clinical manifestations of respiratory tract infection that are associated with this organism, such as the persistent, hacking cough that is so commonly associated with *M. pneumoniae*.

Once *M. pneumoniae* reaches the lower respiratory tract, the organism may be opsonized by complement or antibodies. Macrophages become activated, begin phagocytosis, and undergo chemotactic migration to the site of infection. High percentages of neutrophils and lymphocytes are present in alveolar fluid. $CD4^+$ T lymphocytes, B lymphocytes, and plasma cells infiltrate the lung (65, 318), manifested radiologically as pulmonary infiltrates. Further amplification of the immune response in association with lymphocyte proliferation, production of immunoglobulins, and release of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and various interleukins (including interleukin-1β [IL-1β], IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-18) occurs, based on evidence from clinical and in vitro studies and from animal models (193, 195, 263, 308, 309, 406, 420, 447, 448). Many of these reactive substances will have elevated levels in both alveolar fluid and serum (233, 263, 406, 448). Yang et al. (448) recently reported that *M. pneumoniae* infection of human epithelial carcinoma cells in vitro resulted in increased levels of IL-8 and TNF-α mRNAs and that both proteins were secreted into culture medium. The major proinflammatory cytokine IL-1β mRNA also increased and the corresponding protein was synthesized, but its secretion was cell type specific due to an endogenous caspase-1 inhibitory component in the lung epithelial cells studied (447).

The role of cytokines and other reactive substances in the pathogenesis of *M. pneumoniae* lung disease has been a topic of considerable interest during the past several years, and a number of clinical studies involving humans as well as investigations based on animal models have been reported. Current evidence from human and animal studies suggests that cytokine production and lymphocyte activation may either minimize disease through the enhancement of host defense mechanisms or exacerbate disease through immunological lesion development. Thus, the more vigorous the cell-mediated immune response and cytokine stimulation, the more severe the clinical illness and pulmonary injury (78, 201, 305, 338, 342, 404–406). This concept of immune-mediated lung disease provides a basis for consideration of immunomodulatory therapeutics in addition to conventional antimicrobial therapies in management of disease due to *M. pneumoniae*.

Asthma and Other Chronic Lung Conditions

The release of proinflammatory cytokines in association with *M. pneumoniae* infection has also been implicated as a possible mechanism leading to or exacerbating underlying chronic pulmonary diseases such as bronchial asthma. The concept that chronic infection with *M. pneumoniae* might play a role in the pathogenesis of asthma was speculated on over 30 years ago (42). The relevant questions are whether *M. pneumoniae* is a primary cause of asthma or whether mycoplasmal infection is at least a cofactor in its development. Appreciation of the pathogenesis of chronic murine respiratory mycoplasmosis, a naturally occurring mycoplasmal infection of rodents mediated by *Mycoplasma pulmonis* that is slowly progressive, is greatly influenced by heredity, and has characteristics that are similar in some ways to those of asthma in humans, gives further credence to the potential for mycoplasmas to cause longstanding lung disorders such as asthma (60).

Multiple lines of evidence suggest why *M. pneumoniae* may play a role in the pathogenesis of asthma beyond simple, acute exacerbation. *M. pneumoniae* can be detected by PCR and/or culture more often from the airways of patients with chronic, stable asthma than from matched control patients. Kraft et al. (238) detected *M. pneumoniae* by PCR in respiratory secretions of 10 of 18 stable adult asthmatics (56%) and in only 1 of 11 healthy controls. In another study, throat cultures for *M. pneumoniae* were positive in 24.7% of children and adults with asthma exacerbation, compared with 5.7% of healthy controls (158). However, other studies of children with acute asthma exacerbation showed that while rhinoviruses and respiratory syncytial virus may be detected frequently, *M. pneumoniae* played a minor role and was detected in just a few patients (48, 155, 414). Limitations of some of these studies were the use of complement fixation tests alone to identify patients with *M. pneumoniae* (414) and inclusion of very young children in whom viral bronchiolitis instead of asthma may have been present (155).

Treatment of asthma patients in whom *M. pneumoniae* has been detected with macrolide antimicrobials resulted in improvement in pulmonary function tests in comparison with asthma patients who did not have evidence of *M. pneumoniae* in airways (239), owing perhaps to both the antibacterial and the anti-inflammatory effects of macrolides. Macrolides are known to reduce airway hyperresponsiveness in asthmatic patients, attenuate pulmonary inflammation by protecting ciliated epithelium against oxidative damage, stabilize cell membranes, and decrease sputum purulence (133). Mycoplasmas have been detected by PCR in airways even when cultures and serological results are negative, suggesting that low numbers of organisms may evade detection by the immune system (239). The lack of a measurable serological response may also facilitate the organism's persistence in the lower respiratory tract.

Lung abnormalities, including reduced pulmonary clearance and airway hyperresponsiveness, may persist for weeks to months after an infection with *M. pneumoniae* (227, 279, 295, 359, 377). Marc et al. (279) reported abnormalities in pulmonary function tests in up to 50% of children, and Kim et al. (227) described abnormal computerized axial tomography studies for 37% of children months to years after an episode of *M. pneumoniae* respiratory tract infection, thus establishing the ability of mycoplasmas to induce chronic and possibly permanent lung damage long after resolution of respiratory tract symptoms.

In animal models of *M. pneumoniae* infection, Hardy et al. (178) demonstrated that an initial pneumonia lasted 3 to 4 weeks, similar to the case for human disease, characterized by histological lung inflammation and elevated cytokine and chemokine levels. At 530 days following inoculation of *M. pneumoniae* into the respiratory tract, 78% of mice demonstrated peribronchial and perivascular mononuclear infiltrates that were significantly more pronounced than those in controls by a histopathological severity score, concomitantly with increased airway reactivity and obstruction. Such information provides further evidence for the potential for this organism to produce chronic lung disease of clinical significance.

M. pneumoniae is known to induce a number of the inflammatory mediators implicated in the pathogenesis of asthma that may play a role in exacerbations, which often include wheezing (74, 126, 373, 377). Esposito et al. (126) studied 225 children with an acute episode of wheezing, 16 of whom had mycoplasmal infection as determined by serology and/or PCR on nasopharyngeal secretions, and compared them to 8 asymptomatic children with *M. pneumoniae* and 8 uninfected controls. Children with wheezing and acute *M. pneumoniae* infection had a statistically significant increase in IL-5 compared to children with *M. pneumoniae* who were asymptomatic and to the controls without wheezing. Those authors therefore proposed that *M. pneumoniae* might trigger the wheezing process by means of IL-5 secretion in persons who are genetically predisposed or are otherwise susceptible. This seems plausible since IL-5 is the cytokine that has been shown to be essential for development of airway hyperresponsiveness in association with infection caused by respiratory syncytial virus (370, 371).

Hardy et al. (179) provided further evidence that the presence of *M. pneumoniae* in the lower respiratory tract stimulates production of a wide array of inflammatory mediators, including TNF-α, IFN-γ, IL-6, and IL-8, using a murine model of infection. By plethysmography, intranasal inoculation of live *M. pneumoniae* into mice triggered greater pulmonary airflow resistance or obstruction for a longer duration than what was observed in animals that were inoculated with dead organisms or SP4 broth alone. Significant airflow resistance persisted for the entire 28-day observation period, even after histological evidence of pulmonary inflammation subsided.

M. pneumoniae can be associated with significantly greater numbers of mast cells in patients with chronic asthma, according to one study (284), and experimental evidence from a rodent mast cell line suggests that the organism can induce activation of mast cells with release of serotonin and β-hexosaminidase (193). Elevated serum immunoglobulin E (IgE) levels as well as production of IgE specific to *M. pneumoniae* or common allergens may also occur during mycoplasmal infection in children with onset of asthma. Koh et al. (233) showed that levels of the cytokine IL-4 and the ratio of IL-4 to IFN-γ were significantly higher in children with *M. pneumoniae* than in those with pneumococcal pneumonia or uninfected controls, suggesting that a TH2-like cytokine response represents a favorable condition for IgE production.

There are a relatively small number of very limited studies that have implicated *M. pneumoniae* in other chronic lung conditions. Respiratory tract infections with bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* have been associated with acute exacerbations of chronic obstructive pulmonary disease (COPD) for many years. Over 20 years ago, Gump et al. (176) and later Buscho et al. (54) and Smith et al. (383) made some of the earliest observations that mycoplasmal infections could be associated with some cases of COPD exacerbation. However, little attention was given to this possibility for several years. Since the late 1990s, some investigations have been undertaken to assess the possible contribution of *M. pneumoniae* and *Chlamydophila pneumoniae* in COPD (260, 261, 293). Mogulkoc et al. (293) used serology to assess the presence of *M. pneumoniae* and *C. pneumoniae* in 49 ambulatory patients with acute purulent exacerbations of COPD. They found evidence of acute *C. pneumoniae* in 11 patients (22%), sometimes in association with other bacteria. *M. pneumoniae* infection was detected in only three patients (6%). Lieberman et al. (260) also used

serology to assess the presence of *M. pneumoniae*, evaluating a group of 219 patients hospitalized with acute exacerbations of COPD. A total of 34 patients (14.2%) had serological evidence of acute *M. pneumoniae* infection, making it the third most common bacterial pathogen detected. More than one agent was detected in one-third of the cases. The findings of this study were particularly significant in that they showed that respiratory viruses and atypical bacteria, mainly *Legionella* species and *M. pneumoniae*, were involved in most cases and that the classical bacteria were responsible for a minority of cases. These same investigators (261) further described 34 hospitalizations of COPD patients with evidence of *M. pneumoniae* and showed that 3 had pneumonia, 3 required intensive care management, and 1 died. Since the majority of these patients had serological evidence of an additional pathogen and since the study relied entirely upon serological measurements, it is impossible to determine the precise contribution of *M. pneumoniae* to these clinical conditions. Specific short-term treatment with drugs known to be active against mycoplasmas did not appear to be beneficial in reducing the duration of hospitalization, but this is not too surprising in view of the chronicity of many mycoplasmal infections and the difficulty in their eradication in many instances.

The occurrence of bacterial infections of the respiratory tract is considered the main cause of progressive pulmonary failure in patients with cystic fibrosis (124). Although the role of *M. pneumoniae* in community acquired infections of the lower respiratory tract is well known, very little information is available about its occurrence and pathogenic significance in patients with cystic fibrosis. Petersen et al. (324) detected *M. pneumoniae* antibody by complement fixation (CF) in only 2 of 332 episodes of acute exacerbations in patients with cystic fibrosis. Subsequently, Efthimiou et al. (121) noted a fourfold rise in antibody titers against *M. pneumoniae*, *Coxiella burnetii*, and various viruses in a small number of young adults with cystic fibrosis who experienced deterioration in lung function and increase in lower respiratory tract symptoms. Ong et al. (317) and Pribble et al. (335) detected antibodies against *M. pneumoniae* in 1 of 19 and in 4 of 80 acute pulmonary exacerbations, respectively. Although these studies were limited in the respect that serology was the sole means of assessing the presence of mycoplasmas and the test methods employed present some difficulty in proper interpretation to define a recent infection, taken together they suggest that mycoplasmas may occur but are fairly uncommon causes of these complications in persons with cystic fibrosis. Emre et al. (124) confirmed this assumption in that they were unable to demonstrate the presence of *M. pneumoniae* by PCR of oropharyngeal secretions in 16 patients, and only 1 of 16 showed serological evidence of recent infection. *C. pneumoniae* was detected by nasopharyngeal culture in 4 of 32 cases (12.5%), and three of these four patients had elevated IgM or IgG antibodies that were suggestive of acute infection. Clearly, more work must be done to clarify the importance of *M. pneumoniae* and other atypical bacteria in the epidemiology and pathogenesis of exacerbations of chronic lung diseases, using a more comprehensive diagnostic strategy that would include direct tests for the presence of the organism by PCR so that very low numbers of organisms might be detected in the airways, culture, and serology.

Immune Response and Immunomodulatory Effects

M. pneumoniae possesses both protein and glycolipid antigens that elicit antibody responses in infected individuals. The P1 protein is the target of many of the antibodies that are produced by the host in response to the *M. pneumoniae* infection, and it has also served as a target for development of serological assays. Following an initial infection, the normal immune system responds by rapidly producing antibodies that peak after 3 to 6 weeks, followed by a gradual decline over months to years. As a result of the long incubation period, an antibody response is often evident by the time symptoms appear. Elevation of *M. pneumoniae*-specific IgM alone can often be interpreted as evidence of acute infection, since this antibody typically appears within 1 week of the initial infection and approximately 2 weeks before IgG antibody (286, 380). However, the presence of IgM is considered most significant in pediatric populations, where there have been fewer opportunities for repeated exposures. Adults who have been infected repeatedly over a period of years may not respond to mycoplasma antigens with a brisk IgM response (434). In these cases, reinfection leads directly to an IgG response; therefore, the absence of a positive IgM test does rule out an acute infection. When it does occur, the IgM response may persist for months or years following infection (445), and in these cases a positive IgM test result may not reflect a current or recent infection.

IgA, while often overlooked as a diagnostic antibody class, may actually be a better indicator of recent infections in all age groups (380). IgA antibodies are produced early in the course of disease, rise quickly to peak levels, and decrease earlier than IgM or IgG (166, 440). Research into IgA responses in adult and pediatric populations and assessment of antibody levels in other body fluids such as urine are warranted. The importance of an intact immune response in protection against mycoplasmal disease is apparent in view of prolonged disease and dissemination in persons with hypogammaglobulinemia (410).

In addition to *M. pneumoniae*-specific antibodies, a variety of cross-reactive antibodies may develop in association with *M. pneumoniae* infection. The extensive sequence homology of the *M. pneumoniae* adhesin proteins and glycolipids of the cell membrane with mammalian tissues is a well-known example of molecular mimicry that may trigger autoimmune disorders that involve multiple organ systems through formation of antibodies against substances such as myosin, keratin, fibrinogen, brain, liver, kidney, smooth muscle, and lung tissues (16). Mycoplasmal adhesins also exhibit amino acid sequence homologies with human CD4 and class II major histocompatibility complex lymphocyte proteins, which could generate autoreactive antibodies and trigger cell killing and immunosuppression (353). Cold agglutinins and their historical use as a crude diagnostic test for *M. pneumoniae* infection are discussed in a subsequent section. Circulating immune complexes also occur during acute phases of *M. pneumoniae* diseases (80, 291).

Specific T-cell-mediated immunity is also involved in the host reaction to infection by *M. pneumoniae*. Lymphocytes from persons known to have had a prior mycoplasmal infection will undergo blast transformation when cultured in the presence of *M. pneumoniae* (134). Leukocytes from individuals with *M. pneumoniae* infections will show evidence of chemo-

taxis in the presence of the organism, and these individuals will respond with IFN-α in their blood (285, 302).

A property of many species of mycoplasmas that affects the immune responsiveness of the host is their propensity for mitogenic stimulation of B and T lymphocytes, thereby inducing autoimmune disease through the activation of anti-self T cells or polyclonal B lymphocytes (403). This property is associated with the ability of *M. pneumoniae* to stimulate production of cytokines in the initiation of the acute inflammatory response as described above.

Antigenic Variation

Many mycoplasmal species that infect animals or humans are known for their ability to induce chronic disease states in which clearing of the organism is extremely difficult. Therefore, these organisms must have evolved means by which they can successfully evade or modulate the host immune response. As mentioned above, intracellular localization and immunomodulatory activities are possible means to this end. Another mechanism that has been extensively studied in many other bacteria is variation in surface antigens. High-frequency phase and antigenic variation of surface adhesin proteins made possible by DNA rearrangements in truncated and sequence-related copies of the P1 adhesin genes that are dispersed throughout the genome has been described for *M. pneumoniae* (396, 397). Recombinational events among the repetitive elements themselves and with regions of the three-gene P1 adhesin operon promote diversity and altered specificities and affinities and maximize the coding potential of the limited mycoplasma genome (31, 451).

EPIDEMIOLOGY

Geographic Prevalence and Seasonality of Disease

Soon after the identification of *M. pneumoniae* as the etiological agent of primary atypical pneumonia in the early 1960s, considerable interest arose in elucidation and characterization of its incidence, prevalence, mode of spread, and spectrum of disease. *M. pneumoniae* infections can involve both the upper and the lower respiratory tracts and occur both endemically and epidemically worldwide in children and adults. Climate and geography are not thought to be of major significance. Even though most available data concerning the occurrence of *M. pneumoniae* infections have come from studies performed in the United States, Europe, and Japan, seroprevalence investigations using CF technology in arctic and tropical zones have also indicated the presence of *M. pneumoniae* antibody, suggesting that populations in these regions have had infections due to this organism (164, 214, 398). In the United States, there is no national surveillance system tracking *M. pneumoniae* infections; therefore, much of what we know about rates of endemic disease comes from population-based studies relying primarily on serological measurements (146, 148, 150, 171, 296, 297). Foy (143) used CF antibody determinations and culture to show that *M. pneumoniae* was responsible for 15 to 20% of all cases of community-acquired pneumonia, or two cases per 1,000 persons on an annual basis between 1962 and 1975, in Seattle, Wash. Additional retrospective serological studies performed in Denmark showed a pattern of *M. pneumoniae* infections over a 50-year period from 1946 through 1995 with endemic disease transmission punctuated with cyclic epidemics every 3 to 5 years, similar to what was observed in the United States (143, 267). Additional studies in North America and Europe performed over the past 3 decades have also reported similar trends (106, 143, 144, 146, 181, 207, 252, 266, 267, 313, 340). Although the incidence of disease does not vary greatly by season, the proportion of patients with pneumonia due to *M. pneumoniae* is greatest during the summer in temperate climates due to the lower incidence of other respiratory pathogens at this time (5, 283, 334). Outbreaks of *M. pneumoniae* infections also tend to occur in the summer or early fall (5, 131, 403). The long incubation period and relatively low transmission rate have been implicated in the prolonged duration of epidemics of *M. pneumoniae* infections (137).

Layani-Milon et al. (252) used data obtained by PCR assays on nasal swabs to define the incidence of *M. pneumoniae* and several respiratory viruses among persons presenting with evidence of acute respiratory tract infection in a region of France over a 5-year period. The distributions of organisms varied from year to year, with *M. pneumoniae* ranking second to influenza A virus as the most frequent pathogen encountered during the surveillance period. The rates of *M. pneumoniae* infections varied from year to year, unrelated to rates of other pathogens, suggesting cycles of epidemics as have been described in earlier studies. In some instances, both viral and mycoplasmal organisms were detected simultaneously in the same specimens.

Some recent changes in the incidence of *M. pneumoniae* infections described by Lind et al. (267), in which high numbers of cases occurred between epidemics without a return to lower endemic levels, have led to speculation as to the reasons for this occurrence. Jacobs (202) suggested that the availability of new information on virulence factors and the P1 adhesin along with the deciphering of the complete genome might enhance understanding of why these changes have occurred. Dorigo-Zetsma et al. (107) genotyped *M. pneumoniae* clinical isolates and grouped them into eight subtypes within two genomic groups based on P1 adhesin subtypes. Studies from Germany (207) and Japan (365) have shown that different P1 adhesins subtypes may be operating in the development and cycling times of *M. pneumoniae* epidemics. Such gene divergences within the P1 adhesin and development of subtype-specific antibodies following initial infection might account for the frequency of reinfections, which may be due to another subtype (202). Further work by Cousin-Allery et al. (86) and Dumke et al. (114), using techniques including restriction fragment length polymorphism, rapid amplified polymorphic DNA analysis, multilocus sequence typing, Western blotting, and two-dimensional gel electrophoresis to characterize over 200 *M. pneumoniae* isolates collected over several years from several European countries and the United States, showed that *M. pneumoniae* is a rather uniform microorganism, such that most of the isolates could be classified into two groups or subtypes based on the sequences of the P1 adhesin gene, the ORF6 gene, and the P65 gene and by a typical DNA restriction fragment pattern. Both studies found that one or the other of the two subgroups tended to predominate in specific geograph-

ical regions to some extent and that there were changes over time with respect to which subgroups most of the isolates belonged to. Dumke et al. (114) found four variants, which were identical to one another but did not belong to either subtype. These studies support the earlier findings (207, 365) that suggested that different *M. pneumoniae* subtypes might be operative in the cycling times of epidemics. Ovyn et al. (320) described the use of nucleic acid sequence-based amplification to classify 24 *M. pneumoniae* isolates into two types, yielding results in general agreement with those obtained by the other techniques.

Historically, *M. pneumoniae* has not been considered part of the normal flora of humans, and its detection by culture could usually be considered abnormal and of etiological significance if in a person with a clinical condition known to be caused by the organism. However, it can persist for variable periods in the respiratory tract following infections that have resolved clinically with appropriate antimicrobial therapy (143). The usual explanation for such persistence has been that the organism attaches strongly to and invades epithelial cells and that macrolide or tetracycline antibiotics commonly used for treating mycoplasmal infections are bacteriostatic and unable to kill all of the organisms. Surveillance studies using culture and/or PCR indicate that a prolonged asymptomatic carrier state may occur in some persons, providing a reservoir for spread of the organism to others (109, 143, 162, 254). Gnarpe et al. (162) demonstrated that 13.5% of 758 healthy volunteers harbored the organism. During a subsequent period of 11 months, the incidence of *M. pneumoniae* decreased to 4.6% of 499 volunteers, indicating the fluctuating occurrence of this organism over time.

Powerful molecular techniques such as PCR have extremely high sensitivity, theoretically being able to detect a single organism or a single copy of the targeted gene when purified DNA is used; this greatly exceeds the detection threshold of culture, which is approximately 100 to 1,000 cells under optimum conditions (53, 211, 343, 434). Since the mid-1990s, the widespread use of PCR for studies of *M. pneumoniae* has greatly enhanced, but also complicated, our understanding of its epidemiology, necessitating a reconsideration of the meaning of a "gold standard" for *M. pneumoniae* diagnosis.

Disease Transmission

M. pneumoniae can be transmitted through aerosols from person to person, and disease has been produced experimentally by aerosol inoculation (80). Persons with active mycoplasmal infection will carry the organisms in the nose, throat, trachea, and sputum, indicating diffuse involvement. Spread of organisms is greatly facilitated by the ubiquitous cough. Since the organisms tend to be associated with desquamated cells, relatively large droplets may be required for transmission, as evidenced by the close personal contact typical of outbreak settings, e.g., schools, military barracks, and institutions. *M. pneumoniae* infections commonly spread gradually among family members within a household (109, 137, 403). In view of the intimate contact needed for droplet transmission and the slow (6-h) generation time of *M. pneumoniae*, 1 to 3 weeks of incubation for each case is typical, and several cycles may be necessary before intrafamily transmission is complete. Some studies have reported incubation periods from common-source outbreaks of as short as 4 days (363), whereas others (145) have reported longer incubations, with a median of 23 days with intrafamilial spread, where smaller inocula may be involved and transmission may be less effective until the index case has exhibited symptoms for several days. Foy et al. (145) reported that 39% of family contacts may eventually become infected with *M. pneumoniae*, many asymptomatically. Dorigo-Zetsma et al. (109) found that among 79 asymptomatic household contacts of 30 index cases with acute respiratory tract infection due to *M. pneumoniae*, 15% harbored the organism, with a significantly greater number being children under 15 years of age. A novel mathematical model for investigating the control and spread of disease transmission has recently been applied to assess the effects of interventions in outbreaks of *M. pneumoniae* in closed communities (288).

Disease Outbreaks

Numerous outbreaks of *M. pneumoniae* infections have been documented in the community or in closed or semiclosed settings such as military bases (120, 131, 167, 168, 292), hospitals (141, 221, 229), religious communities (253, 299), and facilities for the mentally or developmentally disabled (197, 228, 391). In outbreaks where many more persons, usually living close together in military barracks or similar situations, are exposed to *M. pneumoniae* aerosols simultaneously, the rate of spread within a facility appears to be higher than in single-family households.

Attack rates of *M. pneumoniae* among military recruits and other closed or semiclosed populations can be quite high, with reports ranging from 25 to 71% in some settings (5, 120, 131, 228). Some studies have shown *M. pneumoniae* to be the leading cause of bacterial pneumonia among hospitalized and nonhospitalized military personnel (168, 170). Although long-term morbidity is uncommon, these outbreaks can be very disruptive and can consume significant resources. Strategies to control these outbreaks have included cohorting and use of antibiotics for symptomatic persons and for prophylaxis.

Demographics and Spectrum of Disease

Serological studies performed in the 1960s and 1970s, evaluating the attack rates of *M. pneumoniae* according to sex and broken down into various age groups, have yielded mixed results, with slight gender differences apparent between some age groups. Overall, there appears to be little reason to suspect that males and females have greatly differing susceptibilities to *M. pneumoniae* infections (145, 280, 314).

M. pneumoniae causes up to 40% or more of cases of community-acquired pneumonias and as many as 18% of cases requiring hospitalization in children (5, 47, 137, 156, 180, 182, 199, 273, 357, 403, 446). Older studies relying upon serology and culture reported *M. pneumoniae* pneumonia to be somewhat uncommon in children aged less than 5 years and greatest among school-aged children 5 to 15 years of age, with a decline after adolescence and on into adulthood (5, 143–145). However, *M. pneumoniae* may occur endemically and occasionally epidemically in older persons, as well as in children under 5 years of age (47, 143, 180, 199, 446). Detection of the organism

in 23% of community-acquired pneumonias in children 3 to 4 years of age in a study performed in the United States during the 1990s (47) and documentation of its occurrence in children less than 4 years of age in France (252), without significant differences in infection rates for other children or adults, may reflect the greater number of young children who attend day care centers on a regular basis than in previous years and the ease with which young children share respiratory secretions with older household members or contacts. These recent findings may also reflect improved detection abilities that were unavailable in the 1960s and 1970s, when the first descriptions of *M. pneumoniae* epidemiology and age distribution were published. Although *M. pneumoniae* is generally not considered to be a neonatal pathogen, Ursi et al. (426) described probable transplacental transmission of *M. pneumoniae*, documented by PCR, in the nasopharyngeal aspirate of a neonate with congenital pneumonia.

Whereas pneumonia may be the most severe type of *M. pneumoniae* infection, the most typical syndrome, especially in children, is tracheobronchitis, often accompanied by a variety of upper respiratory tract manifestations. Esposito et al. (125) demonstrated acute *M. pneumoniae* infection in 44 of 184 children with nonstreptococcal pharyngitis (23%), using the criteria of an elevated IgM antibody titer or a fourfold increase in IgG antibody titer and/or a positive PCR assay on the nasopharyngeal aspirate. Since the testing for *M. pneumoniae* was performed only on specimens that were negative for *Streptococcus pyogenes*, it is possible that an even greater proportion of infections due to *M. pneumoniae* might have been detected, since some cases may be mixed. No other clinical manifestations or laboratory analyses other than a history of recurrent episodes of pharyngitis were useful in predicting the presence of *M. pneumoniae*.

M. pneumoniae infection is ordinarily mild, and many adult cases may be asymptomatic, whereas this is much less common in children, perhaps reflecting some degree of protective immunity for reinfections. Moreover, subsequent infections may be more common following initial mild infections as opposed to infection in which pneumonia develops, perhaps due to lesser stimulation of the immune response (147).

Although most mycoplasma infections occur among outpatients (hence the colloquial term "walking pneumonia"), *M. pneumoniae* is a significant cause of bacterial pneumonia in adults requiring hospitalization in the United States. Marston et al. (283) reported that *M. pneumoniae* was definitely responsible for 5.4% and possibly responsible for 32.5% of 2,776 cases of community-acquired pneumonia in hospitalized adults in a two-county region of Ohio, using CF antibody determinations for detection. Extrapolation of these data nationally provides an estimated 18,700 to 108,000 cases of pneumonia in hospitalized adults due to *M. pneumoniae* annually in the United States. Since the majority of patients with pneumonia in the United States are treated as outpatients, the total number of pneumonias due to *M. pneumoniae* is almost certainly many times greater, and as many as half of all infections in adults may even be asymptomatic. An additional striking finding of the study by Marston et al. (283) was their observation that the incidence of pneumonias due to *M. pneumoniae* in hospitalized adults increased with age, and it was second only to *S. pneumoniae* in elderly persons (Fig. 6).

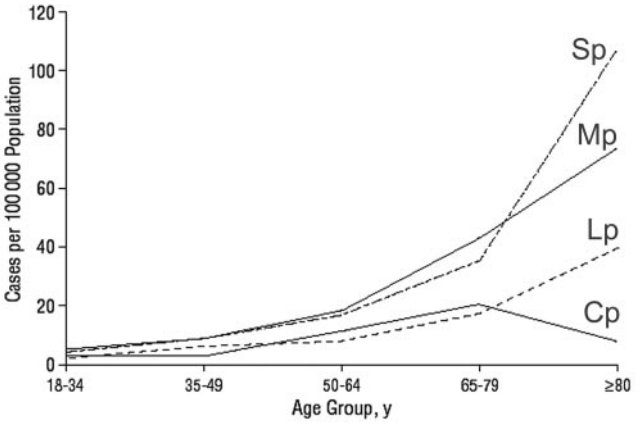

FIG. 6. Data from an active surveillance study performed in Ohio in 1991, showing age-specific rates of community-acquired pneumonia due to the major bacterial pathogens. *M. pneumoniae* infections were diagnosed by seroconversion, using CF tests. These data demonstrate that *M. pneumoniae* causes a relatively large proportion of pneumonias of sufficient severity to warrant hospitalization among persons younger than 50 years but that it is also an important cause of pneumonia in older age groups. Sp, *S. pneumoniae*; Mp, *M. pneumoniae*; Lp, *L. pneumophila*; Cp, *C. pneumoniae*. Reprinted from reference 283 with permission of the publisher.

Another study of hospitalized adults with community-acquired pneumonias performed in Israel (334), which used commercial serological kits to detect antibodies, showed *M. pneumoniae* to be second only to *S. pneumoniae*, and it was responsible for 29.2% of pneumonias overall.

CLINICAL SYNDROMES

Respiratory Tract Infections

The clinical entity of pneumonia eventually proven to be caused by *M. pneumoniae* was recognized many years before the actual identity and nature of the etiological agent were established. The first clues to differentiate pneumonia eventually proven to be due to mycoplasma from classical pneumococcal pneumonia came from the observations that some cases failed to respond to treatment with sulfonamides or penicillin. The lack of response to antimicrobial therapy was deemed "atypical," and the condition was thought likely to be a primary form of lung disease of uncertain etiology; hence, the term "primary atypical pneumonia" was coined. This term, along with "walking pneumonia," has been used widely by physicians and the lay public to denote mycoplasmal respiratory disease.

M. pneumoniae infections may be manifested in the upper respiratory tract, the lower respiratory tract, or both. The frequency of nonspecific upper respiratory tract infection manifestations has varied among numerous studies published since the mid-1960s, with some reports indicating that as many as 50% of patients with *M. pneumoniae* infection present with upper respiratory tract illness (132). Symptomatic disease typically develops gradually over a period of several days, often persisting for weeks to months. The most common manifestations include sore throat, hoarseness, fever, cough which is initially nonproductive but later may yield small to moderate amounts of nonbloody sputum, headache, chills, coryza, myalgias, earache, and general malaise (80, 137, 273, 392, 403).

Dyspnea may be evident in more severe cases, and the cough may take on a pertussis-like character, causing patients to complain of chest soreness from protracted coughing (80). Inflammation of the throat may be present, especially in children, with or without cervical adenopathy, and conjunctivitis and myringitis sometimes occur (7, 125, 152). Children under 5 years of age are most likely to manifest coryza and wheezing, and progression to pneumonia is relatively uncommon, whereas older children aged 5 to 15 years are more likely to develop bronchopneumonia, involving one or more lobes, sometimes requiring hospitalization (137, 273, 392). Mild infections and asymptomatic conditions are particularly common in adults, and bronchopneumonia involving one or more lobes develops in 3 to 10% of infected persons (61). As mentioned above, *M. pneumoniae* is an important cause of pneumonia sufficiently severe to require hospitalization, especially in elderly persons (282, 283, 334). Several studies from the 1960s and 1970s indicate that *M. pneumoniae* may cause up to 5% of cases of bronchiolitis in young children (102, 112, 161, 271).

Chest auscultation may show scattered or localized rhonchi and expiratory wheezes. Since the alveoli are usually spared, rales and frank consolidation are fairly uncommon unless atelectasis is widespread. In uncomplicated cases, the acute febrile period lasts about a week, while the cough and lassitude may persist for 2 weeks or even longer. The duration of symptoms and signs will generally be shorter if antimicrobial treatment is initiated early in the course of illness (80).

It is important for clinicians to understand that the clinical presentation of *M. pneumoniae* respiratory disease is often similar to what is also seen with other atypical pathogens, particularly *C. pneumoniae*, various respiratory viruses, and bacteria such as *S. pneumoniae. M. pneumoniae* may also be present in the respiratory tract concomitantly with other pathogens (47, 109, 137, 180, 182, 252, 446), and there is some evidence from humans and animal models indicating that infection with *M. pneumoniae* may precede and somehow intensify subsequent infections with various respiratory viruses and bacteria, including *S. pyogenes* and *Neisseria meningitidis* (78). Potential explanations for such a synergistic effect include immunosuppression or alteration in respiratory tract flora due to the presence of *M. pneumoniae* (78, 255, 269, 358). Children with functional asplenia and immune system impairment due to sickle cell disease, other conditions such as Down syndrome, and various immunosuppressive states are at risk of developing more fulminant pneumonia due to *M. pneumoniae* (38, 137, 151, 192, 273, 378, 403).

Children with hypogammaglobulinemia are also known to be at greater risk for development of respiratory and joint infections due to *M. pneumoniae*, demonstrating the importance of functional humoral immunity in protection against infections due to this organism (137, 351, 403, 410). Roifman et al. (351) reported that 18 of 23 patients with hypogammaglobulinemia had one or more episodes of acute respiratory illness during which *Ureaplasma urealyticum*, *M. orale*, or *M. pneumoniae* was isolated from sputum. Resolution occurred followed institution of specific antibiotic therapy and elimination of the mycoplasmas. *M. pneumoniae* was isolated from the joint of a patient with arthritis and from six patients with chronic lung disease. Clinical improvement, albeit transient, coincided with negative mycoplasma culture results. There are a few case reports of *M. pneumoniae* infections in pediatric AIDS patients (49, 210), but is not known whether the incidence or severity of pulmonary or extrapulmonary *M. pneumoniae* infections in AIDS patients is increased significantly or how any immunosuppressed state specifically affects host resistance to *M. pneumoniae* infection. Fulminant infections with multiple organ involvement and deaths due to *M. pneumoniae*, usually in otherwise healthy adults and children, have been reported but are uncommon (73, 93, 103, 149, 198, 364, 372, 402, 403, 416, 442).

Extrapulmonary Manifestations

As many as 25% of persons infected with *M. pneumoniae* may experience extrapulmonary complications at variable time periods after onset of or even in the absence of respiratory illness. Autoimmune reactions have been suggested to be responsible for many of the extrapulmonary complications associated with mycoplasmal infection (403). However, the availability of PCR has greatly enhanced understanding of how *M. pneumoniae* can disseminate throughout the body. The presence of *M. pneumoniae* in extrapulmonary sites such as blood, synovial fluid and cerebrospinal fluid, pericardial fluid, and skin lesions has been documented by PCR as well as culture, so direct invasion must always be considered (23, 220, 235, 306, 360). However, the frequency of direct invasion of these sites is unknown because the organism is rarely sought for clinical purposes. It is also important to realize that extrapulmonary complications can be seen before, during, or after pulmonary manifestations or can occur in the complete absence of any respiratory symptoms (62).

Central nervous system (CNS) complications are recognized as among the most common of extrapulmonary manifestations of *M. pneumoniae* infection (384) and have been known to occur since the first report appeared in 1943, even before the true identity of the causative organism was known (56). Approximately 6 to 7% of hospitalized patients with serologically confirmed cases of *M. pneumoniae* pneumonia may experience neurological complications of varying severity (237, 294, 332, 387). Such complications have included encephalitis, cerebellar syndrome and polyradiculitis, cranial nerve palsies, aseptic meningitis or meningoencephalitis, acute disseminated encephalomyelitis, coma, optic neuritis, diplopia, mental confusion, and acute psychosis secondary to encephalitis (39, 98, 159, 226, 264, 331, 386, 392). A number of motor deficiencies have also been described, including cranial nerve palsy, brachial plexus neuropathy, ataxia, choreoathetosis, and ascending paralysis (Guillain-Barré Syndrome) (2, 4, 9, 12, 39, 44, 223, 226, 319). Encephalitis has been the most common neurological manifestation in children (237). Most patients with neurological complications experience them 1 to 2 weeks after the onset of respiratory signs, but 20% of patients or more have no preceding or concomitant diagnosis of respiratory infection (333). This figure may be higher yet in children (413).

Most of the first descriptions of CNS complications were based on serology and later on occasional isolation of *M. pneumoniae* from the respiratory tract rather than the CNS. The lack of clear evidence that mycoplasmas were actually present in neurological tissues led to theories that damage to brain tissue occurred as a result of cross-reacting or autoimmune antibodies (129, 142) and even to concern that neurological

infections by other bacterial pathogens were causing false-positive mycoplasmal serology (230). The potential role of immunological sequelae of *M. pneumoniae* infection that can lead to neurological complications cannot be discounted, and some CNS complications are very likely due to this mechanism as opposed to direct invasion (312, 323). Antibodies against galactocerebroside, a component of CNS myelin, has been detected in 100% of patients with *M. pneumoniae* and CNS involvement and in only 25% of those without CNS involvement (312). Postinfectious leukoencephalopathy due to *M. pneumoniae* also suggests a role for autoimmunity in some cases (325).

Proof that viable organisms or *M. pneumoniae* DNA can be detected directly in neural tissues and CSF provides convincing evidence that this organism does indeed disseminate from the respiratory tract in some instances (3, 105, 198, 251, 307, 401, 403, 415). Neurological manifestations associated with *M. pneumoniae* infections usually resolve completely, but they can result in chronic debilitating deficits in motor or mental function (384). These conditions can be severe and life threatening. Rautonen et al. (341) reported that children with *M. pneumoniae* were seven times more likely to die or have severe neurological sequelae than other children, second only to cases of herpes simplex virus infection. The presence of peripheral neurological sequelae such as radiculitis and transverse myelitis has been identified as a risk factor for chronic CNS sequelae (59, 331). Central nervous system effects due to mycoplasmas have been reviewed in detail by Talkington (401).

Whereas neurological disorders may be the most severe extrapulmonary manifestations of *M. pneumoniae* infections, dermatological disorders, including erythematous maculopapular and vesicular rashes, are perhaps the most common clinically significant complications, occurring in up to 25% of patients. Although the disorders are usually self-limited, severe forms of Stevens-Johnson syndrome, conjunctivitis, ulcerative stomatitis, and bullous exanthems have been reported, and the organism has been detected directly in the cutaneous lesions (70, 71, 256, 276, 395). Clinicians should keep in mind that the presence of erythematous maculopapular rashes in *M. pneumoniae* patients can also be caused by a number of antibiotics commonly used to treat respiratory tract infections.

Nonspecific myalgias, arthralgias, and polyarthropathies occur in approximately 14% of patients with acute *M. pneumoniae* infection and may sometimes persist for long periods (7). Septic arthritis with detection of the organism directly in synovial fluid has been reported numerous times, occurring most commonly in hypogammaglobulinemic patients but sometimes in immunocompetent persons (92, 183, 209, 249, 304, 329, 351, 374, 408, 410, 441). In view of the well-known associations of animal mycoplasmas and their ability to produce chronic, naturally occurring infection of the joints and the experimental models, joint involvement by *M. pneumoniae* is not unexpected. Little attention has been paid to invasive infections of bones due to *M. pneumoniae*, but at least one report of osteomyelitis in a splenectomized patient with hypogammaglobulinemia has been attributed to *M. pneumoniae*, in which the infection was detected by PCR (250).

Cardiac complications associated with *M. pneumoniae* are relatively uncommon, but involvement has been reported at rates of from 1 to 8.5% in persons with serological evidence of infection, somewhat more commonly in adults than in children (287, 330). Pericarditis, myocarditis, and pericardial effusion with and without cardiac tamponade have all been described, and the organism has been detected in pericardial fluid (24, 122, 128, 171, 218, 248, 287, 364, 384, 400). According to one study (330), almost half of the patients with *M. pneumoniae* infection had symptoms or signs of heart abnormalities an average of 16 months later.

Hemolytic anemia is recognized as a rare but severe complication of mycoplasmal pneumonia, occurring more often in children than in adults (70, 122, 381). The mechanism by which *M. pneumoniae* causes this complication has been attributed to cross-reacting cold agglutinins (80, 122, 381, 403, 424). Two cases of aplastic anemia associated with *M. pneumoniae* have also been reported (390). A recent report suggests that thrombotic thrombocytopenic purpura associated with *M. pneumoniae* infection may be the result of cross-reactive antibodies inactivating plasma von Willebrand factor-cleaving protease (23). Fulminant infection leading to fatal disseminated intravascular coagulation has also been reported (73), as has a case of priapism in a 12-year-old boy that was felt to be due to the hypercoagulable state that sometimes occurs in association with *M. pneumoniae* infection (192). If subclinical forms of hemolytic anemia and intravascular coagulation are considered, over 50% of patients with *M. pneumoniae* infections may be affected.

Acute glomerulonephritis, renal failure, tubulointerstitial nephritis, and IgA nephropathy, as well as other conditions, have been sporadically reported in association with *M. pneumoniae* infections (217, 235, 321, 369, 430). Kanayama et al. (217) reported cases of IgA nephropathy in persons in whom the mycoplasma infection was diagnosed serologically. Attempts to demonstrate mycoplasma antigen in damaged renal tissue by immunohistochemical techniques have not been uniformly successful, once again leading to theories that an antibody-mediated pathogenesis is responsible (217, 431). A recent attempt to use PCR to identify mycoplasmas in renal tissue from four children with acute nephritis concomitant with serological evidence of recent *M. pneumoniae* infection also failed (360). However, the presence of mycoplasma antigen has been demonstrated by immunoperoxidase staining in renal tissue in a patient with acute interstitial nephritis (10).

M. pneumoniae infection may be associated with a variety of nonspecific complaints related to the gastrointestinal system. These include nausea, vomiting, and diarrhea. Rarely, cholestatic hepatitis and pancreatitis have been associated with respiratory infections (11, 174, 381, 386).

Up to one-third of patients with *M. pneumoniae* infection may have nonspecific ear symptoms, including otitis externa, otitis media, and myringitis (301, 392). Acute rhabdomyolysis was recently reported in association with *M. pneumoniae* infection in a 15-year-old patient (41). Ocular manifestations have been reported in children occasionally and include conjunctivitis, anterior uveitis, optic neuropathy, retinitis and retinal hemorrhages, iritis, and optic disk swelling, with or without permanent degradation of vision (289, 362). *M. pneumoniae* has been isolated from the urogenital tracts of males and females and has been cultured from a tubo-ovarian abscess (165). Given the apparent ability of the organism to invade the bloodstream, infections in almost any organ system are possi-

ble. Higuchi et al. (184, 185) reported the detection of *M. pneumoniae* by PCR in ruptured atherosclerotic plaques and stenotic heart valves and speculated about its possible association, along with *C. pneumoniae*, as a risk factor for embolization and myocardial infarction. Additional data are necessary to determine the significance of these preliminary reports.

DIAGNOSIS

General Laboratory Features

Clinical laboratory findings are seldom diagnostic for *M. pneumoniae* infection. About one-third of persons with lower respiratory tract infections may have leukocytosis (392). An elevated erythrocyte sedimentation rate may also be observed (45). Gram staining of sputum may show mononuclear cells or neutrophils and normal flora. There are no hepatic or renal abnormalities typical of *M. pneumoniae* infection, although the hemolytic anemia that develops in some patients may be reflected in the hemogram.

Prior to the widespread availability of commercialized antibody assays, and even before the precise bacteriological characterization of the etiological agent was known, clinicians sometimes used the presence of cold agglutinins to confirm their clinical suspicions of primary atypical pneumonia, which was also known as cold-agglutinin disease. Cold agglutinins are actually IgM antibodies that are produced 1 to 2 weeks after initial infection in about 50% of *M. pneumoniae* infections and may persist for several weeks. One theory is that cold agglutinins are the result of cross-reactive autoantibodies developed against the I antigen of human erythrocytes during acute mycoplasmal infection. Another theory is that they develop directly as a result of antigenic alteration of the erythrocytes caused by *M. pneumoniae*. The latter theory is supported by the facts that *M. pneumoniae* is known to interact with and adsorb to human erythrocytes and to produce a hemolysin and because cold agglutinins can be induced in rabbits inoculated with human erythrocytes that were preincubated with *M. pneumoniae* but not in rabbits inoculated with either *M. pneumoniae* or erythrocytes alone (16).

Two varieties of cold agglutinin determination have been used. The simplest version of the test was what is referred to as "bedside cold agglutinins." This consists of collecting blood from a patient suspected of having mycoplasmal pneumonia into a tube containing citrate or another suitable anticoagulant and placing it in ice water in a freezer for approximately 30 s. The tube is then examined for coarse agglutination by being tilted on its side. On warming, the agglutination should resolve, but it can be reproduced by repeating the cooling procedure. A more precise test is to determine the cold agglutinin titer by reacting doubling dilutions of patient sera suspected to contain the cold agglutinins with human type O erythrocytes in the cold and determining the highest dilution at which agglutination occurs. The cold agglutinin response often correlates directly with the severity of pulmonary involvement; patients with extensive lobar involvement will usually have a titer of $\geq$1:32, whereas those with only minimal illness may not develop cold agglutinins. However, cold agglutinins also occur in persons who have various bacterial and rickettsial infections as well as in those with influenza virus and adenovirus infections (16). Due to the availability of antibody assays that are based on immunological reagents specific for *M. pneumoniae*, the popularity of testing serum for the presence of cold agglutinins has declined. If used, this test should be limited to persons in whom mycoplasmal pneumonia is a strong possibility. Under these circumstances, a positive test provides reasonable supportive evidence of a mycoplasmal etiology, on the basis of which clinical management decisions can be made.

Radiographic Findings

Primary atypical pneumonia due to *M. pneumoniae* can be extremely variable and mimic a wide variety of lung diseases. The inflammatory response elicited by *M. pneumoniae* causes interstitial mononuclear inflammation in the lungs that may be manifested radiographically as diffuse, reticular infiltrates of bronchopneumonia in the perihilar regions or lower lobes, usually with a unilateral distribution, and hilar adenopathy. Bilateral involvement may occur in about 20% of cases (137). However, lobar consolidation with bilateral alveolar involvement has been described, and the degree of consolidation may exceed what would be expected based on the severity of clinical manifestations (97). Among patients with *M. pneumoniae* pneumonia who require hospitalization, up to 10.9% will require mechanical ventilation (282).

Pathological Findings

Histopathological examinations from fatal cases of *M. pneumoniae* pneumonia, biopsy material, examination of tissues from animal models, and tracheal organ cultures have shown lesions of the epithelial lining of the mucosal surfaces with ulceration and destruction of ciliated epithelium of bronchi and bronchioles. Edema of bronchial and bronchiolar walls; bronchiolar and alveolar infiltrates of macrophages, lymphocytes, neutrophils, plasma cells, and fibrin; and bronchiolitis obliterans have been described (65, 77, 93, 259, 277, 352, 372, 432). Type II pneumocyte hyperplasia and diffuse alveolar damage have also been reported. Pleura may contain patches of fibrinous exudates. Pleural effusions and diffuse alveolar damage sometimes occur in association with more severe cases, and long-term sequelae such as pleural scarring, bronchiectasis, and pulmonary fibrosis have been reported (40, 72, 97, 222, 338, 372). Lung abscesses may also occur (258, 379). Recent studies utilizing animal models developed to investigate the potential role of *M. pneumoniae* in chronic lung conditions such as asthma (discussed above) have also provided insights into the histopathological aspects of *M. pneumoniae* lung disease (178, 179). Immunosuppressed persons with *M. pneumoniae* infection may lack pulmonary infiltrates, further attesting to the importance of the host immune response in lesion development (151).

Microbiological Tests

The lack of rapid and accurate diagnostic laboratory tests to detect *M. pneumoniae* infection has hampered understanding of the epidemiology as well as contributed to unawareness of the potential clinical significance of this common pathogen by many physicians. From the microbiologist's point of view, one

might argue in favor of the need to routinely use the best means available to identify persons with acute *M. pneumoniae* respiratory tract infection under all circumstances. However, from a practical standpoint, one must also consider therapy that will generally be provided empirically. Thus, the performance of diagnostic tests for *M. pneumoniae* in a typical ambulatory care setting may be unnecessary much of the time. This approach has been taken in the Infectious Disease Society of America diagnosis and treatment guidelines for community-acquired pneumonias (25). However, in the event of an illness in which *M. pneumoniae* is suspected and that is of sufficient severity as to require hospitalization, especially if the patient has any sort of immune deficiency or underlying condition that may make an unfavorable outcome more likely, attempts to detect *M. pneumoniae* infection are justified. Unfortunately, most of the diagnostic methods that are currently available for detection of *M. pneumoniae* infections are somewhat better suited for use in epidemiological studies as opposed to direct management of individual patients due to their prolonged turnaround time, limited availability, and cost. Among currently available test modalities, each has limitations.

Descriptions of the physiological, serological, and morphological procedures for detection and identification of mycoplasmas in clinical specimens have been summarized and updated to include more of the molecular-based methods in *Molecular and Diagnostic Procedures in Mycoplasmology*, vol. 2, published in 1996 (421). Waites et al. (434, 438, 439) have summarized current methods for clinical diagnosis and susceptibility testing of human mycoplasmas and have discussed commercial products used for culture and serology. Razin (343) has also provided an overview of diagnosis of mycoplasmal infections with commentary on various molecular-based methods. Brief summaries and commentary on culture-based and non-culture-based methods for detection of infections due to *M. pneumoniae* are presented here.

Culture. Culture of *M. pneumoniae* from the respiratory tract and other body sites is laborious and expensive, requiring serial blind passages, specialized and expensive growth media, and incubation periods of up to several weeks. Compared to molecular techniques such as PCR, its analytic sensitivity may be no more than 60% in experienced laboratories, even when rigorous adherence to procedures known to enhance cultural isolation are used (199). However, when positive, culture has the advantage of being 100% specific, provided that appropriate additional procedures are used to identify the organism isolated to species level. The persistence of *M. pneumoniae* for variable lengths of time following acute infection also makes it difficult in some cases to assess the significance of a positive culture or assay without additional confirmatory tests such as serology, as discussed above. For the reasons cited above, culture is rarely used for routine diagnosis and/or management of *M. pneumoniae* infections. In view of the many limitations of culture for detection of *M. pneumoniae*, if attempted it should be augmented by additional diagnostic methods such as PCR and/or serology.

The lack of reliable commercially prepared media in the past effectively prevented many clinical laboratories from offering *M. pneumoniae* detection by culture, even before alternative techniques such as PCR existed. If culture is attempted, isolation of *M. pneumoniae* from nasopharyngeal or throat swabs or lower respiratory tract specimens should be considered clinically significant in most instances but should be correlated with the presence of clinical respiratory disease due to the possibility of asymptomatic carriage. Due to the organism's sensitivity to adverse environmental conditions, proper specimen collection, storage, and transport are critical for maintaining viability for culture processing and DNA extraction. Currently recommended methods for specimen collection, transport medium, transport and storage conditions, selection and preparation of growth medium, inoculation, incubation, and organism identification for patients suspected of having *M. pneumoniae* infections have been described in considerable detail in reference texts (434, 438, 439), and only a brief commentary will be provided here.

Isolation of *M. pneumoniae* from essentially any type of clinical specimen from the respiratory tract, as well as from other body fluids and tissues that are suitable for cultivation of conventional bacteria, is possible. SP4 medium, developed over 20 years ago by Tully et al. (422), has become the most successful and widely used broth and agar medium for cultivating *M. pneumoniae* for clinical purposes. It is sold commercially in the United States by Remel Laboratories (Lenexa, Kans.). Detection of *M. pneumoniae* by culture is predicated on its characteristic hydrolysis of glucose, with a resultant acidic shift, after 4 days or more of incubation in broth containing a phenol red pH indicator. Initial specimens, as well as broths with color change and blind subcultures, should be transferred to SP4 agar, incubated, and examined under a stereomicroscope at regular intervals to look for development of spherical colonies of up to 100 μm in diameter (Fig. 2). Incubation of *M. pneumoniae* broth cultures under atmospheric conditions at 37°C is satisfactory, but agar plates will yield the best colonial growth if 5% CO_2 is provided and plates are sealed to prevent loss of moisture during prolonged incubation. A biphasic medium consisting of an agar slant in a screw-cap bottle to which broth is added is sold by Irvine Scientific in the United States under the trade name Mycotrim RS.

Although the common commensal mycoplasmal species from the oropharynx have different metabolic properties, growth rates, and requirements that distinguish them from *M. pneumoniae*, it is necessary to perform additional tests to conclusively prove the identification of a mycoplasma from the respiratory tract or another body site as *M. pneumoniae*. Such procedures for species identification include hemadsorption with guinea pig erythrocytes, reduction of tetrazolium, agar growth inhibition with homologous antisera, epi-immunofluorescence or immunoperoxidase staining, immunoblotting with monoclonal antibodies, metabolism inhibition tests, and PCR assays. Unfortunately, none of these methods has been adapted for development of a commercial product that can be easily purchased and used in a diagnostic laboratory. Hemadsorption assays and tetrazolium reduction can theoretically be accomplished in most clinical laboratories that do not have capabilities for molecular diagnostic testing. However, the cost, the technical expertise required, and the very limited availability of the reagents for the immunologically based methods of species identification effectively preclude their use outside specialized reference or research laboratories.

Antigen detection techniques. Rapid assays for direct antigenic detection of *M. pneumoniae* in respiratory tract speci-

mens have included direct immunofluorescence, counterimmunoelectrophoresis, immunoblotting, and antigen capture enzyme immunoassay (157, 188, 189, 231, 234). The utility and general acceptance of these techniques have been reduced by low sensitivity and cross-reactivity with other mycoplasmas found in the respiratory tract. Considering that the concentration of *M. pneumoniae* cells typically found in sputum specimens from infected patients is approximately 10^2 to 10^6 CFU/ml and that antigen detection techniques may be able to detect about 10^3 to 10^4 CFU/100 μl of specimen, nonamplified antigen detection assays are at the limit of sensitivity for detection and are not recommended for diagnostic purposes when superior technology utilizing nucleic acid amplification is possible. Although not sold in the United States at present, commercial antigen detection tests for *M. pneumoniae* are available in some European countries.

DNA probes. DNA hybridization techniques for the diagnosis of *M. pneumoniae* infection were developed in the early 1980s and had about the same diagnostic sensitivity as antigen detection techniques. These methods have been reviewed by Razin (344). The 16S rRNA genes have been widely used as targets, as have probes consisting of rDNA. GenProbe (San Diego, Calif.) previously sold a ^{125}I-labeled DNA probe for an rRNA sequence specific for *M. pneumoniae*, but the more recently available amplification techniques, such as the PCR assay, that do not involve radioisotopes and have greater sensitivity led to its discontinuation.

PCR. Development of molecular-based testing such as the PCR assay has lessened the importance of culture as a means for detecting *M. pneumoniae*. Studies since the late 1980s using simulated clinical specimens, animal models, and later clinical trials have validated the ability of PCR to detect *M. pneumoniae*, often in conjunction with serology and/or culture (43, 46, 47, 53, 96, 108, 111, 127, 180, 199, 211, 215, 239, 337, 382, 417, 443). The same types of clinical specimens that can undergo culture can also be tested by PCR. The use of two different targets can maximize the ability to detect the organism. The conventional PCR procedure currently used at the Centers for Disease Control and Prevention has been adapted from the procedure originally described by Bernet et al. (43), using primers derived from the *M. pneumoniae* ATPase gene. They have also developed a real-time PCR which targets this gene. Other sequences, primarily the P1 adhesin and conserved regions of 16S rRNA, have also been utilized (96, 211, 236, 275, 344, 402, 427, 429, 442). Table 2 lists selected original references for *M. pneumoniae* nucleic acid amplification assays that describe different target regions of the genome. For additional information and tabulations of various nucleic acid amplification assays according to respiratory specimen tested, or by publication with or without validation data, the reader is referred to three recent publications on this topic (95, 272, 300). Among the other advantages of PCR are that it can be used to detect mycoplasmas in tissue that has already been processed for histological examination or in cultures that are contaminated where culture is impossible, it requires only one specimen, it can be completed in 1 day, it may be positive earlier in infection than serology, and it does not require viable organisms (402, 434, 442). Specific advantages of RNA-based amplification techniques are the high sensitivity that can be achieved due to the large number of rRNA copies per mycoplasmal cell and the fact that its detection is more indicative of viable mycoplasmas in a clinical sample (95).

TABLE 2. Selected original references on *M. pneumoniae* nucleic acid amplification assays

Authors	Reference	Gene target	PCR product size (bp)
Bernet et al.	43	ATPase operon gene	144
Jensen et al.	211	P1 gene	153
Van Kuppeveld et al.	428	16S RNA	277
de Barbeyrac et al.	96	P1 gene	466
Luneberg et al.	275	*tuf* gene	950
Ramirez et al.	339	P1 gene	375

It is difficult to compare results of one study utilizing PCR for epidemiological or diagnostic purposes directly with another because of the varied specimen types, DNA extraction and amplification techniques, primer selection, and reference standards used for comparison. Most techniques are basically similar, but they may differ in targeted sequences and primers. However, comparison of the PCR technique with culture and/or serology has yielded varied results, and large-scale experience with this procedure is still limited for *M. pneumoniae*.

In view of the enhanced analytical sensitivity of the PCR assay over culture, a positive PCR result and negative culture can be easily explained. However, in a case with a negative PCR assay and a positive culture (or serology), the presence of inhibitors or some other technical problem with the PCR assay must be considered (127, 199, 346). Reznikov et al. (346) showed that PCR inhibition was much more likely to occur with nasopharyngeal aspirates than with throat swabs and recommended the latter specimen for diagnostic purposes for *M. pneumoniae*. Dorigo-Zetsma et al. (108) performed a comprehensive examination with 18 patients with *M. pneumoniae* respiratory tract infection detected by PCR or serology and showed that sputum was the specimen that was most likely to be PCR positive (62.5%, versus 41% for nasopharynx, 28% for throat swabs, and 44% for throat washes). Dilution of samples may sometimes overcome inhibition of PCR, but this may also diminish the sensitivity because the nucleic acid is diluted along with any inhibitors that may be present. There are also commercial reagents for nucleic acid purification that are effective in removing most inhibitors of amplification in PCR assays.

There is justified concern when the PCR assay is used as the sole means of detection for surveillance purposes without culture, serology, or clinical data because most studies using PCR have not attempted to do any type of quantitation. Since it is not known with certainty whether there is a specific threshold quantity of *M. pneumoniae* in respiratory tract tissues that can differentiate colonization from infection, a positive result by PCR may overestimate the clinical importance of *M. pneumoniae* as a pathogen if the population sampled has a high carriage rate and because of the propensity of this organism to cocirculate with other bacterial and viral pathogens. Williamson et al. (443) suggested a threshold of 10^4 genomic DNA copies per ml of throat washing as a threshold for differentiating clinical infection and carriage. Dorigo-Zetsma et al. (110) also proposed that the number of organisms may be related to the severity of clinical illness. Using a semiquantitative nested

PCR on dilutions of nucleic acid extracted from throat washings of patients with *M. pneumoniae* respiratory disease, they calculated an organism load of 20 to 3,830 CFU/ml of throat sample. The mean *M. pneumoniae* load in samples from hospitalized patients was significantly higher than that in samples from nonhospitalized subjects. These studies are evidence of progress being made in the characterization of the microecological niche occupied by *M. pneumoniae* in the human respiratory tract and the optimum tools for its assessment, but much more remains to be done to fully appreciate and understand the true meaning of these findings.

Dorigo-Zetsma et al. (108) suggested that elderly adults with pneumonia who are PCR positive and serologically negative for *M. pneumoniae* might be deficient in antibody response due to a natural decline in the humoral immune system as part of the aging process (160). This was also observed in other sero-epidemiological studies (94, 181). A positive PCR test and negative serology could also mean that the specimen was collected too early in the course of illness to allow sufficient time for antibody to develop. In view of the difficulty in the interpretation of serological data because of high background IgG level and sometimes prolonged persistence of IgM, there is also the possibility of false-positive serological data when concomitant PCR testing is negative, depending on the test methods and interpretive criteria. In the case of antibiotic treatment prior to specimen collection, the PCR result may be negative as soon as 24 h following treatment, but serological results may remain positive.

Further refinements to traditional PCR assays, such as combining PCR with a hybridization step, employment of a two-step (nested) procedure in which there is reamplification of a PCR product with a second primer set, real-time detection using specific probes with matched internal controls to evaluate polymerase inhibition, and quantitation of *M. pneumoniae* in acute and subclinical infections, will be very important (1, 177, 202, 403).

Until PCR assays can be standardized, made available at a reasonable cost, and sold commercially as complete diagnostic kits suitable for use in hospital-based or other reference laboratories, this method of diagnosis is unlikely to gain widespread use for detection of *M. pneumoniae* infection for clinical as opposed to epidemiological purposes in the United States. Further development of multiplex PCR tests to detect other atypical pathogens such as *C. pneumoniae* simultaneously with *M. pneumoniae* may make this type of test more attractive and practical for routine use in diagnostic laboratories. Such endeavors have been undertaken already, but no diagnostic products are being sold commercially in the United States thus far (84, 173, 418). Hardegger et al. (177) found that a real-time PCR assay was equal to a conventional nested PCR with regard to sensitivity in detection of *M. pneumoniae* in clinical samples, allowing for quantitation of the amplified product during PCR combined with a reduction in hands-on time. Development of quantitative PCR assays will be beneficial in facilitating better understanding of the carrier state associated with *M. pneumoniae*. Other modifications of the PCR assay for detection of *M. pneumoniae*, in which amplification is carried out in sealed glass capillary tubes, have been described by Honda et al. (194).

Serology. Despite its drawbacks for use with immunosuppressed persons who are unable to mount an antibody response, serological diagnosis of *M. pneumoniae* respiratory infections has long been the cornerstone of *M. pneumoniae* diagnosis and for epidemiological studies because of the relative lack of sensitivity and time-consuming nature of culture. Also, the carrier state that may occur in an unknown percentage of persons in the absence of acute infection can potentially confound interpretation of PCR test results. Serum is easy to collect, store, and ship, but the need for acute- and convalescent-phase specimens and the complex and time-consuming nature of many of the serological assays that have been used in the past have limited acceptance of serology for routine diagnostic testing. Some of the newer and improved commercial assays have overcome some of these limitations. In view of these considerations, it is advisable to test simultaneously for both IgM and IgG in paired specimens collected 2 to 3 weeks apart for the most accurate diagnosis of recent or current *M. pneumoniae* infection, especially in adults (411). A fourfold or greater rise in antibody titer indicates a current or recent infection. The late elevation of IgG that sometimes occurs, the high seroprevalence of IgG antibodies that persist for long periods in persons with a history of *M. pneumoniae* infection, and the lack of an IgM response in adults complicate and impose serious limitations on the use of serology as a sole means for diagnosis of *M. pneumoniae* infections (343). Thus, a logical approach would be to incorporate PCR and serological studies for IgG and IgM for optimum diagnosis of *M. pneumoniae* infections.

Historically, the CF test gained early popularity among laboratories that routinely ran CF tests for viral agents, and it was the sole means for detecting antibodies to *M. pneumoniae* for many years. However, mycoplasmas are much more antigenically complex than viruses, leading to nonspecific reactions. The glycolipids of *M. genitalium* are highly cross-reactive with *M. pneumoniae* due to shared lipid antigens (265), and this also causes problems for CF tests. Sera from patients with bacterial meningitis also tend to have high mycoplasmal CF titers. Cross-reactions of CF antigens with other organ-specific antigens unrelated to microorganisms may also occur.

Kenny et al. (225) reported that among patients with *M. pneumoniae* culture-positive, X-ray-proven pneumonia, 53% showed a fourfold titer increase and 36% showed antibody titers of $\geq$32. With both high titers and high stationary-phase titers as criteria, the sensitivity of the CF test was 90% and the specificity was 88%. Single titers of $>$32 are sometimes considered to be indicative of recent infection. However, this endpoint varies greatly among laboratories, and antibodies to glycolipid antigens may persist for at least a year after infection (343). Confirmation of CF test results with Western immunoblots can aid in interpretation but greatly adds to the time and expense of testing. Since CF measures mainly IgM and to a minor extent IgG, its diagnostic value may be limited to initial *M. pneumoniae* infections (203).

CF tests have largely been replaced by improved methods using alternative technologies that are less time-consuming and labor-intensive. The target antigens vary among tests; some are protein, while others are glycolipid. Whether there is a significant cross-reactivity with these *M. pneumoniae* antibody test systems and *M. genitalium* has not been established

with certainty, but it seems somewhat unlikely. A great many publications describing a number of different techniques for measuring antibody response to *M. pneumoniae* infection have appeared since the early 1980s, but many of the assays have never been developed for commercial distribution. Some have been used for seroepidemiology studies, however. Several of the existing commercial kits have been evaluated by using CF as the reference method. Considering its lack of antibody class distinction and tendency for cross-reactivity with other microorganisms, CF is not really suitable as a reference standard. Appreciation of this fact makes interpretation of the specificity and sensitivity results of comparative studies problematic. Some recent studies evaluating commercial kits have merely compared one method or product with another without consideration of CF data, but since results and conclusions from some studies are based upon assay of a single acute-phase serum sample while others have used paired specimens, direct comparisons and extrapolations from multiple studies become rather complex and are not always feasible.

Assay formats adapted for commercial distribution include indirect immunofluorescence assay (IFA), particle agglutination (PA) assay, and enzyme-linked immunoassay (EIA). A German company, Genzyme Virotech GmbH, has developed a Western blot assay for detection of IgG and IgA, but this test is not sold in the United States. Summaries of the major commercial serological test kits and their various assay formats have been recently published (434, 439), so explicit details of the various methods and their procedures are not provided in this review.

IFAs for *M. pneumoniae* consist of antigen affixed to glass slides. Specific antibody is detected after staining with anti-human IgM or IgG fluorochrome conjugate. These kits provide accurate, quantitative serological data, but their interpretation is subjective and a fluorescence microscope is necessary. Results can be affected by the presence of rheumatoid factor and high *M. pneumoniae*-specific IgG antibody levels, and additional procedures are required to validate IgM results in these settings (403).

The PA tests in use today mainly utilize latex or gelatin as the carrier particles that are incubated with test serum. If the serum contains specific antibodies, the particles agglutinate, resulting in a visible reaction. PA tests use a mixture of *M. pneumoniae* antigens to detect both IgG and IgM simultaneously. Several PA products that provide qualitative results that can be visualized on a card or quantitative data that can be read in a microtiter plate format can be purchased commercially (13, 22, 116, 219, 262, 286), but not all of the products are available in the United States. Lieberman et al. (262) concluded that the Sero *M. pneumoniae* antibody capture EIA kit was better than the Fujirebio Serodia Myco II PA kit for diagnosis of current *M. pneumoniae* infection when a single serum sample was tested, but they stressed that testing of acute- and convalescent-phase sera is required for precise diagnosis, even with the EIA. Barker et al. (22) also compared the Serodia Myco II PA kit with IgM-specific antibody capture EIA and IFA. They determined that this PA test was not as specific for detection of IgM as the other tests. Aubert et al. (14) felt that PA tests performed about as well as EIAs and IFAs, but Matas et al. (286) reported the Meridian ImmunoCard EIA was able to detect lower levels of IgM antibodies than either the Serodia Myco II kit or CF tests. Karppelin et al. (219) evaluated the Serofast (International Microbio, Toulon, France) and found it to be less sensitive than CF and EIA. Echevarria et al. (116), compared the Serofast kit to an antibody capture EIA by using sera that were shown to be either positive or negative by CF. Both tests were shown to be sensitive and specific, but the performance of the antibody capture EIA for detection of IgM diminished with older persons. Based on evidence presented to date, PA assays do not offer any advantages over other techniques such as EIAs or IFAs, except possibly their ease and simplicity of performance.

EIAs are the most widely used commercial mycoplasma serology tests in the United States. They are amenable to a variety of assay conditions suitable for testing large or small numbers of sera, detect very small amounts of antibody, require serum volumes of $\leq$100 μl, can be made isotype specific, and are generally more sensitive than CF (434). EIAs are more sensitive for detecting acute infection than culture and can be comparable in sensitivity to PCR, provided that a sufficient time has elapsed since infection for antibody to develop and that the patient has a functional immune system. Crude multiantigen preparations, purified proteins (including the P1 adhesin), μ-capture approaches, purified glycolipids, and synthetic peptides have all been used as targets (22, 64, 116, 190, 191, 203, 303, 399, 425, 445). Patient sera are incubated with the solid-phase antigen, and bound antibodies are visualized by using substrate and enzyme-labeled conjugates directed against the primary antibody. The amount of conjugate reacting is proportional to the levels of antibody present in the patient's serum, measured quantitatively with a spectrophotometer. Most EIAs are sold in 96-well microtiter plate formats, although some can be obtained as breakaway microwell strips which allow smaller numbers of sera to be tested ecoomically.

Two EIAs are packaged as qualitative membrane-based procedures for the detection of single test specimens. These are truly rapid EIAs (10 min or less) and are simple to perform. The ImmunoCard (Meridian Diagnostics, Cincinnati, Ohio) is an IgM-only assay that is simple to read, and it is especially useful for testing pediatric samples. As are typical of IgM-only assays based on *M. pneumoniae* protein antigens, the specificity may be somewhat compromised for patients with autoimmune disease and there are the limitations in interpreting the results. Studies using sera from confirmed *M. pneumoniae* cases have also shown that the ImmunoCard performed better than the CF test (6, 286, 412). The Remel EIA is a membrane-based assay that detects IgM and IgG simultaneously and has shown good sensitivity and specificity when compared to other tests, including IFA, the GenBio ImmunoWell EIA, the Diasorin EIA, the Meridian ImmunoCard, and CF (130, 412). The manufacturers have endorsed the use of a single assay with the Remel EIA for diagnosis of acute *M. pneumoniae* infections in young persons, and other manufacturers have suggested that high titers of IgM or IgG above a specified cutoff value may be used to indicate acute infection. However, we contend that acute- and convalescent-phase sera are necessary for greatest accuracy, but we acknowledge the fact that the value of the single-point-of-care tests offered by both the Remel and Meridian kits is lost if paired specimens are required. The cost of

materials to perform these rapid EIAs exceeds U.S. $10 for each patient tested.

ANTIMICROBIAL SUSCEPTIBILITIES AND CHEMOTHERAPY

Antimicrobial Susceptibility Profiles

A summary of the in vitro activities of several antimicrobial agents against *M. pneumoniae* is shown in Table 3. *M. pneumoniae* is inhibited by tetracyclines, macrolides, ketolides, and fluoroquinolones, with little variation in MICs among clinical isolates (435, 436). Thus, in vitro susceptibility testing is not indicated for routine patient management purposes for infections of the respiratory tract. Other agents that are active at the bacterial ribosome, such as streptogramins, chloramphenicol, and aminoglycosides, may also show in vitro inhibitory activity against *M. pneumoniae* but are not widely used for therapeutic purposes against this organism. Clindamycin may be effective in vitro, but limited reports suggest that it may not be active in vivo, and it has not been considered a first-line treatment (80). Due to the lack of a cell wall, all mycoplasmas are innately resistant to all beta-lactams and glycopeptides. Sulfonamides, trimethoprim, polymixins, nalidixic acid, and rifampin are also inactive. Linezolid is the prototype agent of the oxazolidinone class. These agents are much less active against *M. pneumoniae* than the other agents that inhibit protein synthesis (224). New quinolones such as moxifloxacin, gatifloxacin, garenoxacin, gemifloxacin, and sparfloxacin tend to have somewhat greater in vitro activity than older agents such as ciprofloxacin, ofloxacin, and levofloxacin, although MICs of all fluoroquinolones are severalfold higher than those of macrolides (224, 435–437). Fluoroquinolones have been shown to be bactericidal for *M. pneumoniae*, whereas macrolides and tetracyclines are primarily bacteriostatic (36, 113, 435, 437).

The extent of naturally occurring, clinically significant acquired antimicrobial resistance of *M. pneumoniae* to any drug class is not known, but it is generally believed to be uncommon. Treatment failures have been rarely reported for microbiologically proven cases of *M. pneumoniae* infection, and recent studies evaluating macrolides, tetracyclines, or fluoroquinolones indicate comparable in vitro activities against isolates obtained from North America and Europe, with no change from data reported earlier other than some minor variations that may be due to differences in testing methods, media, and inocula (32, 34, 311, 435). However, organisms from acute *M. pneumoniae* infections are seldom sought by culture, they are almost never tested for in vitro susceptibilities for clinical purposes, and most in vitro studies reported to date tested relatively small numbers of clinical isolates from limited geographic areas. Organisms can be shed in respiratory secretions for long periods after acute infection, and high-level-macrolide-resistant strains have been isolated following erythromycin treatment, but resistance did not appear to affect clinical outcome (310, 394). Macrolide-resistant *M. pneumoniae* mutants can easily be selected in vitro (311, 393, 394); such mutants typically exhibit the macrolide-lincosamide-streptogramin B-type resistance, rendering lincosamides and streptogramin B inactive in addition to the macrolides.

TABLE 3. MIC ranges of various antimicrobials against *M. pneumoniae*[a]

Drug	MIC (μg/ml)
Tetracycline	0.63–0.25
Doxycycline	0.016–2
Erythromycin	≤0.001–0.016
Roxithromycin	≤0.01
Dirithromycin	0.12–0.5
Clarithromycin	≤0.001–0.125
Azithromycin	≤0.001–0.125
Josamycin	≤0.01–0.125
Telithromycin	0.008–0.06
Cethromycin	≤0.001–0.016
Clindamycin	≤0.008–2
Lincomycin	4–8
Pristinamycin	0.02–0.05
Chloramphenicol	2
Gentamicin	4
Ciprofloxacin	0.5–4
Ofloxacin	0.05–2
Levofloxacin	0.063–2
Sparfloxacin	≤0.008–0.5
Grepafloxacin	0.06–0.25
Trovafloxacin	≤0.008–0.5
Gatifloxacin	≤0.016–0.25
Moxifloxacin	0.06–0.25
Gemifloxacin	≤0.008–0.125
Garenoxacin	0.008–0.125
Gentamicin	4
Quinupristin-dalfopristin	0.008–0.06
Linezolid	>64
Evernimicin	2.0–4.0

[a] Data were compiled from multiple published studies in which different methodologies, and often different antimicrobial concentrations, were used.

Experimental observations of two laboratory-derived erythromycin-resistant *M. pneumoniae* mutants indicated that macrolide resistance can occur due to point mutations leading to A-to-G transitions in the peptidyl transferase loop of domain V of the 23S rRNA gene at positions 2063 and 2064, which reduces the affinity of these antibiotics for the ribosomes (274, 316). The likelihood of *M. pneumoniae* developing resistance to macrolides by this mechanism under natural conditions may be enhanced, since there is only a single rRNA operon in the *M. pneumoniae* genome (163). No plasmids or *erm* genes to mediate ribosomal modification, or any enzymes that break down macrolides, have ever been described for *M. pneumoniae*. The well-documented occurrences of the *tetM* determinant associated with the conjugative transposon Tn*916*, which confers resistance to the tetracyclines, in *M. hominis* and *Ureaplasma* spp. (34, 348, 349) and of mutations in DNA gyrase and topoisomerase that confer fluoroquinolone resistance (34, 37) prove the potential for mycoplasmas to acquire resistance to these drug classes. However, to date no such naturally occurring resistance to tetracyclines or fluoroquinolones in *M. pneumoniae* has been reported in the published medical literature to our knowledge. Whether the bactericidal effect of various fluoroquinolones against *M. pneumoniae* shown in vitro is more effective in preventing persistence of the organism in respiratory secretions after clinical resolution has not been documented.

In Vitro Susceptibility Testing

Even though susceptibility testing is not necessary to guide treatment of *M. pneumoniae* infections, such procedures are needed in order to evaluate new or investigational antimicrobial agents in comparison to existing drugs, occasionally for systemic infections (especially if the host is immunosuppressed), if treatment is not successful clinically, and/or for eradication of the organism from a normally sterile site. There are no official guidelines for performance, interpretation, or quality control of in vitro susceptibility tests for human mycoplasmas published by the National Committee for Clinical Laboratory Standards (NCCLS). However, a subcommittee of the NCCLS was established in 2001 to address this, and work is currently under way to develop in vitro susceptibility protocols and quality control ranges for representative drug classes for *M. pneumoniae*, *M. hominis*, and *Ureaplasma* spp. In the absence of NCCLS-sanctioned methods, the Mycoplasma Chemotherapy Working Team of the International Research Program on Comparative Mycoplasmology has formulated methods for determination of MICs by broth dilution and agar dilution. These methods have been summarized by Waites et al. (434). Procedures for determination of MICs for human mycoplasmas are also available in other publications, which can be used as a basis for development of laboratory protocols (33, 407). Many aspects of susceptibility testing of mycoplasmas are identical to the procedures that have been developed and standardized for conventional bacteria. However, the fastidious nature, complex growth requirements, and incubation length and conditions for mycoplasmal growth have to be considered in optimizing testing for these organisms. Broth- and agar-based methods for determining MICs have been adapted for testing mycoplasmas, and some general comments regarding the procedures to be performed are relevant for both techniques. As with all bacteria, the inoculum of organisms is important. Since mycoplasmas do not produce turbidity in liquid medium, it is somewhat difficult and complex to determine the number of viable cells present in an active culture. Experience has shown that MICs are not greatly affected when *M. pneumoniae* taken from frozen stocks is used. Thus, some laboratories prefer to test a frozen stock culture that was serially diluted previously to determine the number of viable cells and then dilute it to an inoculum containing 10^4 to 10^5 color-changing units/ml for use directly after a 2-h prewarming period (435, 436). SP4 medium and Hayflick's modified medium (pH 7.4 to 7.6) have been used for determining MICs for *M. pneumoniae*, with similar results (35, 434, 435). MIC test mixtures should be incubated at 37°C. Broth-based test mixtures can be incubated under atmospheric conditions, whereas agar plates should be supplemented with 5% CO_2 for optimum colony development. Mandatory controls that must be used for all types of MIC assays include a sterility control, a growth control, and solvent control. Simultaneous testing of an isolate of *M. pneumoniae* for which MICs of the drugs of interest are known and consistent is also advisable. Due to the slow growth of *M. pneumoniae*, MIC test mixtures have to be incubated for 5 days or more and examined daily. No breakpoints to designate susceptibility or resistance have been designated for mycoplasmas. However, some inference about the meaning of the MICs obtained can be made based on breakpoints established for other bacteria and the achievable concentrations within the body for the drugs being tested. Generally speaking, if the MIC is ≤ 1 μg/ml, the drug may potentially be active against *M. pneumoniae*.

The agar dilution method of susceptibility testing has the advantages of having a stable endpoint over time, unlike broth dilution, where color change endpoints tend to shift over time. Agar dilution is suitable for testing large numbers of organisms at once. Testing small numbers of isolates by agar dilution is not very practical, since several individual agar plates must be prepared for each drug to encompass all of the dilutions that may be needed and media must be used when fresh. This method of testing is ideally performed by using a Steers replicator to deliver the inoculum to the agar plate, with a goal of generating 30 to 300 colonies in the growth control (434). The MIC is read as the lowest concentration of drug that prevents colony formation when colonies are evident on the growth control plate.

The broth microdilution technique is based on the principle that a constant number of organisms are added to serial doubling concentrations of antimicrobial agents diluted in broth in a 96-well microtiter plate. Broth microdilution is the most practical and widely used method of susceptibility testing for *M. pneumoniae*. Multiple antimicrobials can be tested in the same microtiter plate at several different dilutions. The inoculated microtiter plates are sealed with an adhesive cover to prevent drying out and are incubated until the growth control shows a color change. The MIC is read as the lowest concentration of drug that inhibits growth at the time the growth control first shows evidence of growth. Minimal bactericidal concentrations can be tested directly by diluting and subculturing wells in the microtiter plate that show no evidence of growth (color change) into fresh medium. When the minimal bactericidal concentration is within two dilutions of the MIC (fourfold), the agent may be considered bactericidal (435).

Waites et al. (435) have described methods for performing time-kill assays in which multiple dilutions of antimicrobials are incubated in broth with inocula of *M. pneumoniae*. Bactericidal activity over time is assessed by removal of small volumes of broth daily for up to a week and subculturing to agar plates. Colony counts performed on the subcultures will demonstrate the extent of bacterial killing and the time of exposure to the antimicrobial that is necessary to achieve this effect. Traditionally, a reduction of 99.9% or 3 $\log_{10}$ dilutions of the original inoculum has been used to define bactericidal as opposed to bacteriostatic activity (435).

Treatment of Infections Due to *M. pneumoniae*

When *M. pneumoniae* infections were first described soon after the organism was characterized and isolated in culture in the 1960s, there was some sentiment that antibiotic therapy was unnecessary since the disease is self-limiting most of the time. We now know that administration of antimicrobials will generally produce satisfactory results, with a marked reduction in duration of respiratory symptoms compared to nontreatment. Treatment of *M. pneumoniae* infections on a case-by-case basis has been guided primarily by its well-known and consistent susceptibilities to drugs in the macrolide, tetracycline, and fluoroquinolone classes that are available for oral

administration. Appreciation of *M. pneumoniae* as a significant respiratory tract pathogen and the numerous new drugs in the macrolide and fluoroquinolone classes that have become available since the early 1990s have led to the performance of several trials involving adults and children with community-acquired pneumonia in which the clinical efficacies of various antimicrobials were evaluated. Many published clinical trials have been able to identify relatively small numbers of community-acquired pneumonias proven to be caused by *M. pneumoniae* and have usually relied upon serological diagnosis, although some recent studies have incorporated culture and/or PCR and thereby improved the numbers of microbiologically proven cases. The use of serology alone as a diagnostic measure precludes determination of whether a treatment regimen actually eradicates the organism, and thus data regarding the microbiological efficacy of any antimicrobial regimen are relatively sparse.

Newer fluoroquinolones are being used extensively for treatment of respiratory tract infections in adults since they can be used empirically to treat infections due to mycoplasmas, chlamydiae, legionellae, *M. catarrhalis*, and *S. pneumoniae* (including penicillin- and macrolide-resistant organisms); they have an acceptable safety profile; and they can be given orally once daily with favorable results. However, fluoroquinolones are not recommended for use in children due to possible toxicity to developing cartilage. Likewise, tetracyclines are not approved for use in children younger than 8 years of age. Macrolides are generally considered the treatments of choice for *M. pneumoniae* in both adults and children.

Table 4 summarizes several comparative and noncomparative studies in which identification of *M. pneumoniae* infections in adults or children with community-acquired pneumonia was specifically sought and in which the clinical and/or bacteriological response was documented for this organism. The results of these studies have generally shown that newer agents such as clarithromycin or azithromycin are as effective clinically as erythromycin, with cure rates consistently exceeding 90% when such data are available. These recent clinical trials evaluating treatment regimens for *M. pneumoniae* have not included untreated controls, so the cure rates for various drugs cannot account for the possibility of spontaneous resolution, which may occur in approximately 10 days (137). However, a treatment arm of one study (139) consisted of beta-lactams alone, and all 22 patients with *M. pneumoniae* infection identified serologically who received these agents achieved clinical cure, suggesting that these infections can indeed resolve spontaneously, since these drugs would have no effect on this organism. However, there could possibly have been another undetected pathogen involved that was inhibited by these agents, thus accounting for a favorable outcome.

Newer macrolides are generally preferred over erythromycin due to their greater tolerability, once- or twice-daily dosing requirements, and shorter treatment duration in the case of azithromycin, even though their costs are considerably greater. The extremely high potency of azithromycin against *M. pneumoniae* and its long half-life probably account for its ability to cure *M. pneumoniae* infections with very short treatment courses, and possibly even with a single 1.5-g dose, despite the relatively slow growth of the organism (367, 368). Overall, azithromycin and the investigational ketolide cethromycin (ABT-773) are the most potent drugs against *M. pneumoniae* in terms of MICs (436). Recently published clinical trials evaluating the investigational ketolide telithromycin alone (57) or in comparison with the fluoroquinolone trovafloxacin (336) for treatment of pneumonia in adults demonstrated that this ketolide produced clinical cure rates of 100 and 93.3%, respectively, for persons whose infection was due to *M. pneumoniae*.

Some recent studies evaluating fluoroquinolones for treatment of community-acquired pneumonias in adults have included diagnostic tests, primarily serology, for *M. pneumoniae* (138–140, 322, 326). The general conclusions of these studies are that newer fluoroquinolones such as levofloxacin and moxifloxacin are effective clinically against *M. pneumoniae* infections, as one might expect from their in vitro potencies. Specific treatment dosage recommendations and therapeutic regiments for *M. pneumoniae* infections in adults and children are provided in reference texts (433).

Relatively little data are available regarding outcomes of antimicrobial treatment of severely ill children or adults requiring hospitalization for *M. pneumoniae* pneumonia or treatment of immunosuppressed persons with *M. pneumoniae* infection. Eradication of *M. pneumonia*e from persons with immunosuppression can be extremely difficult, requiring prolonged therapy, even when the organisms are susceptible to the expected agents. This difficulty highlights the fact that mycoplasmas are inhibited but not killed by most commonly used antimicrobial agents in concentrations achievable in vivo.

Limited information from case reports suggests that high-dose steroid therapy may be effective in reversing neurological symptoms in children with complicated *M. pneumoniae* infection (175), and some clinicians recommend use of steroids in combination with an antibiotic that can penetrate the CNS, such as doxycycline or chloramphenicol (105, 208). Azithromycin and clarithromycin are commonly used for *M. pneumoniae* respiratory infections, and both have been used successfully in *M. pneumoniae* CNS infections, even though macrolides penetrate poorly into the CNS (175, 384). A recent review of steroid therapy in *M. pneumoniae* CNS cases suggests that it is often beneficial, particularly in severe cases. Of 14 patients with severe *M. pneumoniae* CNS disease, 11 (78%) were reported to have a complete or near complete recovery, a better outcome than reported for an earlier series of patients who did not receive steroids (58). The value of using steroids to treat Stevens-Johnson syndrome caused by *M. pneumoniae* has not been clearly established (257).

Both plasmapheresis and intravenous immunoglobulin therapy might be considered if steroid therapy is ineffective for cases of acute disseminated encephalomyelitis, which is considered to have an important immune component (384). A trial of intravenous immunoglobulin in a critically ill patient with encephalitis and *M. pneumoniae* pneumonia was associated with neurological improvement within 48 h of treatment (361). A patient with *M. pneumoniae* infection suffering from bilateral optic neuritis as well as acute Guillain-Barré syndrome recovered after plasmapheresis (325). A survey of commercial intravenous immunoglobulin therapy preparations found that most preparations had significant activity against *M. pneumoniae* (246).

Antibiotic therapy for persons with asthma and documented infection with *M. pneumoniae* or other organisms that are

TABLE 4. Clinical trials for treatment of community-acquired pneumonia in which tests to identify infections with *M. pneumoniae* were included

Authors	Reference	Patients	Drug(s)	*M. pneumoniae* infections		
				No. of cases	Detection method(s)	Outcome and/or comment
Cassell et al.	63	Adults	Clarithromycin (14 days) vs erythromycin (14 days)	15	Culture, serology	Clinical cure was achieved in 4 of 4 (100%) and 3 of 3 (100%) subjects receiving clarithromycin or erythromycin, respectively
Block et al.	47	Children	Clarithromycin (10 days) vs erythromycin (10 days)	69	Culture, PCR, serology	Mycoplasma eradication was achieved in 9 of 9 (100%) culture-positive evaluable subjects who received clarithromycin vs 4 of 4 (100%) who received erythromycin; eradication rates based on PCR alone were 19 of 22 (86%) vs 24 of 26 (92%) for clarithromycin and erythromycin, respectively (however, significance of a positive PCR assay after treatment is unknown)
Wubbel et al.	446	Children	Azithromycin (5 days) vs erythromycin (10 days) or amoxicillin-clavulanate (10 days)	12	Culture, PCR, serology	All subjects with mycoplasma infection were classified as having clinical cure after treatment with any agent; however, 1 subject in the azithromycin group had a positive follow-up culture and 1 had a positive PCR
Harris et al.	180	Children	Azithromycin (5 days) vs erythromycin (10 days) or amoxicillin-clavulanate (10 days)	124	Culture, PCR, serology	Mycoplasma eradication was achieved in 14 of 14 (100%) culture-positive evaluable subjects who received azithromycin vs 4 of 6 (67%) with erythromycin and 0 of 1 with amoxicillin-clavulanate
Schonwald et al.	368	Adults	Azithromycin (3 vs 5 days)	43	Serology	A 1.5-g total dose of azithromycin was equally effective when administered as a 3- or a 5-day regimen and resulted in clinical cure for all mycoplasmal infections that were encountered
Schonwald et al.	367	Adults	Azithromycin (single dose vs 3 days)	24	Serology	A single 1.5-g dose of azithromycin was equally effective as a 3-day regimen for treatment of mycoplasmal infection
Schonwald et al.	366	Adults	Azithromycin (5 days) vs erythromycin (10 days)	55	Serology	Therapeutic success was achieved for all subjects with mycoplasma infection who received either drug
Kaku et al.	216	Adults	Roxithromycin	13	Culture, serology	Clinical efficacy was documented in 12 of 13 (93%) cases; a bacteriological effect was achieved in 4 of 6 (67%) cases
Patel et al.	322	Adults	Moxifloxacin (10 days)	29	Culture, serology	A bacteriological response occurred in 27 of 29 (93%) subjects with mycoplasmal infection
Finch et al.	140	Adults	Moxifloxacin (7–14 days) vs amoxicillin-clavulanate with or without clarithromycin (7–14 days)	30	Serology	Clinical success was documented 21–28 days after the end of treatment in 12 of 13 (93%) subjects who received moxifloxacin and in 16 of 17 (94%) who received the comparator agents
Plouffe et al.	326	Adults	Ofloxacin vs standard treatments with a variety of agents for different durations	38	Serology	Clinical improvement or cure occurred in 13 of 14 (88%) subjects who were serologically positive for mycoplasma and in 20 of 22 (91%) who received various other treatments
File et al.	138	Adults	Gemifloxacin (7 days) vs trovafloxacin (7 days)	88	Culture, serology	Clinical success for mycoplasmal infections was documented in 41 of 44 (94%) subjects who received gemifloxacin and in 38 of 40 (95%) who received trovafloxacin
File et al.	139	Adults	Levofloxacin (7–14 days) vs ceftriaxone (7–14 days) or cefuroxime (7–14 days)	41	Serology	Clinical improvement or cure was documented in 19 of 19 (100%) subjects treated with levofloxacin and in 22 of 22 (100%) who received either ceftriaxone or cefuroxime; the apparent response to cephalosporins may represent self-limited disease or the presence of an undiagnosed copathogen
Carbon et al.	57	Adults	Telithromycin (7–10 days)	8	Serology	All eight patients with mycoplasmal infection had clinical cure
Pullman et al.	336	Adults	Telithromycin, trovafloxacin	93	Serology, culture, PCR	Clinical cure was documented in 4 of 4 subjects with mycoplasmal infection who received telithromycin and in 5 of 5 who received trovafloxacin

amenable to such treatments may prove useful. However, it should be emphasized that antibiotic administration should ideally be reserved for those asthmatic persons who are actually shown to harbor the organism.

A number of trials of prophylactic antibiotics to control outbreaks of *M. pneumoniae* disease have been performed, with varied success. While some earlier studies used tetracyclines (212), recent investigations used the newer macrolides. Azithromycin has been used effectively for prophylaxis against clinical infection with *M. pneumoniae* during outbreaks (197, 228) with a 75% protective efficacy against illnesses resulting in physician visits among hospital employees according to one study (197). Weekly oral azithromycin (500 mg) had a 64% protective efficacy based on data from serological tests against *M. pneumoniae* infections in U.S. marines (169). These findings support the use of prophylactic antibiotics that are highly active against *M. pneumoniae* in outbreak settings.

VACCINES

Interest in development of a vaccine for *M. pneumoniae* has been evident since the early 1960s, soon after the organism was successfully identified and isolated in culture and much of its epidemiology had become apparent. Interest was fostered by the lack of natural protective immunity following primary infection, prolonged carriage, and propensity for outbreaks of infections in military camps, schools, and hospitals. Development of a vaccine also seemed promising in view of the facts that the organism is rather homogeneous antigenically and there appears to be some protection against reinfection (81, 85, 135, 136). Animal models have been used extensively to improve understanding of the natural immune response to *M. pneumoniae* infection and how it might be modified by immunization (17–20, 75–77, 123, 153, 449, 450). Ellison et al. (123) provided an in-depth discussion of protective antigens, protective immune mechanisms, and prior vaccine strategies for *M. pneumoniae*, summarizing the work performed in this area through the early 1990s.

Considering that attachment and initiation of local damage at the cellular and subcellular levels are responsible for *M. pneumoniae* disease, the logical vaccine strategy is to prevent attachment and thereby prevent initiation of disease. The initial studies with human volunteers used formalin-inactivated vaccines, but their protective efficacy results were generally disappointing for a variety of reasons that have been explored in depth by Ellison et al. (123). Perhaps the most significant event related to volunteer immunizations was the experience of some immunized volunteers who developed more severe illness after experimental challenge with live mycoplasmas. This occurrence has also been documented in animal models after administration of inactivated as well as component vaccines (77, 79, 206), signifying that immunization sensitizes the host in some way through a cell-mediated immune response. Development of live attenuated vaccines administered intranasally or by aerosol inhalation in animal models showed some protective efficacy against disease upon rechallenge with virulent strains, suggesting that stimulation of local mucosal defense mechanisms is important (123, 135, 449). Development of a serum antibody response after mucosal immunization may or may not occur, depending on the immunogen (123, 135, 450). Unfortunately, the live attenuated vaccines never made it to human use due to concern over residual virulence of the vaccine strain of *M. pneumoniae*. Similar problems were encountered with temperature-sensitive mutant vaccines (51, 172, 388, 389). Other vaccine candidates have included acellular protein and polysaccharide components and recombinant DNA (50, 52, 123, 204, 206). While the importance of the P1 adhesin in mediating *M. pneumoniae* cytadherence and initiation of disease cannot be denied, animal studies using P1 as a vaccine antigen have not demonstrated protective efficacy (76, 204–206). Experimental animal studies involving mice (449, 450) hamsters (18, 75–77), guinea pigs (206), and chimpanzees (21, 153) have continued through the 1980s and 1990s, but to our knowledge there have been no recent clinical trials in humans with any newer versions of *M. pneumoniae* vaccines, and there is no indication that any type of vaccine will be approved for use against *M. pneumoniae* any time soon.

FUTURE NEEDS

Multifaceted approaches for detection and characterization of *M. pneumoniae* and its associated diseases by using PCR, serology, and culture, augmented by knowledge obtained from the complete genome sequence, have been applied in epidemiological investigations, animal models of disease, evaluation of diagnostic reagents, and clinical trials of antimicrobial agents. As a result, our understanding of this organism's cell biology, interactions with host cells, mechanisms of disease production, evasion of host defenses, disease transmission, and contribution to chronic lung diseases and of the efficacy of new antimicrobial treatments have improved, with much knowledge accumulated during the past decade. Despite these many advances, much is still unknown about this tiny bacterium, which is among the smallest of all free-living life forms. Most mycoplasma infections in clinical settings never have a microbiological diagnosis because rapid, sensitive, specific, and reasonably priced methods for its direct detection are not readily available in many physician offices or hospital laboratories. From a practical standpoint, serological tests are the only means by which *M. pneumoniae* infections are diagnosed on a wide scale, and this method has a number of limitations. A reliable and user-friendly amplified or nonamplified method for detection of the mycoplasma or its nucleic acid in clinical specimens would be of immense importance for patient diagnosis and management, for furthering knowledge of a potential role in chronic lung disease, and for evaluation of microbiological efficacy in clinical trials of antibiotics. Development of a safe vaccine that offers protective immunity might also go a long way towards reducing the extent of *M. pneumoniae* infections, particularly in-high risk populations such as those in the military, schools, hospitals, and other institutions where large numbers of people dwell in close proximity.

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